THE
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PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

SIXTY-THIRD REPORT, FOR THE YEAR 1960—SEVENTY-THIRD YEAR

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R. T. Kempton, Vassar College
L. H. Kleinholz, Reed College
Irving M. Klotz, Northwestern University
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Wm. Randolph Taylor, University of Michigan

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special

meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk:

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

Gentlemen:

I submit herewith the Report of the seventy-third session of the Marine Biological Laboratory.

1. Policy

During the past year there were several extended discussions on the advisability of developing year-round programs in Marine Biology at the Laboratory. Several alternatives were suggested with reservations expressed on the advisability of establishing a year-round program staffed with permanent personnel, if these staff members were to be employed by the Marine Biological Laboratory. It was voted that the Laboratory should do everything possible to assist in establishing in the Woods Hole area an independent institute for basic research in the broad field of Marine Biology.

2. Land Acquisitions

Four parcels of land were acquired during the year, including the Veeder property, the Broderick property and the Tinkham property on Albatross Street. Included were four residences. The small cottage on the Tinkham Property is in very bad condition and unfit for housing purposes. The Breakwater Hotel property on Bar Neck Road was also purchased and the hotel razed, leaving a free area of 1.4 acres designed to be used as a site for a combination dormitory-dining hall for which tentative plans have been developed. The total land area acquired by these purchases increases the land holdings of the Laboratory in the immediate vicinity of its campus by 2.2 acres.

3. New Laboratory Building

One of the feature events of the year was the completion of the new laboratory building in time for summer occupancy. Included in this building are 61 laboratories, 10 being used as general radiobiological service laboratories, 5 constant temperature rooms, 16 dark rooms, 1 aquarium room, 1 conference room, 1 lecture

room accommodating 140 people, 1 photo laboratory and 4 dry rooms. The summer occupancy demonstrated the adaptability of the new building to a wide variety of research activities. Also, the building stood up very effectively through Hurricane Donna and other severe storms, and proved to be a watertight building, contrary to the Laboratory's experience with buildings of brick construction.

4. Personnel Changes

It is the policy of the Laboratory that the heads of the various training programs will serve for a period of five years. Dr. Nelson T. Spratt, Jr., will serve as head of the training program in Experimental Embryology during the summer of 1961 and will be succeeded by Dr. James D. Ebert. Dr. Clark P. Read succeeds Dr. Grover C. Stephens as head of the training program in Invertebrate Zoology. By action of the Executive Committee, three additional training programs at the post-doctoral level will be established starting in the summer of 1962. These will be in Marine Microbiology, Problems of Fertility, and Comparative Physiology, to be headed respectively by Drs. W. D. McElroy, C. B. Metz and C. Ladd Prosser.

5. Laboratory Fees

During the past several years, the fees paid by investigators for laboratory space and the included services have covered only one-sixth of the cost of operation of such a laboratory. It was voted by the Executive Committee to gradually increase these fees over a period of two years so as to finally increase the fees to cover one-third of these costs. The Executive Committee also voted to have a weekly inclusive dormitory charge to cover both board and room, patterned after the usual operation as seen in the colleges and universities.

6. Plant Changes

During the summer of 1960 all of the training programs operated throughout the summer instead of two groups, each group operating for half the summer. In order to accommodate all of the training programs concurrently, the Old Lecture Hall was completely remodeled to accommodate the training program in Experimental Embryology. The ground floor provides a general student laboratory, the second floor a series of laboratories for special research procedures.

7. Grants, Contracts and Contributions, in Support of Laboratory Activities, Including Training Grants

The total income from these sources of support amounts to $373,000 in 1960. This represents 44% of the total income and is made up of the following accounts:

Training grants for the courses from NIH and NSF, support for regular research activities from NIH, NSF, AEC and ONR and gifts from the MBL Associates, Josephine C. Crane Foundation, The Rockefeller Foundation, The George F. Jewett Foundation and the following pharmaceutical companies: The Merck Co. Foundation, Carter Products, Inc., C. I. B. A. Pharm. Products Inc., Abbott Laboratories, Schering Foundation, Inc., Eli Lilly & Company, the Upjohn Company and E. R. Squibb & Sons.

8. Future Plans

With the acquisition of the Breakwater Property the Laboratory now has a proper site for the location of the projected dormitory-dining hall. The Officers of the Corporation are exploring various sources of funds for this construction which is so necessary for the solution of problems of congestion and parking difficulties in our campus. In addition funds are being sought for the construction of additional cottages in the Devil's Lane Tract.

Respectfully submitted,
PHILIP B. ARMSTRONG,
Director

MEMORIALS

ROSS GRANVILLE HARRISON

by

Chester L. Yntema

Ross Granville Harrison died September 30, 1959, after a full life of 89 years. During his lifetime biology became a modern science. His contributions based on critical experimentation were a great factor in this maturation and his example has been an inspiration to biologists.

Dr. Harrison was born January 13, 1870, in Germantown, Pennsylvania. His undergraduate and graduate work was done at Johns Hopkins; he received his Doctorate of Philosophy in 1894. The thesis on the embryological origin of the rays of the fins in teleosts was done with Dr. Brooks as his teacher. Five years later, after intermittent study in Germany, he was awarded the degree of Doctor Medicine by the University of Bonn.

After receiving his Ph.D., Dr. Harrison taught at Bryn Mawr for a year and then studied for a year in Germany. In 1896 he returned to Johns Hopkins to join the department of anatomy headed by Dr. Mall. In 1907 he accepted the Bronson professorship of comparative anatomy at Yale and the remainder of his career continued with Yale as its base.

Early in his stay at Yale, the Osborn Laboratories were built for the Departments of Botany and Zoology and these buildings continue to serve the departments. In addition, the science departments at Yale became University departments as he demanded. This recognition is so generally given today that it is difficult to realize that the issue once had to be made and pressed.

Dr. Woodruff and Dr. Petrunkevitch joined Dr. Coe and Dr. Harrison; these four became central and lasting figures in a zoology department which was outstanding in both its undergraduate and graduate programs. An increasing number of graduate students and foreign fellows came to the Osborn Laboratories and for many years there was a group of students pursuing their thesis work under Dr. Harrison.

In his scientific research Dr. Harrison furthered the concept of an experimental approach to embryology initiated by Roux and Driesch and he devised means of analyzing development. In part, his genius consisted of picking a critical experiment bearing on a basic problem and performing the experiment in an uncomplicated way. This approach is illustrated by his cultivation of neuroblasts from the neural tube of the frog embryo in hanging drops of frog lymph, and following the growth of the processes from these cells by repeated microscopic observations. By this one procedure he settled the controversy over the origin of nerve cell processes, and in addition devised the technique

of tissue culture for animal cells and tissues which has come to be a standard biological procedure. After other pioneer studies with explantation, he developed and refined means of transplantation for amphibian embryos which he and many others have used. The analyses of development he undertook included studies of the lateral line organs, the neural crest, the polarization of the limb and the internal ear, and growth rates in heteroplastic transplants.

In each of his many reports, the same standard of perfection is maintained. In a clear and obvious way, a basic problem is resolved by results from simple experiments ingeniously devised and applied.

Dr. Harrison was granted an honorary master's degree from Yale University in 1907 and honorary doctor's degrees from Yale and Cincinnati in 1920. These honors were followed by similar recognition from several other institutions in this country and in Europe. He was a member of many learned societies and the recipient of awards given in recognition of his achievements.

Dr. Harrison's memberships in societies and academies of other countries indicate the regard held for him. He was a member of the Royal Society of Lund, the Royal Society of Uppsala, and the German Anatomical Society, of which he was a president. He was a corresponding member of the Göttingen Academy of Science, the German Academy of Sciences, the Bavarian Academy of Science, the Academy of Sciences of the Institute of France, and the Society of Biology of Paris. He was an honorary member of the Royal Academy of Turin and the Royal Academy of Belgium. He was a foreign correspondent of the Academy of Science of the Institute of Bologna. He was a foreign associate of the Academy of Medicine of Paris. He was a foreign member of the Royal Netherlands Academy of Science, the Norwegian Academy of Science, the National Academy of Rome, the Royal Swedish Academy of Stockholm, the Zoological Society of London and the Royal Society of London.

The professional activities of Dr. Harrison included many administrative responsibilities in addition to those that he met at Yale. His interest in marine laboratories is evident from his connections with such institutions. He served as a Trustee of this Laboratory from 1908 to 1940 and then became a Trustee Emeritus. In addition he was a Trustee of the Woods Hole Oceanographic Institution and the Bermuda Biological Station. He served as president of several scientific societies. He was a member of the boards of the Rockefeller Institute of Medical Research and the Jane Coffin Childs Fund for Medical Research.

From 1903 to 1946, Dr. Harrison was Managing Editor of the Journal of Experimental Zoology; he imprinted upon this publication a standard of excellence which is a challenge to contributors.

For many years, Dr. Harrison was a councilor and member of the executive committee of the National Academy of Sciences. After his retirement from Yale he was chairman of the National Research Council from 1938 to 1946. During this period, which included the Second World War, he handled responsibilities for the national government with which the National Academy and the National Research Council were faced. During the same period he was a member of the science committee of the National Resources Planning Board. Later he served on the United States National Committee for the United Nations Educational Scientific and Cultural Organization.

With all his accomplishments, Dr. Harrison was modest, self-contained and retiring. He had a deep regard for the individualities of others. This was particularly evident to those who were graduate students under him. He himself set an example of application and devotion to his work; others could determine their own pace and ways without comment or persuasion. He had no understanding of incompetence but he overlooked human foolishness and foolhardiness. His practice of sharing his luncheon hour with students was of great value which came to be appreciated more fully with passing years. During

this hour of sandwiches and tea no mention of scientific interests was recognized. Consequently a variety of topics was covered under the wise and sympathetic aegis of Dr. Harrison. We came to appreciate and be influenced by his wide range of knowledge and interests, his great understanding, and his whimsical humor.

Dr. Harrison is survived by his wife, Mrs. Ida Lange Harrison, whom he met in Germany and married in 1896. Also surviving him are their three daughters and two sons. In honoring the memory of Dr. Harrison we wish to convey to his family a sense of our indebtedness and appreciation for his many years with us.

Lewis Victor Heilbrunn

by

H. B. Steinbach

This is a note in memory of Lewis Victor Heilbrunn who died in an automobile accident early last fall. If a memorial could echo the nature of the man, it would be vigorous, terse, highly intelligent and very human.

Lewis Victor Heilbrunn was one of the most influential figures of modern cellular biology, not only through his books and scientific papers but through his impact on his students and associates. He had the special knack of bringing out and fostering the intellectual best of those who worked with him. In large part this must have been due to the fact that he spent his life as an eager searcher after truth, not as a repository of the truth. Thus, those who talked to him of their problems, scientific and otherwise, found themselves discussing the problems and arriving at conclusions rather than being told answers. Surely this is at the heart of all good teaching, and Heilbrunn was its best exponent.

His life was intimately associated with the Marine Biological Laboratory, and he loved the institution with a fierce devotion.

The records show him first appearing here as a student investigator in 1912 at the age of twenty. He was elected a Member of the Corporation in 1915. The Director's report for this year records that Heilbrunn, with a few others, was responsible for raising the sum of twelve dollars to enable the library to subscribe to the British Journal of Physiology.

He was elected a Trustee in 1931 and to the Executive Committee in 1934, and over the years continued to serve the MBL in a variety of capacities. While his services to the Laboratory may lose their sharpness with the death of the man, they do not cease. At least eighteen active members of the Corporation received their doctorate degrees under his direction, as did four who have served or are serving on the Executive Committee. An equal number of workers active in the interests of the Laboratory gladly would acknowledge their direct debt to his training.

In 1917 and 1918 his name does not appear on the attendance records of the Laboratory. During these years he served as a pilot in the then new air force of his country. In a parenthetical way it could be noted that it is quite consistent with the essential daring of the man that he should be an accomplished pilot of an aircraft fifteen years before he learned to drive a car.

In 1919 the record shows him in attendance as an Independent Investigator from Brooklyn, New York, his home town. The record does not spell out the circumstances but one can be sure that he paused but briefly at home upon demobilization and then took off at once for his beloved Woods Hole.

Heilbrunn's professional career is recorded in other places and need not be repeated here. He was a great man, not to be illustrated by a recital of data. Time may dim the memory but his influence will be great for years to come. In the absence of ade-

quate words the true nature of the man will be found residing in the memories of those who had the pleasures and the jolts of working with him. He was a catalyst, an arouser. Some awoke to anger, to difference—but this was productive; some he awoke to curiosity, to the equable search; some he awoke to fire, to the necessity of looking and thinking and doing day and night, in dreams as in waking, for the truth that man must seek in the laboratory, in the university, in life.

To work with Heilbrunn was to be a part of his family. The interests of the world were the subjects of his cosmic classroom, housed alike in the laboratory, ice cream socials and the soft-ball field. We extend to his widow, Ellen Donovan Heilbrunn and to his daughter Constance our understanding sympathy and our gratitude for sharing him with us.

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University
Libbie H. Hyman, American Museum of Natural History
A. C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Grover C. Stephens, Associate Professor of Zoology, University of Minnesota, in charge of the course
Milton Fingerman, Assistant Professor of Zoology, Newcomb College, Tulane University
Bernard L. Strehler, Chief, Cellular and Comparative Physiology, Division of Gerontology, National Institutes of Health
Paul P. Weinstein, Laboratory of Tropical Disease, National Institutes of Health
Richard C. Sanborn, Professor of Zoology, Department of Biological Sciences, Purdue University
James Case, Associate Professor of Zoology, State University of Iowa
A. Farmanfarmaian, Research Associate, University of California
G. F. Gwilliam, Assistant Professor of Biology, Reed College

III. Assistants

Robert Ashman, Wabash College
Jonathan P. Green, University of Minnesota

EMBRYOLOGY

I. Instructors

Mac V. Edds, Jr., Professor of Biology, Brown University, in charge of the course
Philip Grant, Assistant Professor of Pathobiology, The Johns Hopkins University
Lionel I. Rebhun, Assistant Professor of Biology, Princeton University
John W. Saunders, Jr., Professor of Zoology, Marquette University
Nelson T. Spratt, Jr., Professor of Zoology, University of Minnesota
Maurice Sussman, Associate Professor of Biology, Brandeis University

II. Laboratory Assistants

David Sonneborn, Brandeis University
Richard Whittaker, Yale University

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Otto Loewi, Professor of Pharmacology, New York University School of Medicine
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Györgyi, Director, Institute for Muscle Research, Marine Biological Laboratory

II. Instructors

W. D. McElroy, Director, McCollum-Pratt Institute, The Johns Hopkins University; in charge of the course
Francis D. Carlson, Associate Professor of Biophysics, The Johns Hopkins University
Bernard D. Davis, Professor of Pharmacology, Harvard Medical School
Donald R. Griffin, Professor of Zoology, Harvard University
Timothy H. Goldsmith, Society of Fellows, Harvard University
Robert B. Loftfield, Massachusetts General Hospital

BOTANY

I. Consultant

William Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Richard C. Starr, Associate Professor of Botany, Indiana University, in charge of the course
Walter R. Herndon, Associate Professor of Botany, University of Alabama
John M. Kingsbury, Assistant Professor of Botany, Cornell University

III. Collector

Joyce Fletcher, New York Botanical Garden

IV. Laboratory Assistants

Melvin Goldstein, Indiana University
Philip Cook, Botany Department, Indiana University

ECOLOGY

I. Consultants

Paul Galtsoff, U. S. Fish and Wildlife Service, Woods Hole
Alfred S. Redfield, Woods Hole Oceanographic Institution
Bostwick H. Ketchum, Woods Hole Oceanographic Institution
Edwin T. Moul, Rutgers University
Charles E. Jenner, University of North Carolina
Howard T. Odum, University of Texas

II. Instructors

Eugene P. Odum, Alumni Foundation Professor of Zoology, University of Georgia, in charge of the course
John H. Ryther, Marine Biologist, Woods Hole Oceanographic Institution
Howard L. Sanders, Woods Hole Oceanographic Institution
Walter R. Taylor, Chesapeake Bay Institute, The Johns Hopkins University

III. Laboratory Assistants

Richard B. Williams, Harvard University and Marine Institute, University of Georgia
Elijah V. Swift, Swarthmore College

1. The Laboratory Staff, 1960

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
Carl O. Schweidenback, Manager of the Supply Department
Irvine L. Broadbent, Office Manager
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Development of Research Service

GENERAL OFFICE

Mrs. Lila S. Myers
Mrs. Vivien R. Brown
Mrs. Virginia M. Morehouse
Mrs. Marion C. Chase
Mrs. Vivian I. Manson
Mrs. Ruth Mayo

LIBRARY

Mrs. Gwendolyn S. Blomberg
Albert K. Neal
Janice Parent

MAINTENANCE OF BUILDINGS AND GROUNDS

Robert Adams
Eldon P. Allen
Gardner Gayton
Robert Gunning
Robert W. Hampton
Walter J. Jaskun
Donald B. Lehy
Ralph H. Lewis
Russell F. Lewis
Alan G. Lunn
Alton J. Pierce
Robert H. Walker, Jr.
James S. Thayer

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
Seaver R. Harlow
Caroline McDaniel

SUPPLY DEPARTMENT

Donald P. Burnham
Milton B. Gray
Geoffrey J. Lehy
Robert O. Lehy
Robert M. Perry
Bruno F. Trapasso
Mrs. Patricia Travares
John J. Valois
Jared L. Vincent
Hallett S. Wagstaff

2. Investigators; Lalor and Lillie Fellows; and Students

Independent Investigators, 1960

Adams, Ralph G., National Institutes of Health
Adelman, William J., Jr., Physiologist, National Institutes of Health
Allen, M. Jean, Professor of Biology and Chairman, Wilson College
Amatniek, Ernest, Biophysicist, College of Physicians and Surgeons
Amberson, William R., Marine Biological Laboratory
Anderson, John Maxwell, Professor of Zoology, Cornell University
Armstrong, Philip B., Professor and Chairman, Department of Anatomy, Upstate Medical Center
Atwood, Kimball C., Associate Professor of Medical Genetics, University of Chicago
Baltus, Elyane, Research Associate, Free University of Brussels, Belgium
Bang, Frederik B., Chairman, Department of Pathobiology, Johns Hopkins School of Hygiene
Barth, L. G., Professor of Zoology, Columbia University
Baylor, Martha B., Marine Biological Laboratory
Beevers, Harry, Professor of Biology, Purdue University
Belton, Peter, College of Physicians and Surgeons, Columbia University
Bennett, Michael V. L., Assistant Professor of Neurology, College of Physicians and Surgeons
Berendsen, Herman J. C., Staff Member, Massachusetts Institute of Technology
Berger, Charles A., Chairman, Biology Department, Fordham University
Bernstein, Maurice H., Assistant Professor, Wayne State University
Borgese, Thomas A., Research Fellow in Medicine, Harvard Medical School
Bradley, Dan F., Scientist, Commissioned Corps of U. S. Public Health Service
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Goldsmith, Timothy H., Junior Fellow, Harvard University
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Laurie, John S., Assistant Professor of Experimental Biology, University of Utah
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Levy, Milton, Professor and Chairman, Department of Biochemistry, New York University College of Dentistry
Lochhead, John H., Professor of Zoology, University of Vermont
Loewenstein, Werner R., Associate Professor of Physiology, Columbia University
Loftfield, Robert B., Associate Biochemist, Massachusetts General Hospital
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Loomis, William F., Professor of Biochemistry, Brandeis University
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Mahler, Henry R., Professor of Chemistry, Indiana University
Mann, David E., Professor of Pharmacology, Temple University
Mann, Thaddeus R. R., Director, Unit of Reproductive Physiology and Biochemistry, University of Cambridge, England
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Polgar, George, Research Fellow, University of Pennsylvania, Graduate School of Medicine
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Reuben, John P., Research Associate, Columbia University

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Scott, Allan C., Professor of Biology, Colby College
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Scott, George T., Professor and Chairman, Department of Zoology, Oberlin College
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Speidel, Carl C., Professor of Anatomy, University of Virginia, School of Medicine
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Spindell, William, Associate Professor of Chemistry, Rutgers University
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Stone, William, Jr., Director, Ophthalmic Plastics Laboratory, Massachusetts Eye and Ear Infirmary
Strehler, Bernard L., Chief, Cellular and Comparative Physiology Section, National Institutes of Health
Strittmatter, Cornelius F., Assistant Professor of Biological Chemistry, Harvard Medical School
Strittmatter, Philipp, Assistant Professor of Biochemistry, Washington University Medical School
Strumwasser, Felix, Neurophysiologist, National Institutes of Health
Stunkard, Horace W., Research Associate, The American Museum of Natural History
Sturtevant, A. H., Professor of Genetics, California Institute of Technology
Sudak, Frederick N., Assistant Professor of Physiology, Albert Einstein College of Medicine
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deTerra, Noel, Post-doctoral Fellow, Rockefeller Institute

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Trager, William, Associate Professor, Rockefeller Institute
Travis, David M., Assistant Professor of Pharmacology, University of Florida
Troll, Walter, Associate Professor, New York University
Tweedell, Kenyon S., Assistant Professor of Biology, University of Notre Dame
Van Norman, Earl, Sr., Princeton University
deVillafranca, George W., Associate Professor of Zoology, Smith College
Villee, Claude A., Associate Professor of Biological Chemistry, Harvard University
Vincent, Walter S., Assistant Professor of Anatomy, Upstate Medical Center
Warren, Leonard, Visiting Scientist, National Institutes of Health
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Wilson, Walter L., Assistant Professor of Physiology, University of Vermont College of Medicine
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Zigman, Seymour, Research Associate, Massachusetts Eye and Ear Infirmary
Zimmerman, Arthur M., Instructor in Pharmacology, Downstate Medical Center
Zwilling, Edgar, Professor of Biology, Brandeis University

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Flaks, Joel G., University of Pennsylvania School of Medicine
Hurwitz, Jerard, New York University College of Medicine
Strittmatter, Cornelius F., Harvard Medical School
Warren, Leonard, National Institutes of Health

Lillie Fellow, 1960

Moscona, A. A., University of Chicago

Grass Fellows, 1960

Kitai, Stephen T., Massachusetts General Hospital
Luco, J. V., Catholic University Medical School, Santiago, Chile
Reynolds, Leslie B., Medical College of South Carolina

Beginning Investigators, 1960

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Beebe, Curt, University of Vermont, College of Medicine
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COSTELLO, ROBERT CHARLES, University of North Carolina
CURTIS, DAVID R., Australian National University
DOOLITTLE, RUSSELL F., Harvard University
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DUDEL, JOSEF, Harvard Medical School
DUNHAM, PHILIP B., University of Chicago
EISENBERG, ROBERT S., Harvard College
FAUST, ROBERT G., Princeton University
FILOSA, MICHAEL F., Johns Hopkins University
FRUMENTO, A. S., University of Buenos Aires
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HANLORE, MARY S., University of California
HENSHAW, EDGAR C., Harvard Medical School
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Chanutin, Alfred, Professor of Biochemistry, University of Virginia School of Medicine
Chase, Aurin M., Associate Professor of Biology, Princeton University
Clark, Eliot R., University of Pennsylvania
Cohen, Seymour S., Professor of Biochemistry, University of Pennsylvania
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Flavin, Martin, National Heart Institute, National Institutes of Health
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Karush, Fred, Professor of Immunochemistry, University of Pennsylvania School of Medicine
Klein, Morton, Professor of Microbiology, Temple University School of Medicine
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Stetten, DeWitt, Associate Director in Charge of Research, National Institutes of Health
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WEXLER, HARRY, Director of Research, U. S. Weather Bureau
WHEELER, GEORGE E., Assistant Professor of Biology, Brooklyn College
YNTEMA, CHESTER L., Professor of Anatomy, Upstate Medical Center at Syracuse

Students, 1960

All students listed completed formal course program, June 21–July 30. Asterisk indicates students completed Post Course Research Program, August 1–September 3.

BOTANY

AUYANG, SHIH-CHEN, Clark University
*BONAMO, PATRICIA M., Cornell University
BROOKS, AUSTIN E., Wabash College
*CYRUS, RODNEY V., University of Michigan
DEUTSCH, ELIZABETH J., Radcliffe College
ERICKSON, PAUL A., Clark University
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FRANKLIN, SANDRA E., Acadia University
*HALL, NANCY V., Vassar College
KOETZNER, KENNETH L., Lycoming College
KREMER, PETER R., Cornell University
LANG, NORMA J., Indiana University
MITCHELL, ROBERT A., Cornell University
MULLIN, MICHAEL M., Harvard University
*NICHOLS, HERBERT W., University of Alabama
NOODEN, LARRY D., Harvard University
*WATERS, ANNETTE, Indiana University
*WESTERDALE, THOMAS H., University of Michigan

EMBRYOLOGY

*ALLISON, WILLIAM S., Brandeis University
ARNOLD, JOHN M., University of Minnesota
*CLARKE, RICHARD B., University of Illinois
*COHEN, NICHOLAS, University of Rochester
EISENSTADT, JEROME, Brandeis University
*ESPER, HILDEGARD, Columbia University
*GREEN, SANDRA J., University of Minnesota
HOVINGH, PETER, Johns Hopkins University
*JAFFEE, ROBERT L., University of Rochester
*KIMMEL, DONALD L., JR., Temple University
LICHTENBERG, INGEBORG, University of Chicago
MARSHALL, LEE ANN, University of Michigan
ORLOFF, SERVE, Brussels, Belgium
*PLATT, JOHN R., University of Chicago
RACE, JAMES, JR., State University of Iowa
*RITTENHOUSE, ELIZABETH W., University of Michigan
SLATER, DONALD W., Indiana University
SWEENY, PHILLIP R., Brown University
WILLE, JOHN J., JR., Indiana University
*WINESDORFER, JOHN E., Johns Hopkins University

ECOLOGY

*Alexander, Douglas G., University of North Carolina
Beardow, Jane M., Drew University
*Broughton, William S., University of Georgia
*de la Cruz, Armando, University of the Philippines, Pasay City, Philippines
Gold, Kenneth, New York University
*Gustafson, Alton H., Bowdoin College
*Gutknecht, John W., University of North Carolina
*Kramer, Dana D., City College of New York
*Platzman, Sara J., Yale University
*Sterns, Carol W., Peekskill, New York
Vandenack, Sister Julia Marie, Catholic University of America
*Wilkens, Jerrel L., Tulane University
*Zieg, Roger G., University of Nebraska

PHYSIOLOGY

Alberts, Bruce M., Harvard College
*Boass, Agna, Radcliffe College
*Brody, Stuart, Stanford University
*Collier, Robert J., Harvard University
*Dolan, Michael F., Johns Hopkins University
*Forrest, Helen F., Rutgers University
*Freeman, Alan R., Hahnemann Medical College
Fridovich, Irwin, Duke University
*Hall, Zach W., Emory University
*Hempfling, Walter P., Yale University
Holtzman, Eric, Columbia University
*Maddux, William S., Princeton University
McEwen, Bruce S., Rockefeller Institute
Nading, Louis K., Oberlin College
*Nathenson, Stanley G., Washington University
*Norris, John L., Vanderbilt University
Orr, Charles W. M., Johns Hopkins University
*Patrick, Noel V., Columbia University
Richardson, G. S., Harvard Medical School
Rosenfield, Carol, New York University College of Medicine
*Roze, Uldis, Washington University Medical School
Schindler, Frederick J., University of Pennsylvania
*Schwartz, Norman M., Syracuse University
Snipes, Charles A., Duke University
*Stone, Henry O., Duke University
Tang, Jien-nan Jordan, Oklahoma Medical Research Foundation
*Turney, Tully, Jr., University of North Carolina
Webb, George D., University of Colorado Medical School

INVERTEBRATE ZOOLOGY

Alexander, Kathleen, University of North Carolina
*Ballard, Juliet L., Drew University
Bottomly, Gail, University of Massachusetts
Briggs, Richard G., Cornell University
*Broch, Edmund S., Cornell University
*Bruno, Merle S., Syracuse University
Chaicharn, Aimorn, University of New Hampshire
Clarridge, Jill E., University of Michigan

*Cleland, Charles F., Wabash College
Costello, Robert C., University of North Carolina
*D'Agostino, Anthony S., New York University
Drummond, Sister Therese, Catholic University of America
Eagleson, Louise J., Spellman College
Edlin, Gordon J., University of Oregon
Emlen, John M., University of Wisconsin
Farrell, Carolyn Rose, Marquette University
*Fenner, Barbara, Vassar College
Fourcade, Miguel, S. J., Fordham University
Gauthier, Geraldine F., Harvard Medical School
*Haddad, Lamia, Brown University
Harman, Mary, Radcliffe College
Holland, Nicholas D., Carleton College
Holt, Portia, Colorado College
*Hopper, Fred A., Jr., University of Oklahoma
Jarvis, Sister Julie, Catholic University of America
Kaneshiro, Edna S., Syracuse University
Keck, Carl W., Lafayette College
Kee, James W., Jr., Massachusetts Institute of Technology
*Kirchenberg, Ralph J., DePaul University
Knowlton, Robert E., Bowdoin College
Krewson, Carrie R., Vassar College
Luckenbill, Louise M., Washington University
Mahowald, Anthony P., S. J., Johns Hopkins University
Morrison, Roberta A., Smith College
Porcaro, Carol A., Marymount College
*Schopf, Thomas J. M., Oberlin College
Smith, Stephen D., Wesleyan University
Squadroni, Jose, S. J., Fordham University
Therous, Roger B., Bureau of Commercial Fisheries
Vogel, Steven, Tufts University
Waugh, Mary, Wilson College
Westhoff, David D., St. Louis University
Zweig, Charles H., Brandeis University

3. Fellowships and Scholarships, 1960

Calkins Scholarship:
Richard Briggs, Invertebrate Zoology Course

Bio Club Scholarship:
Dana Kramer, Ecology Course

Lucretia Crocker Fellowships:
Kenneth Gold, Ecology Course
Gisela Falcon, Botany Course

4. Tabular View of Attendance, 1956–1960

	1956	*1957*	*1958*	*1959*	*1960*
Investigators—Total	304	326	410	427	458
Independent	184	186	203	215	231
Under Instruction	20	23	39	45	42
Library Readers	50	42	54	51	50
Research Assistants	50	75	114	116	135

Students—Total	140	139	138	134	122
Invertebrate Zoology	55	55	55	49	43
Embryology	28	27	22	23	20
Physiology	30	30	27	27	28
Botany	18	18	18	20	18
Ecology	9	9	16	15	13
Total Attendance	444	465	548	561	580
Less persons represented as both investigators and students	2	3	5	4	2
	442	462	543	557	578
Institutions Represented—Total	130	129	142	143	144
By Investigators	97	94	110	98	83
By Students	33	35	74	73	61
Schools and Academies Represented					
By Investigators	1	1	2	8	5
By Students	3	5		12	2
Foreign Institutions Represented					
By Investigators	9	11	20	29	11
By Students	6	5	6	9	3

5. Institutions Represented, 1960

Acadia University
American Museum of Natural History
American University
Amherst College
Bowdoin College
Brandeis University
Brown University
Carleton College
Catholic University of America
City College of New York
Clark University
Colby College
Colorado College
Columbia University
Columbia University College of Physicians and Surgeons
Cornell University
Dartmouth College
DePaul University
Drew University
Duke University
D'Youville College
Drexel Institute of Technology
Emory University
Florida State University
Fordham University
George Washington University
Goucher College
Hahnemann Medical School
Harvard University
Harvard University Medical School
Indiana University
Institute for Muscle Research
Johns Hopkins University
Lafayette College
Loyola College
Marquette University
Marymount College
Massachusetts Institute of Technology
McMaster University
Medical College of South Carolina
National Institutes of Health
New York University—Heights
New York University, College of Dentistry
New York University, College of Medicine
New York University, Washington Square College
North Carolina State College
Notre Dame University
Oberlin College
Oklahoma Medical Research Foundation
Princeton University
Purdue University
Queens College
Radcliffe College
Reed College
Rice Institute
Rockefeller Institute
Rockefeller Foundation
Russell Sage College
Rutgers University
Saint Joseph College
Saint Louis University
Seton Hill College
Single Cell Research Foundation
Smith College
Spellman College
State University of Iowa

State University of New York, Upstate Medical College
State University of New York, Downstate Medical College
Syracuse University
Temple University
Tufts College
Tulane University
University of Alabama
University of California
University of Chicago
University of Colorado Medical School
University of Delaware
University of Florida
University of Georgia
University of Illinois
University of Massachusetts
University of Michigan
University of Minnesota
University of Nebraska
University of New Hampshire
University of North Carolina
University of Oregon
University of Pennsylvania
University of Pennsylvania School of Medicine
University of Pittsburgh School of Medicine
University of Utah
University of Vermont
U. S. Bureau of Commercial Fisheries
Vassar College
V. A. Administration Hospital at Brooklyn
Wabash College
Washington University
Washington University Medical School
Wayne State University
Wesleyan University
Western Reserve University School of Medicine
Wilson College
Woods Hole Oceanographic Institution
Yale University

Foreign Institutions Represented, 1960

Free University of Brussels, Belgium
Faculté Des Sciences, Lyon, France
University College, London
University of Witwatersrand, Johannesburg, South Africa
Catholic University Medical School, Santiago, Chile
University of Cambridge, England
University of Edinburgh, Scotland
Kyoto University, Kyoto, Japan
McMaster University, Canada
Tohoku University School of Medicine, Sendai, Japan
University of Buenos Aires
University of the Philippines, Pasay City, Philippines
Instituto Investigaciones, Barcelona 3, Spain

Supporting Institutions and Agencies, 1960

Associates of the Marine Biological Laboratory
Atomic Energy Commission
Josephine B. Crane Foundation
The Grass Foundation
The Lalor Foundation
The Merck Company Foundation
National Institutes of Health
National Science Foundation
Office of Naval Research
The Rockefeller Foundation
Schering Foundation, Inc.

Corporate Associates

Abbott Laboratories
CIBA Pharmaceutical Products, Inc.
Carter Products, Inc.
Eli Lilly and Company
E. R. Squibb & Sons
The Upjohn Company

6. Evening Lectures, 1960

June 24	G. Adrian Horridge St. Andrews University, Scotland	"Electrophysiological and anatomical analysis of primitive ganglia"
July 1	A. A. Moscona University of Chicago, Frank R. Lille Fellow at MBL	"Experimental studies on tissue synthesis: problems and prospects"

July 8	Thaddeus R. R. Mann Molteno Institute, University of Cambridge, Senior Lalor Fellow at MBL	"Comparative aspects of sperm physiology"
July 15	Clifford V. Harding Columbia University, College of Physicians & Surgeons	"The control of cell division"
July 22	J. V. Luco Catholic University of Chile, Alexander Forbes Lecturer at MBL	"Physiological studies during Wallerian degeneration"
July 25	J. V. Luco	"The trophic effect of neuron activity"
July 29	DeWitt Stetten, Jr. National Institutes of Health	"The metabolism of gout"
August 5	Laszlo Lorand Northwestern University	"The chemical basis of the clotting of blood"
August 12	Severo Ochoa New York University School of Medicine	"Metabolism of propionic acid in animal tissues"
August 19	Ernst A. Scharrer Albert Einstein College of Medicine	"Neurosecretion"
August 26	George L. Clarke Harvard University	"Ecological aspects of daylight and bioluminescence in the sea"

7. Tuesday Evening Seminars, 1960

July 5	Martin B. Mathews	"Some comparative biochemistry of connective tissue ground substance"
	Harold F. Blum	"Complexity and organization"
July 12	Ronald C. Rustad	"X-ray induced dissociation of the mitotic and micromere 'clocks'"
	Donald P. Costello	"The giant cleavage spindle of the egg of *Polychoerus carmelensis*"
	C. C. Speidel R. H. Cheney	"Motion pictures of radiation-induced modifications of fertilization and early development of the sea urchin Arbacia"
July 19	Albert Szent-Györgyi	"Energy and charge transfer"
	Irvin Isenberg	"Spin resonance studies of riboflavin semiquinones and riboflavin complexes"
	Benjamin Kaminer	"Contractile responses in the presence of charge transfer complexes"
	Andrew Hegyeli	"Detection of electron donors"
July 26	W. Robert Middlebrook	"The action of trypsin on acetylated myosin"
	Herman J. C. Berendsen	"The structure of water in tissue, as studied by nuclear magnetic resonance"
	Alex B. Novikoff	"Phagocytosis, pinocytosis and lysosomes: Cytochemical and electron microscopic studies"
August 2	Philip Person Albert Fine	"The role of free radical formation during indophenol blue synthesis by respiratory enzymes"
	Klaus Schwarz	"A role of trivalent chromium in glucose utilization"
	S. Ehrenpreis	"A receptor protein: Isolation and drug binding properties"

August 9	James Case	"Excitation of firefly light organ"
	John Buck	
	R. A. Sjodin	"Cation permeability in muscle"
	P. Belton	"Effects of ions on potential in lepidopteran muscle fibers"
August 16	F. D. Carlson	"A scheme for the mechanochemistry of muscle"
	A. G. Szent-Györgyi	"Studies on actin. I. Reversibility of actin depolymerization in presence of KI"
	T. Hayashi	"Studies on actin. II. Polymerization and the bound nucleotide"
	Raja Rosenbluth	

8. Members of the Corporation, 1960

1. Life Members

Brodie, Mr. Donald M., 522 Fifth Avenue, New York 18, New York
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania
Carver, Dr. Gail L., Mercer University, Macon, Georgia
Cole, Dr. Elbert C., 2 Chipman Park, Middlebury, Vermont
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri
Crane, Mrs. W. Murray, Woods Hole, Massachusetts
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Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana
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Schrader, Dr. Franz, Duke University, Durham, N. C.
Schrader, Dr. Sally, Duke University, Durham, N. C.
Turner, Dr. C. L., Northwestern University, Evanston, Illinois
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Wallace, Dr. Louise B., 359 Lytton Avenue, Palo Alto, California
Warren, Dr. Herbert S., 610 Montgomery Avenue, Bryn Mawr, Pennsylvania

2. Regular Members

Abell, Dr. Richard G., 7 Cooper Road, New York City, New York
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Addison, Dr. W. H. F., 286 East Sidney Avenue, Mount Vernon, New York
Adolph, Dr. Edward F., University of Rochester School of Medicine and Dentistry, Rochester, New York
Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota
Allen, Dr. M. Jean, Department of Biology, Wilson College, Chambersburg, Pennsylvania

ALLEN, DR. ROBERT D., Department of Biology, Princeton University, Princeton, New Jersey

ALSCHER, DR. RUTH, Department of Physiology, Manhattanville College, Purchase, New York

AMATNIEK, DR. ERNEST, Department of Neurology, College of Physicians and Surgeons, New York City, New York

AMBERSON, DR. WILLIAM R., Woods Hole, Massachusetts

ANDERSON, DR. J. M., Department of Zoology, Cornell University, Ithaca, New York

ANDERSON, DR. RUBERT S., Medical Laboratories, Army Chemical Center, Maryland (Box 632, Edgewood, Maryland)

ANDERSON, DR. T. F., Institute for Cancer Research, Fox Chase, Philadelphia 11, Pennsylvania

ARMSTRONG, DR. PHILIP B., Department of Anatomy, State University of New York College of Medicine, Syracuse 10, New York

ARNOLD, DR. WILLIAM A., Division of Biology, Oak Ridge National Laboratory, Oak Ridge, Tennessee

ATWOOD, DR. KIMBALL C., Department of Microbiology, University of Illinois, Urbana, Illinois

AUSTIN, DR. MARY L., Wellesley College, Wellesley, Massachusetts

AYERS, DR. JOHN C., Department of Zoology, University of Michigan, Ann Arbor, Michigan

BAITSELL, DR. GEORGE A., Osborn Zoological Laboratories, Yale University, New Haven, Connecticut

BALL, DR. ERIC G., Department of Biological Chemistry, Harvard University Medical School, Boston 15, Massachusetts

BALLARD, DR. WILLIAM W., Department of Zoology, Dartmouth College, Hanover, New Hampshire

BALTUS, DR. ELYANE, Laboratory of Animal Morphology, Brussels, Belgium

BANG, DR. F. B., Department of Pathobiology, Johns Hopkins University School of Hygiene, Baltimore 5, Maryland

BARD, DR. PHILLIP, Johns Hopkins Medical School, Baltimore, Maryland

BARTH, DR. L. G., Department of Zoology, Columbia University, New York 27, New York

BARTH, DR. LUCENA, Department of Zoology, Barnard College, New York 27, New York

BARTLETT, DR. JAMES H., Department of Physics, University of Illinois, Urbana, Illinois

BEAMS, DR. HAROLD W., Department of Zoology, State University of Iowa, Iowa City, Iowa

BECK, DR. L. V., Department of Physiology and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania

BEHRE, DR. ELINOR M., Black Mountain, North Carolina

BENESCH, DR. REINHOLD, College of Physicians and Surgeons, New York 32, New York

BENESCH, DR. RUTH, College of Physicians and Surgeons, New York 32, New York

Bennett, Dr. Michael V., Department of Neurology, College of Physicians and Surgeons, New York 32, New York

Bennett, Dr. Miriam F., Department of Biology, Sweet Briar College, Sweet Briar, Virginia

Berg, Dr. William E., Department of Zoology, University of California, Berkeley 4, California

Berman, Dr. Mones, Institute for Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, Maryland

Bernheimer, Dr. Alan W., New York University College of Medicine, New York 16, New York

Bernstein, Dr. Maurice, Department of Anatomy, Wayne State University College of Medicine, Detroit 7, Michigan

Bertholf, Dr. Lloyd M., Illinois Wesleyan University, Bloomington, Illinois

Bevelander, Dr. Gerrit, New York University School of Dentistry, 477 First Avenue, New York 16, New York

Bigelow, Dr. Henry B., Museum of Comparative Zoology, Harvard University, Cambridge 38, Massachusetts

Bishop, Dr. David W., Department of Embryology, Carnegie Institution of Washington, Baltimore 5, Maryland

Blanchard, Dr. K. C., Johns Hopkins Medical School, Baltimore, Maryland

Bloch, Dr. Robert, 518 South 42nd Street, Apartment C7, Philadelphia 4, Pennsylvania

Blum, Dr. Harold F., Department of Biology, Princeton University, Princeton, New Jersey

Bodansky, Dr. Oscar, Department of Biochemistry, Memorial Cancer Center, 444 East 68th Street, New York 21, New York

Bodian, Dr. David, Department of Anatomy, Johns Hopkins University, 709 North Wolfe Street, Baltimore 5, Maryland

Boell, Dr. Edgar J., Osborn Zoological Laboratories, Yale University, New Haven, Connecticut

Boettiger, Dr. Edward G., Department of Zoology, University of Connecticut, Storrs, Connecticut

Bold, Dr. Harold C., Department of Botany, University of Texas, Austin 12, Texas

Borei, Dr. Hans, Department of Zoology, University of Pennsylvania, Philadelphia 4, Pennsylvania

Bowen, Dr. Vaughan T., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

Bradley, Dr. Harold C., 2639 Durant Avenue, Berkeley 4, California

Bridgman, Dr. Anna J., Department of Biology, Agnes Scott College, Decatur, Georgia

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Brown, Dr. Dugald E. S., Department of Zoology, University of Michigan, Ann Arbor, Michigan

Brown, Dr. Frank A., Jr., Department of Biological Sciences, Northwestern University, Evanston, Illinois

Brownell, Dr. Katherine A., Department of Physiology, Ohio State University, Columbus, Ohio

Buck, Dr. John B., Laboratory of Physical Biology, National Institutes of Health, Bethesda 14, Maryland

Bullington, Dr. W. E., Randolph-Macon College, Ashland, Virginia

Bullock, Dr. T. H., Department of Zoology, University of California, Los Angeles 24, California

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Cantoni, Dr. Giullio, National Institutes of Health, Mental Health, Bethesda 14, Maryland

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Carpenter, Dr. Russell L., Tufts University, Medford 55, Massachusetts

Carson, Miss Rachel, 11701 Berwick Road, Silver Spring, Maryland

Case, Dr. James, Department of Zoology, State University of Iowa, Iowa City, Iowa

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Cattell, Mr. Ware, Cosmos Club, Washington 5, District of Columbia

Chaet, Dr. Alfred B., Department of Biology, American University, Washington 16, District of Columbia

Chambers, Dr. Edward, Department of Physiology, University of Miami Medical School, Coral Gables, Florida

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CRANE, MR. JOHN O., Woods Hole, Massachusetts

CRANE, DR. ROBERT K., Department of Biological Chemistry, Washington University Medical School, St. Louis, Missouri

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CURTIS, DR. W. C., University of Missouri, Columbia, Missouri

DAN, DR. JEAN CLARK, Misaki Biological Station, Misaki, Japan

DAN, DR. KATSUMA, Misaki Biological Station, Misaki, Japan

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FISHER, DR. KENNETH C., Department of Biology, University of Toronto, Toronto, Canada

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GOODRICH, DR. H. B., Department of Biology, Wesleyan University, Middletown, Connecticut

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GRAHAM, DR. HERBERT, U. S. Fish and Wildlife Service, Woods Hole, Massachusetts

GRAND, MR. C. G., Cancer Institute of Miami, 1155 N. W. 15th Street, Miami, Florida

GRANT, DR. M. P., Sarah Lawrence College, Bronxville, New York

GRANT, DR. PHILIP, Department of Pathobiology, Johns Hopkins University School of Hygiene, Baltimore 5, Maryland

GRAY, DR. IRVING E., Department of Zoology, Duke University, Durham, North Carolina

GREEN, DR. JAMES W., Department of Physiology, Rutgers University, New Brunswick, New Jersey

GREEN, DR. MAURICE, Department of Microbiology, St. Louis University Medical School, St. Louis, Missouri

GREGG, DR. JAMES H., Department of Biological Sciences, University of Florida, Gainesville, Florida

GREGG, DR. JOHN R., Department of Zoology, Duke University, Durham, North Carolina

GREIF, DR. ROGER L., Department of Physiology, Cornell University Medical College, New York 21, New York

GRIFFIN, DR. DONALD R., Biological Laboratories, Harvard University, Cambridge 38, Massachusetts

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GROSS, DR. PAUL, Department of Biology, New York University, University Heights, New York 53, New York

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Crane, Mr. Stephen
Crane, Mrs. W. Carey
Crossley, Mr. and Mrs. Archibald M.
Crowell, Mr. and Mrs. Prince S.
Curtis, Dr. and Mrs. W. D.
Daniels, Mr. and Mrs. F. Harold
Day, Mr. and Mrs. Pomeroy
Draper, Mrs. Mary C.
Dreyer, Mr. and Mrs. Frank A.
Elsmith, Mrs. Dorothy
Enders, Mrs. Frederick
Ewing, Mr. and Mrs. Frederic
Ewing, Mr. William
Fay, Mr. and Mrs. Henry H.
Fisher, Mr. and Mrs. B. C.
Francis, Mrs. Lewis H., Jr.
Frost, Mrs. Frank J.
Galtsoff, Mrs. Paul S.
Gifford, Mr. and Mrs. John A.
Gifford, Mr. and Mrs. Prosser
Gilchrist, Mr. and Mrs. John M.
Gildea, Dr. and Mrs. E. F.
Green, Miss Gladys M.
Gulesian, Mrs. Paul J.
Haig, Mrs. R. H.
Hamlen, Mr. and Mrs. J. Monroe
Harrell, Mr. and Mrs. Joel E.
Harrington, Mr. and Mrs. Robert
Harvey, Dr. Ethel B.
Herrington, Mrs. A. W. S.
Hervey, Dr. and Mrs. John P.
Hirschfeld, Mrs. Nathan B.
Houston, Mr. and Mrs. Howard
Jewett, Mrs. G. F.
Johlin, Mrs. Jacob M.
Keith, Mr. and Mrs. Harold C.
King, Mr. and Mrs. Franklin
Koller, Mr. and Mrs. Lewis
Laurence, Mr. and Mrs. Thomas E.
Lemann, Mrs. Benjamin
Lineaweaver, Mr. Thomas, III
Lobb, Mrs. John
Loeb, Dr. and Mrs. Robert F.
McCusker, Mr. and Mrs. Paul J.
McKelvy, Mr. John E.
Marsland, Mrs. Douglas A.
Marvin, Mrs. Walter T.
Mast, Mrs. S. O.
Meigs, Dr. and Mrs. J. Wister
Mitchell, Mrs. James McC.
Mixter, Mrs. W. Jason
Mosser, Mrs. Benjamin D.
Motley, Mrs. Thomas
Newton, Miss Helen
Nichols, Mrs. George
Nims, Mrs. E. D.
Packard, Mrs. Charles

Park, Mr. and Mrs. M. S.
Pennington, Miss Anne H.
Redfield, Dr. and Mrs. Alfred C.
Reznikoff, Dr. and Mrs. Paul
Riggs, Mr. and Mrs. Lawrason
Rivinus, Mrs. F. M., Jr.
Robinson, Dr. Miles
Rudd, Mr. and Mrs. H. W. Dwight
Sands, Miss Adelaide G.
Saunders, Mr. and Mrs. Lawrence
Shiverick, Mrs. Arthur
Sinclair, Mr. and Mrs. W. Richardson
Speidel, Dr. and Mrs. Carl
Stone, Mr. and Mrs. Leo
Stone, Mr. and Mrs. S. M.
Strauss, Dr. and Mrs. Donald B.
Stunkard, Mrs. Horace W.
Swift, Mr. E. Kent
Swope, Mr. and Mrs. Gerard, Jr.
Swope, Miss Henrietta
Tompkins, Mr. and Mrs. B. A.
Webster, Mrs. Edwin S.
Whiteley, Miss Mabel W.
Wickersham, Mr. and Mrs. James H.
Wilhelm, Dr. and Mrs. Hilmer J.
Williston, Mr. Samuel
Williston, Miss Emily
Wilson, Mrs. Edmund B.
Wolfinsohn, Mrs. Wolfe

V. REPORT OF THE LIBRARIAN

At the close of the year, the Library received currently 1717 journals, 56 new titles having been added in 1960. Of the total, the Marine Biological Laboratory subscribed to 502, received 653 in exchange and 190 as gifts. The Woods Hole Oceanographic Institution subscribed to 109, received 200 in exchange and 63 as gifts.

The Laboratory purchased 121 books, received 92 complimentary copies (9 from authors and 83 from publishers), and accepted 74 miscellaneous gifts. The Institution purchased 38 books and received 9 as gifts. The total number of books accessioned totalled 334.

Through purchase, exchange and gift, the Laboratory completed 12 journal sets and partially completed 13. The Institution completed two sets and partially completed one. There were 3825 reprints added to the collection, of which 1749 were of current issue.

At the close of the year there were 77,525 bound volumes and 216,452 reprints. The number of requests for inter-library loans increased over 1959. There were 469 volumes sent out and 57 were borrowed. About 1000 volumes were bound.

Many valuable books and reprints were received from Drs. P. W. Whiting, L. H. Hyman, Walter S. Root, Roberts Rugh, Ethel B. Harvey, Irvine H. Page, Robt. F. Loeb, H. J. Humm, and the Department of Microbiology, University of Pennsylvania Medical School. The Library extends grateful acknowledgment to these generous contributors.

Many duplicate books and reprints were sent to the Department of Cytology, Warsaw University, thus furthering our exchange relationship abroad.

The year was an exceptionally busy one due to the increase in acquisitions, to the larger number of scientists using the library, and to several changes in the staff.

Respectfully submitted,

Deborah L. Harlow,
Librarian

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1960, amounted to $1,796,571 as against a book value of $1,146,393. This compares with values of $1,786,262 and $1,023,297 at the end of the preceding year. The average yield on the securities was 3.60% of the market value and 5.65% of book value. The total uninvested principal cash in the above accounts as of December 31, 1960, was $255.16. Classification of the Securities held in the Endowment Funds appears in the Auditor's report.

The market value of the pooled securities as of December 31, 1960, was $333,218 with uninvested principal cash of $120.35; the market value at December 31, 1959, being $309,251. The book value of the securities in this account was $274,294 on December 31, 1960, compared with $257,576 a year earlier. The average yield on market value was 3.75% and 4.56% of book value.

The proportionate interest in the Pool Fund Account of the various Funds as of December 31, 1960, is as follows:

Pension Funds	23.979%
General Laboratory Investment	53.681
Other:	
Bio Club Scholarship Fund	1.536
Rev. Arsenius Boyer Scholarship Fund	1.881
Gary N. Calkins Fund	1.760
Allen R. Memhard Fund	.342
F. R. Lillie Memorial Fund	5.933
Lucretia Crocker Fund	6.423
E. G. Conklin Fund	1.088
M. H. Jacobs Scholarship Fund	.774
Jewett Memorial Fund	.572
Anonymous Gift	2.031

The special custodian account yielded an income last year of $9,619 and this amount is being reserved for capital improvements.

Donations from the M. B. L. Associates for 1960 were $4,320 as compared with $4,170 for 1959. Unrestricted gifts from foundations, societies and companies amounted to $18,035.

We are administering 15 grants for investigators in addition to those grants made directly to the Marine Biological Laboratory. The amounts of grants vary in accordance with the investigator's project of research. An amount of 15% based on amount expended is allowed the Laboratory as overhead.

The Lillie Fellowship Fund with a market value of $88,415 and a book value of $92,789, as well as the investment in the General Biological Supply House with a book value of $12,700, is carried in the Balance Sheet, item "Other Investments." The General Biological Supply House for the fiscal year ended June 30, 1960, had a profit after taxes of $314,034 as compared to $303,300 in 1959 and $218,210 in 1958, and $123,430 in 1957. In the fiscal year 1960, the Marine Biological Laboratory received dividends from the General Biological Supply House of $30,480 as against $30,480 in 1959 and $25,400 in 1958.

Following is a statement of the auditors:

To the Trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of the Marine Biological Laboratory as at December 31, 1960 and 1959, the related statements of operation expenditures, income and current fund for the years then ended, and statement of funds for the year ended December 31, 1960. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the account records and such other auditing procedures as we considered necessary in the circumstances.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of the Marine Biological Laboratory at December 31, 1960, and the results of its operations for the year then ended.

Boston, Massachusetts
May 31, 1961

Lybrand, Ross Bros. & Montgomery

James H. Wickersham,
Treasurer

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1960 and 1959

	1960	1959
Investments		
Investments held by Trustee:		
Securities, at cost (approximate market quotation 1960—$1,796,000)	$1,146,393	$1,023,297
Cash	255	2,990
	$1,146,648	$1,026,287
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation 1960, $333,218; less $5,728 temporary investment of current fund cash)	$ 268,566	$ 251,848
Other investments	137,742	132,882
Cash	10,839	13,973
Accounts receivable	21	1,510
	$1,563,816	$1,426,500
Plant Assets		
Land, buildings, library and equipment (note)	$3,280,059	$3,204,017
Less allowance for depreciation (note)	1,142,879	1,109,716
	$2,137,180	$2,094,301
Construction in progress	1,455,811	776,628
Cash	82,042	3,699
Accounts receivable		1,196
U. S. Government obligations, at cost:		
$275,000 Treasury bills, due 1/15/60		273,258
	$3,675,033	$3,149,082
Current Assets		
Cash	$ 77,546	$ 113,588
U. S. Government obligations, at cost:		
$75,000 Treasury bills, due 1/15/60		74,525
Temporary investment in pooled securities	5,728	5,728
Accounts receivable (U. S. Government, 1960, $43,443; 1959, $35,554)	59,889	61,629
Inventories of specimens and Bulletins	47,641	45,400
Prepaid insurance and other	16,778	12,953
	$ 207,582	$ 313,823
	$5,446,431	$4,889,405

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1960 and 1959

Endowment Funds

	1960	*1959*
Endowment funds given in trust for benefit of the Marine Biological Laboratory	$1,146,648	$1,026,287
Endowment funds for awards and scholarships:		
Principal	$ 126,302	$ 126,193
Unexpended income	7,285	5,399
	$ 133,587	$ 131,592
Unrestricted funds functioning as endowment	206,378	206,378
Retirement fund	71,449	61,640
Pooled investments—accumulated gain	5,754	603
	$1,563,816	$1,426,500

Plant Liability and Funds

Funds expended for plant, less retirements	$4,668,475	$3,832,639
Less allowance for depreciation charged thereto	1,142,879	1,109,716
	$3,525,596	$2,722,923
Unexpended plant funds	82,042	276,957
	$3,607,638	$2,999,880
Accounts payable	67,395	149,202
	$3,675,033	$3,149,082

Current Liabilities and Funds

Accounts payable	$ 41,106	$ 46,154
Unexpended research grants	51,726	131,146
Unexpended balances of gifts for designated purposes	9,663	10,799
Current fund	105,087	125,724
	$ 207,582	$ 313,823
	$5,446,431	$4,889,405

Note—The Laboratory has since January 1, 1916, provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

Statements of Operating Expenditures, Income and Current Fund

Years Ended December 31, 1960 and 1959

Operating Expenditures

	1960	*1959 **
Research and accessory services	$ 250,578	$ 222,624
Instruction	219,234	79,396
Library and publications	61,462	58,190
Direct costs on research grants	182,899	221,436
	$ 714,173	$ 581,646
Administration and general	70,037	63,947
Plant operation and maintenance	117,980	113,454
Dormitories and dining	162,713	154,405
Additions to plant from current income	78,654	23,448
	$1,143,557	$ 936,900
Less depreciation included in plant operation and dormitories and dining above but charged to plant funds	48,086	46,604
	$1,095,471	$ 890,296

Income

Research fees	$ 56,408	$ 50,242
Accessory services (including sales of biological specimens, 1960, $48,817, 1959, $66,742)	151,109	141,022
Instruction fees	23,905	21,395
Grants for instruction and research training	185,571	40,478
Library fees, Bulletin subscriptions and other	35,174	25,225
Reimbursements and allowances for direct and indirect costs on research grants	221,197	246,326
Dormitories and dining income	105,086	106,424
	$778,450	$ 631,112
Gifts used for current expenses	48,300	36,590
Grants used for current expenses	143,018	107,500
Investment income used for current expenses	105,066	100,432
Total current income	$1,074,834	$ 875,634
Excess of operating expenditures over current income	$ 20,637	$ 14,662
Current fund balance January 1	125,724	140,386
Current fund balance December 31	$ 105,087	$ 125,724

* 1959 amounts have been reclassified for purposes of comparison.

MARINE BIOLOGICAL LABORATORY

Statement of Funds

Year Ended December 31, 1960

	Balance Jan. 1, 1960	*Gifts and Other Receipts*	*Investment Income*	*Used for Current Expenses*	*Other Expenditures*	*Balance Dec. 31, 1960*
Invested funds	$1,426,500	$ 142,749	$110,875	$103,074	$ 13,234	$1,563,816
Unexpended plant funds	$ 276,957	556,456	9,619		760,990	82,042
Unexpended research grants	$ 131,146	470,366		549,786		$ 51,726
Unexpended gifts for designated purposes	$ 10,799	48,355		48,300	1,191	$ 9,663
Current fund	$ 125,724			20,637		$ 105,087
		$1,217,926	$120,494	$721,797	$775,415	
Gifts		$ 603,616				
Grants for research, training and support		470,366				
Net gain on sales of securities		125,620				
Appropriated from current income and other		18,324				
		$1,217,926				
Expended for construction of new building					$760,990	
Scholarship awards					3,185	
Payments to pensioners					10,049	
Other					1,191	
					$775,415	

MARINE BIOLOGICAL LABORATORY

Summary of Investments of Endowment Funds

December 31, 1960

	Cost	% of Total	Market Quotations	% of Total	Investment Income 1960
Securities held by Trustee:					
General endowment fund:					
U. S. Government Securities	$ 35,164	3.6	$ 36,619	2.5	$ 1,272
Corporate bonds	572,187	59.9	547,653	37.3	18,984
Preferred stocks	84,778	8.9	70,138	4.8	3,370
Common stocks	263,894	27.6	813,572	55.4	29,474
	$ 956,023	100.0	$1,467,982	100.0	$ 53,100
General educational board endowment fund:					
U. S. Government securities	$ 31,060	16.3	$ 32,434	9.9	$ 1,205
Other bonds	94,888	49.8	88,450	26.9	3,480
Preferred stocks	26,745	14.0	24,058	7.3	1,063
Common stocks	37,677	19.9	183,647	55.9	5,972
	$ 190,370	100.0	$ 328,589	100.0	$ 11,720
Total securities held by Trustee	$1,146,393		$1,796,571		$ 64,820
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government securities	$ 1,018	.4	$ 1,048	.3	$ 180
Corporate bonds	149,866	54.6	147,782	44.3	6,575
Preferred stocks	3,214	1.2	3,075	1.0	112
Common stocks	120,196	43.8	181,314	54.4	5,645
	$ 274,294	100.0	$ 333,219	100.0	$ 12,512
Other investments:					
U. S. Government securities	$ 7,000				$ 981
Other bonds	47,971				1,675
Preferred stocks	3,728				131
Common stocks	46,530				31,552
Real estate	32,513				
	$ 137,742				$ 34,339
Total investments of other endowment and restricted funds	$ 412,036				$ 46,851
Total investment income					$111,671
Custodian's fees charged thereto					(546)
Income of current funds temporarily invested in pooled securities					(250)
Investment income distributed to funds					$110,875

REACTION TO INJURY IN THE OYSTER (CRASSOSTREA VIRGINICA)

FREDERIK B. BANG

Marine Biological Laboratory, Woods Hole, Mass., and the Department of Pathobiology, The Johns Hopkins School of Hygiene and Public Health, Baltimore 5, Md.

The comparative approach to pathology, which uses both cold-blooded vertebrates and invertebrates to advantage, was pioneered brilliantly by Metchnikov (1891) and has since been continued, somewhat sporadically, in France (Cantacuzène, 1923) and elsewhere (Cameron, 1932; Schlumberger, 1952). There still remains a tremendous dearth of information concerning the reaction of various invertebrates to injury and infection. A partial exception to this is the study of insect pathology (Steinhaus, 1949).

In a continuing study of pathological processes in the oyster we have found that the initial phase of phagocytosis of bacteria by the oyster amebocyte is often preceded by adhesion of the bacteria to the amebocyte so that the cell surface is literally covered with bacteria, and the sticking may be limited to the contact of the flagellum of the organism with the amebocyte so that the still motile bacterium becomes anchored. Secondly, the classical cellular clot formed by the agglutination of these amebocytes may be accompanied by an extracellular clot which immobilizes bacteria. And finally, the cellular clot may be directly observed within the vascular system of the living oyster and may be produced by the injection of an extract of oyster tissue.

Material and Methods

Most of the experiments were done on a so-called half shell preparation in which, after an edge of shell was knocked off, the shell was pried open with a knife until the adductor muscle was seen; then, with as little trauma as possible, the adductor muscle was cut and the upper shell removed. In good preparations this meant that a portion of the mantle and the muscle was cut, and the pericardium was left intact. Such preparations (Fig. 1) were kept in running sea water and used during the next several days. Some of these lived as long as a week or ten days, but had by that time gradually deteriorated, showing a loss of leucocytes from the blood and progressive infection and disintegration of the muscle. Heart blood was readily obtained from them at any time, and direct examination of the various vessels of the mantle, palps and gills was satisfactory under a Zeiss dissecting microscope (40 ×). Intracardiac injections were usually done directly into the ventricle, and blood was withdrawn from the auricle.

During these operations, the animals must obviously be damaged to a greater degree than were Stauber's preparations (1950) in which a window was made directly over the heart. However, they allowed direct examination of the entire gill and vascular system, and were used only as acute preparations. A limited

number of observations were made on oysters in which a hole was carefully drilled near the pericardium and the shell was then picked away until the sheath was exposed.

Observations on phagocytosis were made with a Zeiss phase microscope both at 500 × and 1250 ×. A drop of freshly obtained blood was placed on a slide, then either a drop of bacterial suspensions from a freshly grown culture of marine bacteria was added to it, or a small portion of the colony itself was added with a loop directly to the drop of blood. The preparation was covered with a #1 cover-

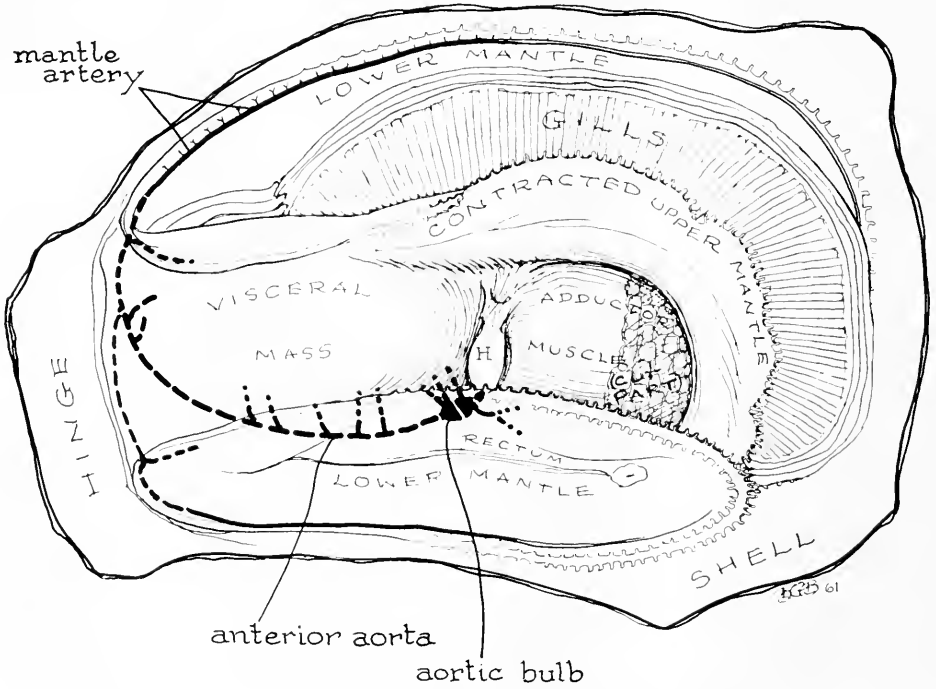


FIGURE 1. Diagram of half-shell preparation of oyster. Circulatory system indicated in heavy black line. Injections were made directly into the heart (H). Observations were made principally on mantle arteries.

slip, and if observations were to be continued, the entire preparation was ringed with Vaseline to prevent evaporation. Amebocytes remained viable for 12 hours or more under these conditions.

RESULTS

Phagocytosis

The amebocyte, which has been extensively studied in this and other molluscs (Fauré-Fremiet, 1927), is a granular round cell floating freely in the blood stream. On contact with glass it flattens out and moves continually over the surface of the slide. This motion under phase may clearly be seen to begin by the extrusion of a

series of filamentous pseudopodia which may be resolved with high power phase microscopy (Fig. 2) and which is shown in the accompanying electron microscope pictures. The spread of ectoplasm, illustrated in the accompanying figures (Figs. 3, 4, 5), then flows afterward, filling up the spaces between. Granules and other portions of the cell then flow into this region. A variety of cell forms may be observed on these slides; some of them lack granules, others contain large wavy frills of pseudopodia, other large amorphous but refractile inclusions. Since the amebocyte may both lose its granules and may ingest large amounts of material, we are unable to say whether these represent different types of cell or physiological variants of one type. Most of the cells observed during the process of phagocytosis were granular cells.

One of the most remarkable facts which was observed early in the study was the absence of phagocytosis. Frequently an amebocyte was seen to approach a bacterium with its fibrous processes, then either to reverse its flow or turn aside. During the course of several hours this behavior was repeated continuously and no phagocytosis was observed. Since it is so obviously contrary to established ideas of the importance of phagocytosis, and specific studies on phagocystosis of food particles by the oyster, the observations were repeated with a number of bacteria, and it was found that excellent phagocytosis might be obtained with a certain bacterium, yet little if any phagocytosis was observed in amebocytes from the same oyster if another preparation of bacteria was introduced. Repeated attempts were made to determine whether such failure of phagocytosis was due to the strain of bacteria, or to a combination of certain bacteria with amebocytes from certain oysters. It was not possible from day to day to find a combination of amebocytes and bacteria which did not phagocytize, but no observations were repeated within a few hours of each other and it remains likely that there is an undiscovered factor important in phagocytosis which is responsible for this variation.

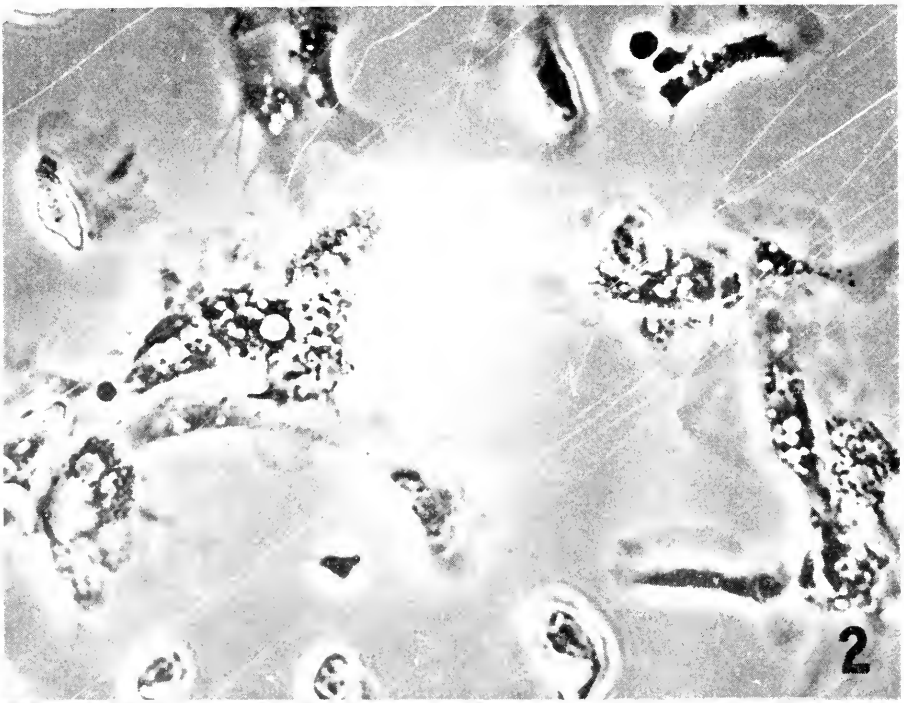
When a successful combination was obtained, and particularly when the bacterium used was motile, phagocytosis was usually preceded by a massive sticking of the bacteria to the amebocyte, so that the amebocyte resembled a porcupine. Ingestion followed this phase (Fig. 4). Some incidental observations on phagocytosis by amebocytes of the marine worm, *Urechis,* showed the adhesion of bacteria to be limited to the portion of the cell which had spread out on the glass.[1] In the oyster, however, since extrusions of the cell appeared from all sides it was not possible to determine whether all portions of the amebocyte were equally sticky.

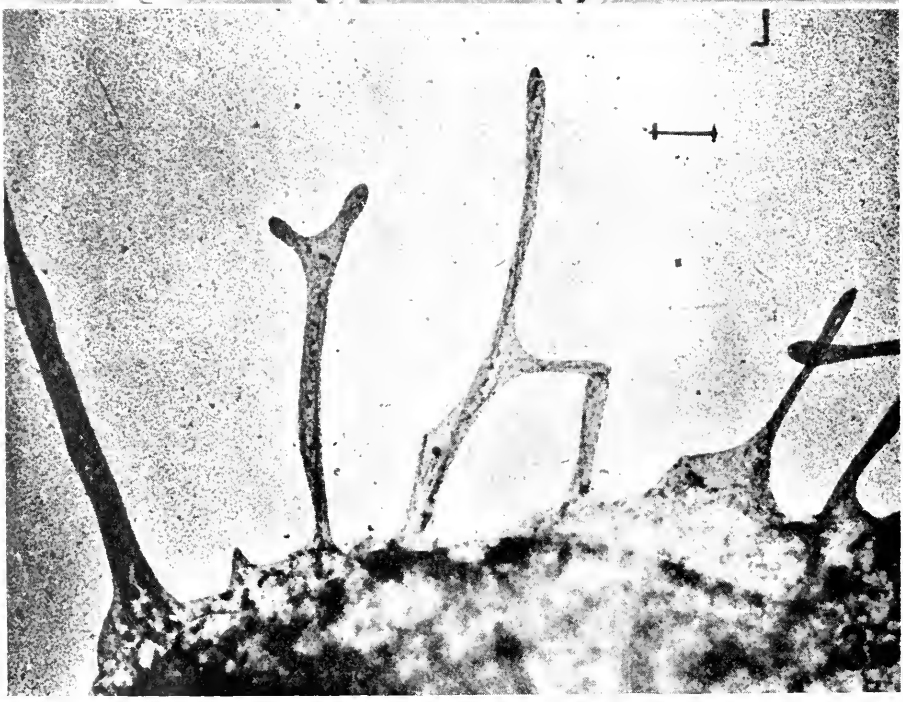
The curious anchoring of motile bacteria to amebocytes which renders them unable to leave the amebocyte while they seem at the same time to have no contact with it, was explained by a fortunate electron microscope picture (Fig. 5). The presence of the unipolar flagellum wrapped around the filamentous pseudopodia fully explains the continual tugging and jerking at an invisible anchor.

Extracellular clot formation

During the summer of 1956, and in the two succeeding summers, the formation of a definite extracellular clot was observed (Fig. 6). It could be seen only under phase microscopy and seemed similar in texture and formation to a clot described

[1] These observations were made originally by Mr. Stuart Krassner, to whom we are indebted for permission to include this material.





FIGURES 2–3.

by Grégoire (1952) in a variety of insect bloods. The clot was found in only about one-third of the oysters which were examined, and was not present in the same oyster at all times. Furthermore, we have been unable to determine the conditions under which it may be consistently formed in an individual oyster. However, we were able to reproduce this extracellular clot throughout the summer months of the last three years, and have been clearly able to rule out artifacts of preparation.

The clot was first noticed in slides which had been kept for continued observation of phagocytosis. It tended to occur near the occasional small air bubble which was entrapped beneath the coverslip in the sealed slide. It was not present immediately but usually appeared in 15 minutes to half an hour, so that it was suspected of being a local drying phenomenon until it was seen in slides which had no appreciable air bubbles, and in sealed hanging drops and direct preparations. Attempts to relate it to the time of day, time since opening of the oyster, amount of trauma, and sex of oyster, failed. A mixture of pericardial fluid and blood did not affect the process. A higher percentage of extracellular clots seemed to be obtained from oysters which had been forced-starved by keeping them out of the sea water, and then replacing them; but this produced positive results in only about half of the cases. Since the clot had been originally observed in a preparation to which bacteria had been added, repeated comparisons were made in the presence and absence of the bacteria. In most cases, when the clot was obtained in the presence of the bacteria, it was also found in the control slide to which bacteria had not been added, though it was usually less extensive.

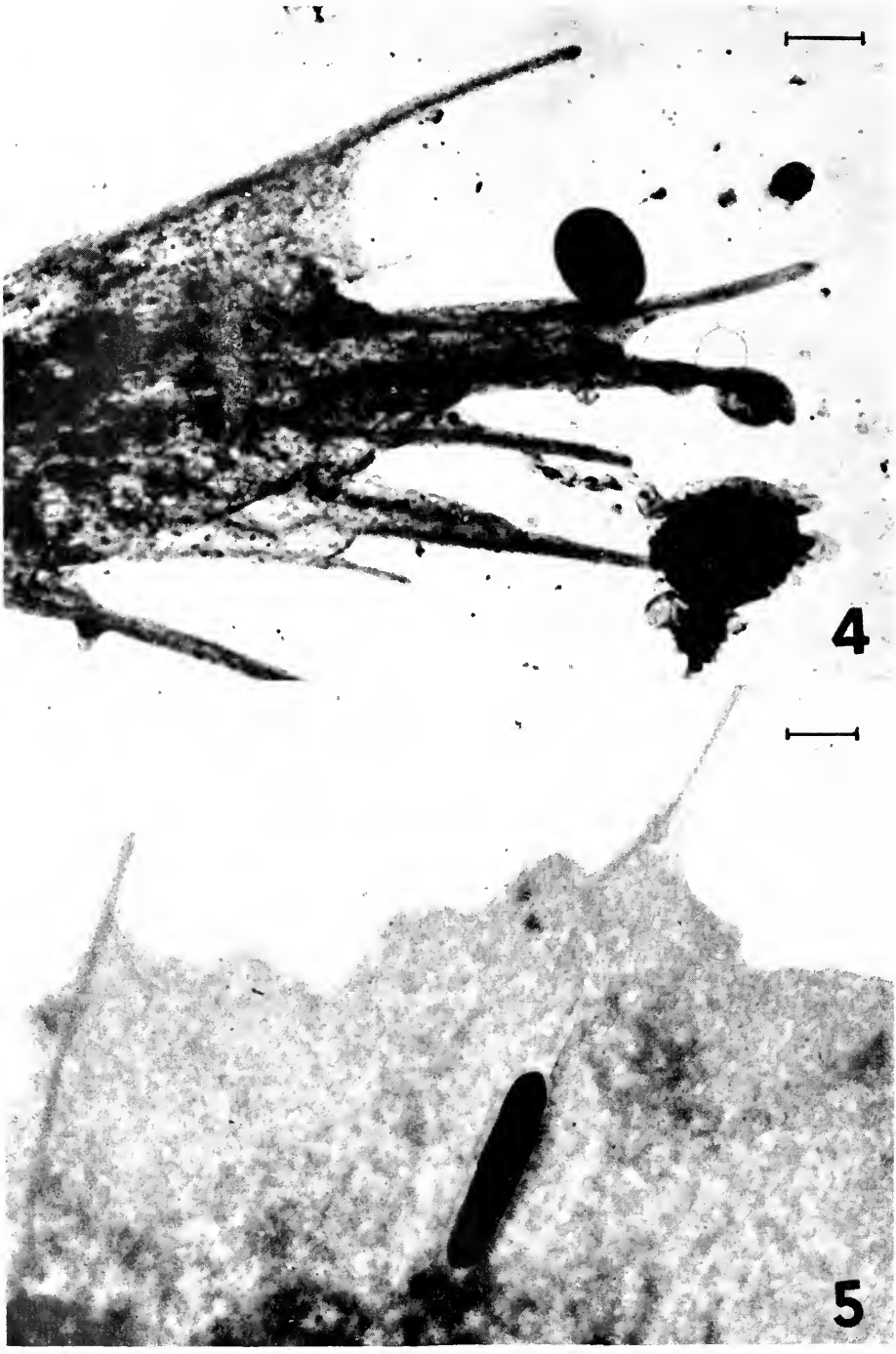
It seems to have a real rôle in the repair of traumatized tissue, for: 1) it was found already developing in small cellular clots taken directly from the heart; 2) it occurred predominantly around clumps of cells; 3) bacteria were immobilized by its development (Fig. 7); and, 4) it was obtained both immediately after opening an oyster and from some preparations which had been on the half shell as long as 24 hours. Its possible relation to cycles of feeding by the amebocytes is unknown.

Intravascular clots

There is a rapid clumping of cells when oyster blood is withdrawn in glass vessels, which is also the case with many other invertebrate bloods. Direct observation of traumatized blood vessels shows the formation of the same type of cellular clot at the open end of the vessel, so that within a few minutes of cutting, the clotted cells have effectively sealed the end. Similar clumps of amebocytes are observed directly covering the cut end of the adductor muscle in our preparations, and oysters which had been repeatedly bled develop a shaggy pericarditis which consists of masses of these clumped cells. However, in oysters which have had minimal trauma and have remained in clean running sea water for several hours after being opened, the circulation may be fully effective and direct examination of the distended vascular system was possible. In such a view, the cells are seen moving to and fro,

FIGURE 2. Phase micrograph of oyster amebocyte on glass, approximately 600 times.

FIGURE 3. Electron micrograph of whole cell preparation of amebocyte. Filamentous pseudopods extend away from the cell edge. This and succeeding electron micrographs were made by allowing the amebocytes to spread out on a collodion film. The cells were fixed with osmium vapor, washed and the film placed on grids; 9,000 times.



FIGURES 4–5.

and relatively few of them are clumped. When the circulation is sluggish they may be found lining the lower side of a vessel, but they readily move from one portion to another as the oyster is tilted. The obvious question whether a particular portion of the tissue was responsible for the formation of the cellular clot was tested by making a sea-water extract of gill tissue, centrifuging the extract and injecting about 0.1 cc. of the relatively clear supernatant directly into the heart. The material immediately spread throughout the animal and an interesting series of events set in. If the oyster had relatively large numbers of cells so that the blood was milky in appearance, the first reaction was the formation of large curd-like clumps of loosely aggregated cells. These became more dense, soon ceased to flow back and forth, and within 10 to 15 minutes were stuck in tight clumps to the edge of the vessel, and the fluid itself appeared perfectly clear. The vessel frequently decreased in size, particularly if the heart happened to cease beating. In many cases, some flow back and forth in the mantle vessels continued even though there was no visible heart beat, presumably from the action of the accessory heart (Fig. 1). Within about two hours after the injection, most of the effects had worn off: the heart was beating, the blood was again flowing freely, and relatively few clumps were seen. Individual cells were observed flowing freely in the large vessels or moving in and out of the fine branches of the palps or the gills. When these oysters were reinjected with the original extract, an apparently full-fledged repetition of the reaction was observed.

The reaction was not obtained by the injection of sea water, of suspensions of carmine, or of bacteria of several sorts, though a moderate "curdling" of the blood was seen after the injection of heavy suspensions of bacteria.

India ink of two sorts was then injected in suspensions of sea water. The usual preparation of colloidal ink when injected caused prompt clumping of cells, a cessation of heart beat, and the probable development of intravascular clumps like those seen following the injection of tissue extracts. However, the black masses of material which were partially phagocytized, as described by Stauber (1950), obscured the observation. A preparation of "Pelican" India ink, which lacks the gum coating present in most commercial India inks, produced a much milder reaction (Muller, 1927a, 1927b). The particles were soon phagocytized as small particles or clumps without major changes in the circulation itself, just as carmine particles had been.

Discussion

The capacity to react to injury, an essential function of living cells, is basic to studies in pathology. Following Metchnikov (1891), who began with a marine echinoderm embryo, the greatest attention of pathologists when studying invertebrates has concentrated on the wandering cells or amebocytes. From a comparative pathological point of view, at least three phenomena are contained in the oyster in this one cell. These are phagocytosis of invading bacteria, inflammation, and thrombosis. Since the amebocyte is the only circulating cell of the blood in the oyster, and since cellular clots are the common mechanism of closing gaps in the

Figure 4. Beginning phagocytosis of uniflagellate bacterium. The flagellum is coiled around several pseudopods of the amebocyte; 14,000 times.

Figure 5. An electron micrograph showing later stage in phagocytosis of bacterium; 9000 times.

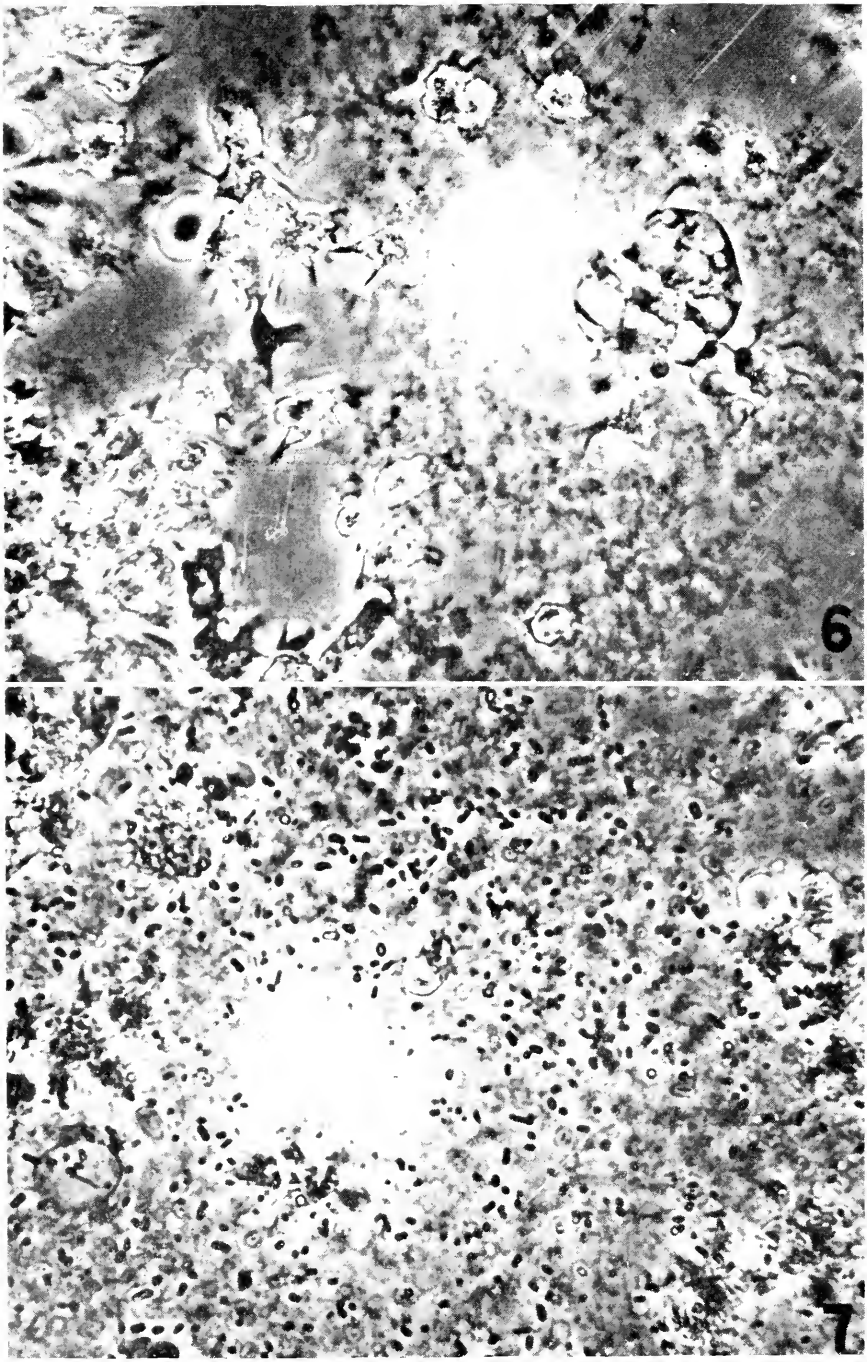


FIGURE 6. Phase micrograph showing extracellular clotting of amebocytes; 1000 times.
FIGURE 7. Similar extracellular clot with bacteria involved in clot; 1000 times.

vascular system among invertebrates (Geddes, 1880; Cuénot, 1891), it is of course impossible to separate the function of inflammation whereby a white cell in vertebrates becomes adherent to a vessel wall and migrates through it, and that of the adherence of many amebocytes together to form a tight clump which blocks the free flow of blood.

An ideal invertebrate in which to follow the above processes would allow direct observation of the vascular channels without trauma, and would from the bacteriological point of view allow for external sterilization and thus obtaining of blood without contamination by the surrounding fluid or air. In this regard the oyster and other molluscs have no external surface which may be sterilized and then punctured, and have no extension of the vascular system that may be observed without the introduction of trauma. Thus, though a variety of studies have been done on diseased oysters (Herdman and Boyce, 1899; Roughley, 1926; Stauber, 1945; Mackin, 1951; Mackin, *et al.,* 1952), there is little direct information on the pathogenesis of any of the disease states.

Phagocytosis of food material for transport through the oyster, and of particulate matter has been studied rather extensively (Yonge, 1926; Takatsuki, 1934), primarily by following the events in sequence by histological sections.

Phagocytosis itself was first observed about a hundred years ago, by Haeckel (1862), who injected particulate dyes into molluscs so that the distribution of the vascular system might be determined. He pointed to the potential importance of the phenomenon in nutrition. Then came the disclosure by Metchnikov of the role of phagocytosis as a defense mechanism (1884, 1891). In the oyster and other molluscs the importance of the amebocyte in digestion, transfer of food, and repair, has been firmly established (Yonge, 1928; Wagge, 1955). Recently Tripp (1960), has followed the fate of several species of bacteria in oyster tissue following intracardiac injection of large numbers. He has shown that phagocytosis may be apparent in the cells circulating within the vessels and subsequently in the tissues. Intracellular digestion appeared to be a major mechanism of disposal of the bacteria. In several infectious diseases of the oyster the presumptive agent is thought to be disseminated by the amebocyte (Orton, 1923). It was therefore a surprise to us to find that there was a marked variation in the phagocytosis of different preparations of bacteria by leucocytes of the same oyster. Attempts to show increased phagocytosis in the presence of mucus from the gill, of disintegrating crystalline style material, or of extracts of the hepato-pancreas failed.

Although "surface phagocytosis" (Wood, 1951–1952) took place in the process of the flow of amebocytic protoplasm around bacterium, it was not always the explanation, for masses of bacteria were found stuck to the surface of amebocytes in most of the cases where phagocytosis was apparent. The direct adherence of the amebocyte to the bacterium itself was highlighted by the observation of the flagellar adherence of the bacteria to the amebocyte so that it was unable to escape from the amebocyte.

The evolutionary need for extracellular clot formation becomes greater when the amebocytes or leucocytes have much less direct contact with each other because of the presence of large numbers of red cells. However, extracellular clot or gel formation is well developed in several invertebrates (Grégoire and Florkin, 1950; Loeb, 1910; Grégoire, 1952; Bang, 1956) in which the predominant circulating

cells are directly involved in clot formation (Yonge, 1926). The presence of this extracellular gel, which seemed fully able to limit bacterial motion in many of the oysters which we examined, may indicate that additional advantage is to be gained from such mechanisms of thrombosis which extend beyond the cell. The possible role of this extracellular material in rendering bacteria more susceptible to phagocytosis needs further study. The origin of this extracellular gel from the extrusion of the many cellular granules is an obvious possibility which has not been investigated.

Direct observations of the formation of the cellular clot at a point of traumatic rupture of a vessel, the accumulation of great numbers of these cells on the heart when it is exposed to sea water by opening the pericardium, and the accumulation of amebocytes at the cut edge of the adductor muscle, led to the question as to the effect of tissue extracts. It was soon found that a fresh crude sea-water extract of ground gill tissue, when injected directly into the heart, caused a rapid clumping of cells and the tight adherence of these cells to the vessel wall, so that the circulation was greatly slowed or stopped. Injection of sea water, of bacterial suspensions, and of carmine, failed to cause similar marked effects. Thrombosis accompanied by phagocytosis was rapidly produced by the injection of certain preparations of

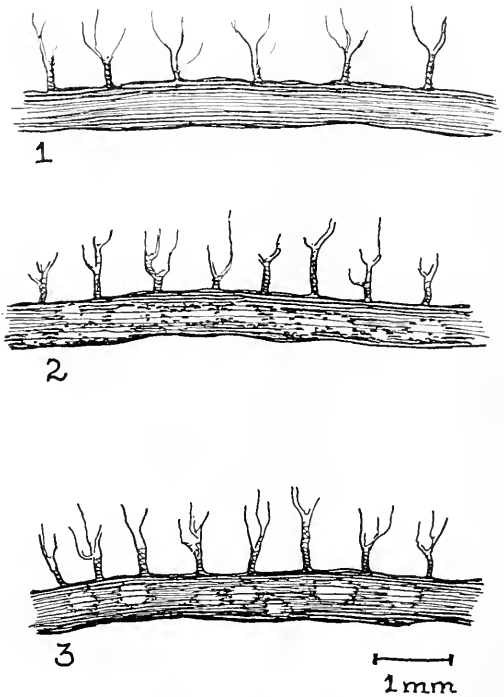


FIGURE 8. Diagram of observations of mantle artery: (1) shows the clear appearance of the vessel under normal conditions. Individual amebocytes may be seen poorly and are not indicated here. (2) Beginning clumping amebocytes within the vessel. They are loosely clumped and move rather freely in the vessel. (3) Amebocyte clumps which have contracted into tight balls of thrombus and are adherent to the vessel wall.

India ink, but not by a preparation which is stated to lack the shellac coating which in itself causes extensive thrombosis.

Our experiments have been limited to acute short term experiments. Several other molluscs have been used in the study of chronic processes (Drew and de Morgan, 1910; Zawarzin, 1927), and the importance of epithelial tissues, mucous sheets and chronic fibrous tissue "repair" needs extensive exploration (Kedrowsky, 1925; Labbe, 1929).

Summary

1. *In vitro* phagocytosis of marine bacteria by fresh oyster leucocytes, though readily demonstrable in most cases, was by no means an invariable phenomenon. When it occurred, it was frequently accompanied by a massive sticking of bacteria to the leucocytes. The flagellar portion of the bacterium might be so caught by the amebocyte that the bacterium was unable to escape, even though the body was not in contact with the amebocyte.

2. An irregular but repeated formation of an extracellular clot is described as seen *in vitro* by phase microscopy. Reasons for believing that it is a true phenomenon in the oyster are given.

3. Intravascular clotting or thrombosis was produced by the intracardiac injection of tissue extracts. The clotting disappeared spontaneously within two hours after the injection.

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THE OBLIGATE COMMENSAL CILIATES OF STRONGYLOCENTROTUS DRÖBACHIENSIS: OCCURRENCE AND DIVISION IN URCHINS OF DIVERSE AGES; SURVIVAL IN SEA WATER IN RELATION TO INFECTIVITY

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Seven species of ciliated protozoa have been reported from the alimentary tract of the sea urchin *Strongylocentrotus dröbachiensis* (O. F. Müller) in the coastal waters of Mt. Desert Island, Maine (Powers, 1933a). Three of them are holotrichs which have no known free-living congeners and are restricted to echinoid hosts. They are *Entodiscus borealis* (Hentschel, 1924) Madsen, 1931; *Madsenia indomita* (Madsen, 1931) Kahl, 1934; and *Biggaria gracilis* (Powers, 1933) Kahl, 1934. In the words of Kirby (1941, p. 921), such ciliates "may be supposed to have evolved in the shelter of these hosts" and they are thus regarded as obligate commensals. The relation of the remaining four to their host is not entirely clear, owing to inadequate study. Powers (1933a, p. 119) regards them "as chance or vagrant ciliates, which, after being engulfed with food, are able to survive" and multiply as entozoic commensals. Two of the four are holotrichs, namely, *Plagiopyla minuta* Powers, 1933, and *Cyclidium stercoris* Powers, 1935; one is a hypotrich which Beers (1954) identified as *Euplotes balteatus* (Dujardin, 1841) Kahl, 1932, and the final and least common is an undetermined species of the peritrich *Trichodina*. Reference may be made to Beers (1948) for further details concerning the taxonomy of the ciliates. In order to avoid the constant repetition of the unwieldy binomial *Strongylocentrotus dröbachiensis,* the terms "urchin" and "urchins" are substituted in the following pages and refer without exception to this echinoid.

To return to the three obligate commensals, which are the subject of the present study, Power (1933a) notes that adult urchins at Mt. Desert Island are almost invariably infected with them and indeed may harbor them in almost incredible abundance. In the summer of 1947, Beers (1948) extended Powers' investigations by making a quantitative study of the occurrence and morphogenetic condition of the ciliates in 182 urchins, the tests of which varied in diameter from 30 to 60 mm. All the urchins were infected with *E. borealis* and *M. indomita,* and 181 of them with *B. gracilis*. Counts of the ciliates in fresh samples of enteric fluid showed that the vast majority of the urchins harbored infections of each species that varied in intensity from "moderate" (M) to "heavy" (H), M meaning 50–500 individuals of the species per 0.1 ml. of fluid and H meaning 500–1000 or more per 0.1 ml. The remaining infections were designated as "light" (L), meaning fewer than 50 individuals of a species per sample. A regional distribution of the ciliates was also

noted, in that *E. borealis* occurred primarily in the stomach (inferior spiral or intestine of some authors), *M. indomita* in the intestine (superior spiral or large intestine), and *B. gracilis* in the rectum. However, the foregoing distribution of *E. borealis* and *M. indomita* prevailed as a rule only in well-fed urchins; in inadequately fed urchins they tended to shift aborally and in extreme cases of hunger to commingle with *B. gracilis*. The factors that were responsible for the regional distribution of the ciliates were unexplained.

In any flourishing population of ciliates, whether free-living or associated in any way with a host, one might reasonably expect to find at almost any time a significant percentage of individuals that are dividing. It is therefore remarkable that dividing specimens of *E. borealis* and *M. indomita* are extremely rare, even in ciliate populations of great density. With reference to the division of *E. borealis,* Powers (1933b, p. 130) comments as follows: "A study of about 600 specimens fixed during the day gave but three individuals showing any signs of fission." In 1947 the writer made a special effort to find dividing specimens of *E. borealis* and *M. indomita* in the 182 urchins that have been mentioned. The urchins were collected and examined without delay at practically all hours of the day and night, but only six of them revealed dividing individuals of *E. borealis*. Concerning *M. indomita,* neither Madsen (1931) nor Powers (1933a) mentioned its division, and the task of finding dividing specimens was especially difficult. In the 88 urchins that were examined in July, only one dividing individual was found; in 94 studied in August, dividing specimens were found in only three. It was concluded that division in *E. borealis* and *M. indomita* was a periodic phenomenon: that long intervals of non-divisional life alternated with brief periods of intense divisional activity. In retrospect it became apparent that both Powers and the writer, in their efforts to find dividing ciliates, inadvertently restricted their studies to relatively large mature urchins, in which the ciliate populations were already well-established and probably somewhat stabilized. It will be seen in the following pages that when some of the younger urchins are examined, divisional stages can be found in abundance. Turning finally to *B. gracilis,* it was evident that this ciliate differed markedly in its reproductive activities from the preceding two. Of the 181 infected urchins, all contained dividing specimens, and there was thus no evidence of long periods of non-divisional life.

The relation of the size of the urchins to the condition of their respective ciliate infections was not considered in the earlier study (Beers, 1948). Actually, there is often great diversity of size in an aggregation of urchins on a rocky ledge or in a tide pool. For example, urchins taken by the writer from a single tide pool at Long Ledge, Mt. Desert Island, on July 10, 1960, varied in diameter from 8 to 65 mm. To some extent these differences merely reflected different rates of growth, but Grieg (1928) concluded that size (diameter of test) is a fairly reliable measure of the age of the urchins. Basing his studies on urchins taken from the Folden Fjord and the Bals Fjord of Norway, and on other materials, he concluded that the following relations of size to age prevail, at least in a general way: diameter 0.5 mm., metamorphosis just completed; 1–2.5 mm., "the same year-group" as the foregoing, meaning urchins in their first summer of life; 5–6 mm., about 1 year old; 15 mm., about 2 years old; 24 mm., 3 years old; 40 mm., 4 years; 50 mm., 5 years; 60 mm., 6 years; 78 mm. (the largest specimen), "probably about 8 years

old." In the region of Mt. Desert Island, the spawning of urchins begins in February and ends in April. Since the urchins of northern Europe have a similar spawning period (Mortensen, 1943, p. 211), there is little doubt that Grieg's estimates of age are equally applicable to Mt. Desert Island urchins.

The present paper is a record of further observations on *E. borealis, M. indomita* and *B. gracilis* as found in 152 urchins taken at Mt. Desert Island in the summer of 1960. It is based on seven collections or small populations of urchins, each of which consisted of specimens of as many different sizes (age-groups) as were available at the respective sites of collection. The study concerns in particular the following aspects of the biology of the ciliates.

(1) Their occurrence and morphogenetic condition (whether dividing or not) in "small" urchins, meaning urchins 8–14 mm. in diameter and presumably about 1.5 years old. (A minimal size of 8 mm. was fixed solely by the unavailability of any urchins of smaller size.) This aspect attempts to answer these questions: At what age do urchins become infected with the respective ciliates? Once established in the urchin, do the ciliate populations build up immediately or does a delay ensue following their ingestion by the host?

(2) Their occurrence and morphogenetic condition in "larger" urchins, meaning urchins 15–65 mm. in diameter and representing five age-groups, namely, 2.5 to 6.5 years, in increments of one year. This aspect attempts to answer these questions: Do the infections become progressively more intense (ciliates more plentiful) as the urchins increase in age? Is the division of the ciliates, in particular that of *E. borealis* and *M. indomita,* correlated in any way with the age of the urchins?

(3) Their morphogenetic condition throughout a population of urchins. That is to say, does division of the ciliates occur simultaneously in all the urchins of a population or does it affect only certain age-groups or random individuals?

(4) Their capability to survive in sea water outside the body of the urchin, bearing in mind that cysts are unknown in all echinoid ciliates and that young urchins undoubtedly become infected by the ingestion of the usual trophic forms; thus, such survival affects directly their transmission from urchin to urchin.

Material and Methods

Of the seven collections of urchins, three were taken at low tide from the rocks of Emery Cove Ledge on July 2, 13 and 24. The remaining four were taken from four different tide pools at Long Ledge on July 10, August 8 and 26, and September 1. Each collection consisted of about 40 individuals. Of the urchins of each collection, 10 to 15, representing as many age-groups (sizes) as were available, were opened and examined without delay on the day of collection, and a like number was examined on the following day. The remaining ones were excluded from consideration, since it seemed advisable to use only urchins that were relatively recently collected.

The total number of individuals of each species of ciliate was actually counted in the small urchins, but this procedure was usually impracticable with reference to larger urchins, in view of the enormous numbers of ciliates in them. Thus, 0.05-ml. or 0.1-ml. samples of enteric fluid were taken from these urchins, and

the number of ciliates of each species was estimated in the samples. If the size of the urchin permitted, five 0.1-ml. samples were taken from the stomach, five from the intestine, and two from the rectum. In 0.1-ml. samples, the three degrees of infection that have been defined were again distinguished with reference to each species. In 0.05-ml. samples, half the aforementioned numbers of individuals was employed to distinguish the respective degrees of infection.

With reference to the survival of the ciliates in pure sea water, details of the procedure will follow.

Results

1. Occurrence and morphogenetic condition of the ciliates in small urchins (diameter of test, 8–14 mm.)

Unfortunately, only nine urchins of this size were available for study. Nevertheless, it is believed that they furnish information that is significant (Table I).

Table I

Total numbers of ciliates of three species in each of nine small urchins (age about 1.5 years) taken at Long Ledge, Mt. Desert Island, in 1960

Diameter of test in mm.	Date collected	*Entodiscus borealis*	*Madsenia indomita*	*Biggaria gracilis*
8	July 10	0	0	0
8	August 26	0	0	0
8	September 1	0	0	0
9	July 10	3	0	1
9	August 26	9	12	2
9	September 1	1	8	0
12	August 8	10	8	1
13	August 26	26	15	4
14	July 10	9	28	2

Since urchins attain a diameter of 5–6 mm. at the end of one year and of 15 mm. at the end of two years, it is assumed that these nine urchins emerged as plutei in February or March of 1959 and were thus about 1.5 years old in the summer of 1960.

A very careful examination of the contents of the digestive tracts of three 8-mm. urchins revealed no ciliates whatsoever, although the digestive tract of each was well filled with algal food. A similar examination of three 9-mm. urchins that were collected on the same dates as the foregoing revealed only small numbers of ciliates, though *M. indomita* was absent in one of them and *B. gracilis* in another. None of the ciliates was dividing. Evidently these three urchins, at the time in their second summer of life, were in the process of acquiring their respective ciliate infections. Finally, an examination of three urchins that had diameters of 12, 13, and 14 mm., respectively, showed ciliates of all three species in each urchin. On the average, these urchins contained two to three times as many individuals of each species as the 9-mm. urchins, even though the infections with *B. gracilis* were extremely light. Again, no dividing specimens were observed.

2. Occurrence and morphogenetic condition of the ciliates in larger urchins (diameter of test, 15–65 mm.)

Urchins 15–23 mm. in diam. Twelve urchins of this size, assumed to be about 2.5 years old and thus in their third summer of life, were available for study. Whereas the urchins of the preceding age-group (1.5 years) were either uninfected or at best only lightly infected, all the urchins of the present group were infected with the three ciliates, and about half of the infections qualified either as moderate or heavy. The status of the respective infections in the 12 urchins was as follows: *E. borealis,* 1 H, 5 M, 6 L; *M. indomita,* 1 H, 4 M, 7 L; *B. gracilis,* 6 M, 6 L. Thus, a marked increase in the intensity of infection was clearly demonstrable in the 2.5-year-old urchins. It is evident that such an increase could have come about either by the ingestion of additional individuals or by the division of those already ingested. Manifestly, no comment can be made concerning the ingestion

TABLE II

Incidence of division of three species of ciliates in urchins of five different age-groups taken at Mt. Desert Island, summer 1960. All the urchins were infected with the three species.

Number of urchins examined	Diameter of test in mm.	Approximate age of urchins in years	Number (and percentage) of urchins that contained dividing ciliates of species indicated		
			Entodiscus borealis	*Madsenia indomita*	*Biggaria gracilis*
12	15–23	2.5	9 (75.0)	7 (58.3)	12 (100)
32	24–39	3.5	2 (6.3)	1 (3.1)	32 (100)
35	40–49	4.5	2 (5.7)	2 (5.7)	35 (100)
37	50–59	5.5	3 (8.1)	3 (8.1)	37 (100)
27	60–65	6.5	2 (7.4)	1 (3.7)	27 (100)

of ciliates during the one-year interim, but it is significant that a remarkably high percentage of the 2.5-year-old urchins contained dividing individuals, showing conclusively that the respective ciliate populations were undergoing rapid augmentation by binary fission. The data concerning division in these urchins are summarized in Line 1 of Table II, reference to which shows that *E. borealis* was dividing in 9 of the 12 urchins, *M. indomita* in 7 of them, and *B. gracilis* in all of them. Furthermore, dividing specimens were relatively abundant, the incidence amounting to about one in every 25–50 individuals of each species. With reference to the division of *E. borealis* and *M. indomita,* it may be said now for purposes of emphasis that in none of the remaining age-groups was there such a high percentage of urchins that contained the two ciliates in division. Concerning *B. gracilis,* it has been pointed out that this ciliate differs in its reproductive activities from the aforementioned two, in that long periods of non-divisional life are absent. Thus, *B. gracilis* was dividing in all 12 urchins.

Urchins 24–39 mm. in diam. Urchins of this size, assumed to be about 3.5 years old, were available in almost unlimited numbers at both collecting sites, as were indeed those of all succeeding age-groups. Of 32 urchins of this size that were examined, all were infected with the three ciliates, as were all the urchins of the age-groups subsequently to be discussed. The respective degrees of infec-

tion among the 32 hosts were as follows: *E. borealis,* 20 H, 10 M, 2 L; *M. indomita,* 18 H, 11 M, 3 L; *B. gracilis,* 7 H, 23 M, 2 L. In terms of percentages, 91 to 94% of the urchins harbored infections of each species that qualified as moderate to heavy. Thus, these urchins were distinctly more heavily infected than those of the preceding two groups, and it will be seen, when older age-groups are considered, that the infections had now attained their maximal intensities.

The findings relative to division are summarized in Table II, Line 2. Of the 32 urchins, only two contained dividing specimens of *E. borealis,* and even in them division was somewhat sparse and affected no more than one specimen in every 100. In spite of an exceptionally thorough examination of the samples, dividing individuals of *M. indomita* could be found in only one of the urchins (a different one from the foregoing two). In accordance with expectations, *B. gracilis* was dividing in all the urchins of the group.

Urchins 40–49 mm. in diam. Thirty-five urchins of this size, assumed to be about 4.5 years old, were examined. The respective degrees of infection follow: *E. borealis,* 15 H, 17 M, 3 L; *M. indomita,* 17 H, 16 M, 2 L; *B. gracilis,* 5 H, 27 M, 3 L. Again, 91 to 94% of the urchins harbored infections that varied from moderate to heavy. Data relative to the occurrence of division are summarized in Table II, Line 3, where it is seen that only two urchins contained dividing specimens of *E. borealis* and a like number (actually another two) those of *M. indomita.* All contained dividing specimens of *B. gracilis.*

Urchins 50–59 mm. in diam. An examination of 37 urchins of this size, assumed to be about 5.5 years old, yielded the following degrees of infection: *E. borealis,* 12 H, 22 M, 3 L; *M. indomita,* 19 H, 16 M, 2 L; *B. gracilis,* 12 H, 21 M, 4 L. With reference to each of the species, 90 to 95% of the urchins had infections that varied in intensity from moderate to heavy. The data concerning the incidence of division, summarized in Table II, Line 4, show that three of the urchins had dividing specimens of *E. borealis* and three (one of the foregoing plus two others) had *M. indomita* in division. As usual, all the urchins contained dividing forms of *B. gracilis.*

Urchins 60–65 mm. in diam. Twenty-seven urchins of this size (age about 6.5 years) were examined. Their respective degrees of infection were the following: *E. borealis,* 13 H, 12 M, 2 L; *M. indomita,* 15 H, 11 M, 1 L; *B. gracilis,* 9 H, 16 M, 2 L. Again, with reference to each species, moderate to high infections comprised more than 90% of the total. Of the 27 urchins, two harbored divisional stages of *E. borealis* and a third one contained *M. indomita* in division (Table II, Line 5), whereas *B. gracilis* was dividing, as expected, in all of them.

3. Morphogenetic condition of the ciliates in the respective collections of urchins

It has been mentioned that the urchins of the present study comprised seven collections, each of which may be regarded as a small population; each at least is believed to be a fairly representative sample of a natural population. And it has been shown, within the limits of the available material, (1) that urchins 8–14 mm. in diameter (age 1.5 years) may or may not be infected, but that if infected, they contain no dividing ciliates (Table I); and (2) that all urchins 15–23 mm. in diameter or larger (2.5 years of age or older) are infected with the ciliates, that *B. gracilis* is constantly dividing in all of them, but that *E. borealis* and *M. indomita*

can be found in division in only a limited, though variable, number of them (Table II). However, the data concerning the division of *E. borealis* and *M. indomita* in certain urchins of ages 2.5–6.5 years, as presented in Table II, tell nothing about the distribution of these particular urchins in the respective collections or population samples. Thus, one may ask: If *E. borealis* and *M. indomita* are dividing in most of the urchins of one age-group of a collection—for example, the 2.5-year group—are they also dividing in a like percentage of urchins of the remaining age-groups of the same collection?

This aspect can be adequately presented by considering in detail the composition of two typical collections of urchins of ages 2.5–6.5 years and the condition of the two ciliates therein. The collections are those taken at Long Ledge on July 10 and August 8. The results are presented in Table III, in which the left column under the headings beginning "No. of urchins" refers to the collection of

TABLE III

Incidence of division of two species of ciliates in two collections of urchins taken at Long Ledge, Mt. Desert Island, on July 10 and August 8, 1960. All the urchins were infected with both ciliates.

No. of urchins examined		Range in size of urchins in mm.	Approximate age of urchins in years	No. of urchins that contained dividing ciliates of species indicated			
				Entodiscus borealis		*Madsenia indomita*	
5	4	15–23	2.5	4	3	4	3
5	5	24–39	3.5	1	1	0	0
5	5	40–49	4.5	0	0	2	0
5	5	50–59	5.5	2	0	1	1
5	4	60–65	6.5	0	1	1	0

July 10, the right to that of August 8. Line 1 shows that five urchins of the size and age indicated were taken on July 10 and four on August 8. Of the five, four contained *E. borealis* in division and four had *M. indomita* in division. (Three of the five contained dividing individuals of both species.) Of the four urchins taken August 8, three had *E. borealis* and three had *M. indomita* in division. (Two had both species.) If the nine urchins are considered as a group, seven of them or 77.7% contained dividing individuals of *E. borealis* and seven contained *M. indomita* in division.

What was the condition of the two ciliates in the remaining age-groups of the two collections? Was division as widespread in the urchins of these groups? The answer is conclusively in the negative, as shown in the remaining four lines of Table III. For example, Line 2 shows that five 3.5-year-old urchins of each collection were examined. Only one urchin of each collection contained *E. borealis* in division; in none of the ten was *M. indomita* dividing. The urchins of the remaining age-groups revealed essentially similar findings (Lines 3–5). Thus, division in *E. borealis* and *M. indomita,* when it occurs in a population of urchins, does not necessarily affect uniformly all the urchins of the different age-groups of the population.

4. Survival of the ciliates in sea water and its relation to infectivity

It has been pointed out that cysts are unknown in ciliates of echinoids and that young hosts undoubtedly acquire their faunules by the ingestion of trophic forms that escape among the fecal pellets. This conclusion implies that echinoid ciliates can live in sea water outside the body of the host, although information on their survival is meager. Powers (1933b, p. 123) states that specimens of *E. borealis* when transferred to sea water "appear normal" and "live for various lengths of time," and he was able to keep specimens in hanging-drop preparations at 7° C for periods that varied from 15 to 23 days. It is doubtful that the survival of a large entozoic ciliate in the restricted confines of a small hanging drop reveals anything of special significance about its survival under natural conditions, and Powers himself states that the animals seemed "merely to exist." Since the capability of echinoid ciliates to survive in sea water is inseparably related to the infection of new hosts, a study of the survival of the three entocommensals of *S. dröbachiensis* was undertaken, but the procedure differed radically from that of Powers.

The sea water was taken from Frenchman Bay (mean annual salinity, 31.8) well beyond the intertidal zone and was passed through Whatman No. 43 filter paper to remove the predatory or unwanted plankters. Each of the three species was dealt with separately in the following manner, as illustrated by *E. borealis*. A clean pre-cooled Syracuse watch glass was placed on the stage of a dissecting binocular and filled with 10 ml. of sea water (temperature 15° C., approximately that of Salisbury Cove sea water in the summer of 1960). Then, about 75 specimens of *E. borealis* from a recently collected urchin were placed in the watch glass near its right margin. The ciliates usually dispersed rapidly, so that many of them soon arrived in relatively pure sea water at the left margin of the watch glass, whereupon 25 of them were transferred by means of a small pipette to 1 ml. of fresh sea water in a Columbia culture dish (square plate-glass depression slide, measuring 42 mm. on a side). The culture dish was placed in a covered Stender dish which was outfitted as a small moist chamber and kept in a tray of running sea water to maintain the temperature at 15° C. The condition of the ciliates was observed and recorded at the end of 6, 9, 24, 48, 72, and 96 hours, reckoning from the beginning. The experiment as just described was repeated some 20 times, using ciliates from more than a dozen different urchins. The method was decidedly superior to the use of hanging-drop preparations, in that the ciliates were first allowed to wash themselves relatively free of intestinal materials and were then transferred to 1 ml. of fresh sea water, which is a relatively large volume for only 25 ciliates. In most of the experiments the final culture dishes were exposed to the natural light of the laboratory, but in some they were kept in darkness (Stender dishes painted black on the outside) except during the brief intervals of observation. Since the histories of the cultures were identical, there was no evidence that moderate illumination was detrimental to the ciliates or that darkness was beneficial.

The procedure that has been described for *E. borealis* was likewise employed with *M. indomita* and *B. gracilis*. To facilitate comparisons, the results obtained with 300 individuals of each species, representing 12 culture-dish experiments, will be considered (Table IV).

E. borealis. Upon transfer to sea water, the ciliates, in agreement with Powers' findings, showed little or no heightened irritability and suffered no observable distortion in shape. At the end of 6 hours, 297 of the original 300 were present in the cultures, and at end of 9 hours, 296. It is likely that the death and disappearance of a few resulted from injuries that accompanied the process of washing and transfer. At the end of 24 hours, 281 were present (survival, 93.7%). Some were swimming normally and others were creeping on the bottom of the dish or against the surface film. However, the many food vacuoles which they originally contained had disappeared, and thus the cytosome was relatively transparent. At the end of 48 hours, 256 (85.3% of the original number) were still present, but they were distinctly smaller, quite transparent, very slow of movement, and evidently much weakened from lack of food. During the succeeding 24-hour period

TABLE IV

Survival of three species of urchin ciliates in sea water. Total number of individuals of each species at beginning of experiment was 300. Hours cited are reckoned from the beginning.

	Ciliate		
	Entodiscus borealis	*Madsenia indomita*	*Biggaria gracilis*
No. of survivors after			
6 hours	297	298	217
9 hours	296	298	103
24 hours	281	295	0
48 hours	256	272	
72 hours	17	36	
96 hours	0	0	

the animals suffered drastic mortality, since only 17 (5.7%) remained at the end of 72 hours. These few survivors were much smaller than formerly and were barely able to swim. At the end of 96 hours, there were no survivors.

M. indomita. Unlike *E. borealis,* this ciliate when transferred to sea water displayed greatly heightened irritability, for the animals swam rapidly and erratically. However, their intense activity subsided within 5 to 10 minutes, and with no ill effects, to judge by their survival. In general, the results paralleled those obtained with *E. borealis,* although there were slightly more survivors throughout the first three days. At the end of 24 hours all the food vacuoles had disappeared from the cytoplasm, but the animals were still swimming normally. At the end of 48 hours they were considerably diminished in size and were very transparent, and their locomotion was extremely sluggish. Again, a high mortality occurred during the third 24-hour period, such that only 36 were present after 72 hours. No survivors remained at the end of 96 hours.

B. gracilis. The outcome of the experiments with this ciliate was entirely unexpected. Upon transfer to sea water, *B. gracilis* swam rapidly and quite erratically, as if the medium were distinctly unfavorable. Of the original 300 specimens, only 217 (72.3%) were present after 6 hours, and at the end of 9 hours this number was reduced to 103. Many of these were vacuolated and clearly

abnormal in structure, and the remains of others were visible in the culture dishes. Since nearly all the survivors contained food vacuoles, the many deaths among the animals could not be attributed to starvation, but must have resulted from the properties of the medium. At the end of 24 hours there were no survivors.

Discussion

A comprehensive investigation of the relation of the three ciliates to their host in the Mt. Desert Island region would require a study of urchins of practically all sizes taken during all the months of the year. Unfortunately, such a study has not been feasible, and the present one is admittedly incomplete. Nevertheless, the results are of special interest and are fully adequate, it is believed, to support the conclusions that are advanced in the following sections.

1. Acquisition of infections by young urchins and the delayed onset of division

The absence of ciliates in 8-mm. urchins (age about 1.5 years) indicates that young urchins do not acquire their infections during their first summer of life, or even during the first year. The presence of relatively small numbers of ciliates in urchins 9–14 mm. in diameter (age likewise about 1.5 years, but no doubt somewhat older than the foregoing) indicates that the urchins first acquire their ciliates during their second summer when they are at least 9 mm. in diameter and about 1.5 years old.

It might reasonably be assumed that all urchins would become infected not long after metamorphosis and that all would contain fairly dense populations of ciliates by the middle of their second summer. Actually, at least four factors militate against the early acquisition of infections by young urchins at Mt. Desert Island. The first three are of general occurrence; the fourth is to some extent peculiar to the region of the Island. They are the following. (1) The ciliate losses that accompany the extrusion of fecal pellets are relatively small, to judge by earlier experience (Beers, 1948), as if each ciliate resists dislodgement from its preferred segment of the gut. Thus, urchin ciliates are extremely scarce and very difficult to find in the waters of the urchin's natural habitat, though they can be found with no difficulty in the bottom sediments of an aquarium that is well-stocked with urchins. (2) The period of survival of the ciliates in a healthy condition in sea water outside the body of the host is relatively short, varying from 6 to 48 hours. Although the ciliates tend to adhere loosely to any substratum and to creep upon it, thereby facilitating to some extent their ingestion by a new host, the length of time available for their chance discovery and ingestion by an urchin is distinctly limited. (3) Some of the ciliates are no doubt destroyed by predators. (4) Tidal extremes are great, the mean tidal range being 10.6 ft. (3.23 m.) at Salisbury Cove. Thus, enormous quantities of water ebb and flow twice daily over the urchins and undoubtedly carry away many of the extruded ciliates. In view of the existence of these inimical factors, it is perhaps not so surprising to find that the infection of the young urchins is appreciably delayed.

The absence of dividing ciliates in urchins 9–14 mm. in size suggests that the respective ciliate populations do not undergo augmentation by cell division immediately after the infection of the host, but are increased during the second summer

only by the ingestion of additional specimens. Significant augmentation by division appears to be delayed until the third summer, when the urchins are about 2.5 years old.

2. Establishment of the ciliate populations; division of the ciliates in urchins of diverse ages and in populations of urchins

Whereas none of the infected 1.5-year-old urchins contained dividing ciliates, it has been seen that of 12 infected urchins of age 2.5 years, 9 had *E. borealis* in division, 7 had *M. indomita*, and all had *B. gracilis*. These findings indicate, as has been said, that it is during the third summer of the urchin's life that the respective ciliate populations first experience augmentation by division, resulting in the establishment of populations of maximum density.

Once the populations of *E. borealis* and *M. indomita* are established, infrequent eruptions of divisional activity seem adequate to maintain them in the host; thus, relatively few (3.1 to 8.1%) of the older urchins (age 3.5 to 6.5 years) harbor them in division. The factors that are responsible for the seemingly long intervals of non-divisional life and the occasional, intense outbreaks of division are unexplained, as has been said. It has been seen that division in the two cannot be correlated with the age of the host; it seems to occur randomly in older urchins, irrespective of their age. Neither does their division affect *en masse* the individuals of an urchin population, even though the urchins seem to be living under similar conditions. Various possibilities present themselves by way of explanation, for example: (1) There is an inherent rhythm of long frequency in the reproductive activities of the ciliates. (2) Division is correlated, either qualitatively or quantitatively, with the food of the urchin and with the nature of the intestinal flora. Although sea-weeds are the preferred food of *S. dröbachiensis*, it is actually omnivorous, and the nature of the intestinal contents is somewhat unpredictable. Thus, urchins of a collection from one and the same tide pool at Long Ledge were found to have fed on a variety of materials. Some contained principally filamentous green algae in their alimentary tracts; others, bladder wrack (*Fucus* and the like) and sea lettuce (*Ulva*); still others, calcareous algae; and finally some contained nondescript materials that seemed to consist of barnacle remains and bottom sediments. To what extent these diverse food materials affect the ciliate fauna has not been ascertained. (3) Division is correlated with the physiological state of the urchin, though practically nothing can be said at present concerning this point. Of the foregoing possibilities, the second would seem to be the most readily amenable to experimental analysis, and it is planned that progress in this direction will be attempted within the near future.

The probable significance of the constant and uninterrupted division of *B. gracilis* is mentioned below.

3. Survival of the ciliates in sea water in relation to infectivity

It has been seen that under the conditions of the experiments both *E. borealis* and *M. indomita* can tolerate pure sea water for about 48 hours. Evidently this interval of time, assuming that it also prevails under natural conditions, is adequate to insure the eventual ingestion of a sufficient number of individuals to perpetuate the two species in the host.

Much in contrast with the two-day survival of the foregoing species is the seeming incapability of *B. gracilis* to tolerate sea water longer than 6–12 hours. Although little can be said with certainty in explanation of this pecularity, certain aspects of the autecology of *B. gracilis* seem worthy of mention. Of all the species of ciliates that occur in *S. dröbachiensis* at Mt. Desert Island, *B. gracilis* is the only one that is primarily an inhabitant of the rectum. In this disadvantageous site, it is expelled in greater numbers than any of its confreres (Beers, 1948). But it is also the only one of the ciliates, with the exception of *Euplotes balteatus,* which is probably nothing more than a facultative commensal (Beers, 1954), that is constantly dividing within the urchin. Thus, its loss in greater numbers is offset by frequent division, and its continued survival in the host is reasonably assured. In sea water outside the body of the host, *B. gracilis* experiences a further disadvantage from the standpoint of survival, in that it has relatively little tolerance for sea water. But it is lost in greater numbers from its host, as has just been said. The escape of larger numbers of individuals into the external world would seem to compensate adequately for the briefer period of survival of each; thus, a relatively constant number of individuals is presumably maintained in the external environment, where they can be ingested by new hosts. Though vulnerable to excessive losses both within the urchin and without, *B. gracilis* nonetheless maintains itself by the agency of constant division.

Summary

1. The first part of the study concerns certain relationships of the ciliates *Entodiscus borealis, Madsenia indomita* and *Biggaria gracilis* to their host, the sea urchin *Strongylocentrotus dröbachiensis.* It is based on an examination of 152 urchins taken at Mt. Desert Island, Maine, in the summer of 1960. The respective ages of the urchins are estimates based on size (diameter of test). The second part concerns the survival of the ciliates in sea water, since their survival is inseparably related to the infection of new hosts.

2. Nine urchins measuring 8–14 mm. in diameter (age 1.5 years) were either uninfected or very lightly infected, and none of the ciliates was dividing. Urchins evidently acquire their ciliates at this age (second summer).

3. All the urchins of the remaining age-groups were infected with all 3 ciliates. Of 12 urchins that measured 15–23 mm. in diameter, all contained dividing specimens of *B. gracilis,* 9 contained dividing individuals of *E. borealis,* and 7 contained *M. indomita* in division. The results indicate that the respective ciliate populatons build up rapidly to maximal densities in the third summer of the urchin's life (age about 2.5 years).

4. The remaining urchins were assigned by size to 4 age-groups. The number of urchins in each group, their range in size, and their estimated ages follow: 32 urchins, 24–39 mm., 3.5 years; 35, 40–49 mm., 4.5; 37, 50–59 mm., 5.5; 27, 60–65 mm., 6.5. All the urchins harbored dividing specimens of *B. gracilis;* thus this ciliate remains in constant division once infection is well established. But in each group only a small percentage of the urchins (3 to 8%) contained dividing specimens of *E. borealis* and *M. indomita.* Thus, their division, though evidently cyclical, could not be correlated with the age of the urchins.

5. In a natural population of urchins, the division of *E. borealis* and *M. indomita* does not affect simultaneously any large percentage of the urchins. Except in 2.5-

year-old urchins, it appears to occur randomly. Since the urchins of a population practice dissimilar food habits, it is possible that division is correlated with the nature of the food and the subsequent intestinal flora.

6. In pure sea water most specimens of *E. borealis* and *M. indomita* can survive about 48 hours, and their death is due to starvation. Individuals of *B. gracilis* can survive no longer than 6–12 hours, and death does not result from starvation but seemingly from the properties of the medium. It is suggested that the constant voiding of *B. gracilis* among the fecal pellets of the host compensates for its relatively brief period of survival in sea water.

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OBSERVATIONS ON THE RESPIRATION OF THE SABELLID POLYCHAETE SCHIZOBRANCHIA INSIGNIS

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Schizobranchia insignis Bush lives in tough fibrous tubes, mostly 10–20 cm. long and 5–10 mm. in diameter, attached to the underside of floating wharves, to pilings and to rocks on the Pacific northwest coast of America (Fig. 1, A). It may also be dredged from muddy bottoms.

The dense crown of orange, purple or grey branched filaments, which is used both for feeding and for respiration, may be expanded beyond the opening of the tube for long periods when the worm is undisturbed. For shorter periods the worm lies wholly within the tube. Worms also irrigate their tubes by waves of muscular contraction of the body wall which may pass in either direction. Irrigation occurs when the crown is expanded as well as when the worm is retracted within the tube.

That the crown of all sabellids is used for feeding may readily be confirmed by simple observation, but its importance in respiration appears to vary from one species to another. Zoond (1931) found a 63% fall in oxygen uptake after amputation of the crown in *Bispira volutacornis* (Montagu) and Fox (1938) found the same decrease when *Sabella spallanzanii* (Viviani) was similarly treated. On the other hand, Wells (1952) found that bisected *Sabella pavonina* Savigny showed no significant fall in total rate of oxygen uptake of the two parts, but that *Myxicola infundibulum* Rénier did, there being a sharp drop in total uptake when bisected, and the posterior part giving relatively lower values than those of *Sabella pavonina*. He concluded that in *Sabella*, while the current caused by the crown provides for the crown's own respiratory needs, it is the irrigation current which is of importance to the rest of the body. *Myxicola*, on the other hand, does not irrigate its tube and is wholly dependent on the crown which functions not only in feeding but as a gill.

These differences suggested that it might be of interest to investigate the activities of another sabellid under conditions as natural as possible. The importance of the crown in respiration has only hitherto been assessed by the drastic procedure of amputation, and the rate of oxygen uptake has never been measured with the rate of water transport through the crown. Consequently, I have made measurements of oxygen uptake by the worm when expanded and when wholly withdrawn within the tube. The volumes of water passed (1) through the tube and (2) through the crown have also been measured, and the percentage utilization of oxygen by the crown and by the remainder of the body estimated under normal circumstances. All measurements have been at 12–13° C.

All the observations were made on animals from wharves in the vicinity of Friday Harbor, Washington. I am glad of this opportunity to thank Dr. Robert L. Fernald and the Staff of the Friday Harbor Laboratories of the University of

Washington for their hospitality and help. I also wish to thank Professor H. Munro Fox, F.R.S., for helpful criticism of this paper.

Worms were stripped of their own tubes and accommodated in pieces of transparent plastic or transparent rubber tubing of suitable length and diameter. Such tubes reveal the activities of the worm, readily enable the tube to be linked to recording apparatus, and permit measurement of oxygen uptake under nearly

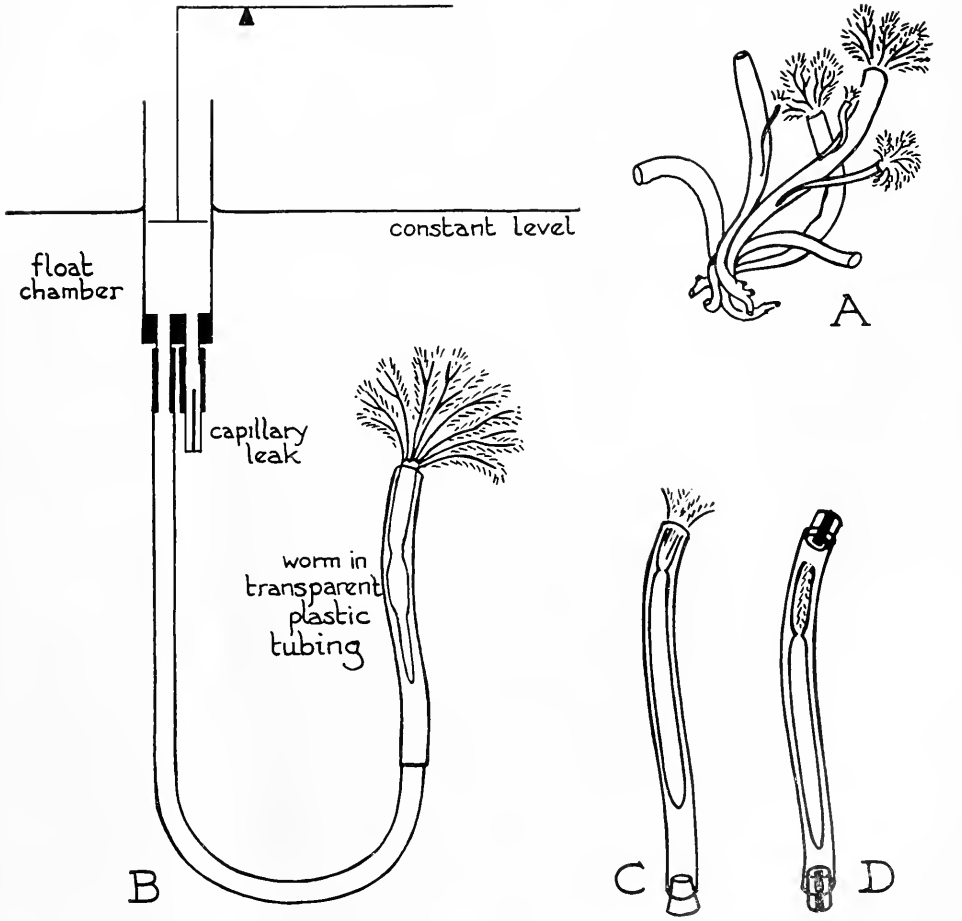

FIGURE 1. A, appearance of colony of *Schizobranchia* in nature; B, apparatus used to record irrigation; C, irrigation prevented; D, expansion prevented.

normal conditions. The work of Hyman (1932) and Fox (1938) emphasises the importance of simulating natural conditions as far as possible. Worms used in the experiments had been acclimatised to plastic or rubber tubes for at least one or two weeks.

EXTENSION AND WITHDRAWAL

To obtain some idea of the amount of time spent by the worm with the crown expanded, and the amount of time passed wholly withdrawn within the tube, worms

accommodated in plastic tubes were attached to a recording apparatus (Fig. 1, B) similar to that used by Wells (1951) for *Sabella*. The apparatus was immersed in a tank through which a circulation of sea water was maintained and in which the water level (1) remained constant. By adjusting the size of the capillary leak (a) the movement of the worm could be recorded on a slowly revolving kymograph by the lever actuated by changes in the level of the water in the float chamber (f). By selecting a larger capillary which allowed a more rapid flow than could be maintained by the worm irrigating under normal conditions, it was possible to adjust the capillary so that the float would be affected only by relatively rapid movements of the whole body, as in extension or withdrawal. Two typical traces, each of 12 hours duration, made by different worms are shown in Figure 2. It will

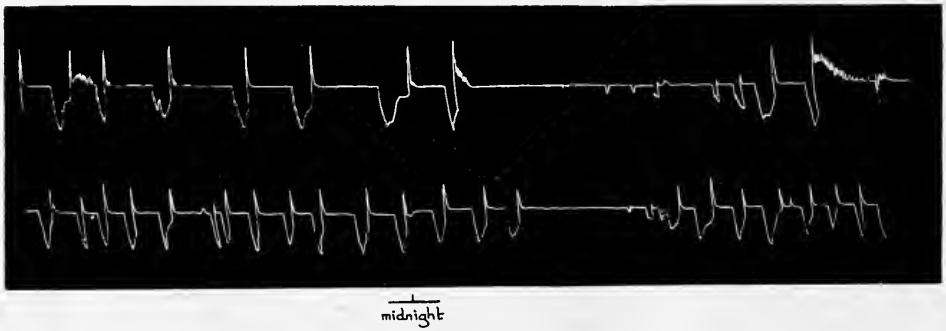

FIGURE 2. Continuous record of expansion and withdrawal by two different worms. Duration of each trace, 12 hours. Read from left to right. Upward spikes represent withdrawal within the tube, downward spikes represent extension from the tube with expansion of the crown. Extension is more gradual than withdrawal, as is shown by the stepped trace. The horizontal parts of the trace represent the times when the worm remained with the crown expanded beyond the opening of the tube.

be seen that extension and withdrawal occur at intervals of some regularity. The long period when the crown is expanded following extension after midnight was common to many worms and records. It may have been due merely to absence of stimulation by workers in the laboratory, although many other sabellids have been seen to expand their crowns more at night (McIntosh, 1922, Fox, 1938). It will be noticed that each period of expansion exceeded each period spent wholly withdrawn. The time spent retracted on any one occasion did not exceed 10–15 minuts, while periods of expansion were 20–60 minutes or longer. The interpretation of the records was confirmed by frequent observation.

IRRIGATION

Each tube, though firmly attached by mucus at or near the base to wharves or pilings, has one or more small openings 1 mm. or so in diameter near the hind end (Fig. 1, A). Mucus can be secreted through these to regain attachment, or new apertures made as occasion demands, and the orientation of the tube somewhat changed, as Fox (1938) observed in *Sabella spallanzanii*. Apart from these possibilities, which enable a small amount of re-orientation within the clump of animals so that each has room to expand the crown, *Schizobranchia* is completely sessile and

is unable to turn around in the tube except on its own longitudinal axis. Unlike *Sabella,* however, it rarely does so.

Irrigation of the tube is effected by muscular swellings passing down the body, most commonly from head to tail. Occasionally the direction of irrigation is reversed. These activities may occur when the crown is expanded or when the worm is withdrawn into the tube. The volume of the tube containing a worm of average size (2 grams fresh weight) is about 1.5 ml., the volume of such a tube empty being about 3.5 ml. Some idea of the irrigation rate may be obtained by injecting a suspension of carbon into the tube by means of a hypodermic syringe, and observing the rate of travel of the particles along a horizontally fixed graduated tube sealed on to the hind end. Under otherwise normal conditions the fluid in the tube may be completely renewed in 30–60 seconds of activity.

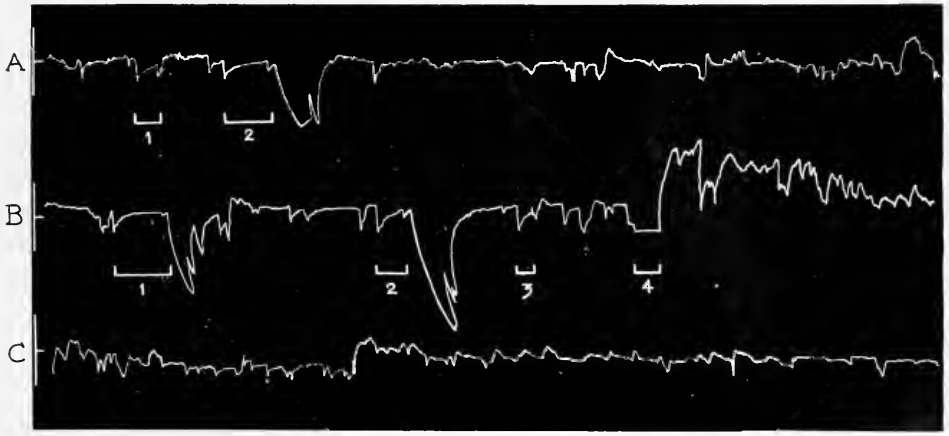

FIGURE 3. A–C, continuous record of irrigatory activity of a single worm over a period of 36 hours. Read from left to right. Each line represents 12 hours. Further explanation in text.

A continuous record of irrigation may be obtained with the apparatus already described by attaching a finer capillary at (a), such that the flow into or from the tank causes a slight rise or fall of 1–3 mm. to occur in the float chamber. A pressure difference of this magnitude may easily be recorded, but is unlikely to be great enough to modify the behaviour of the worm. A record of such activity is shown in Figure 3, C. The details of such traces were interpreted by watching worms from time to time while the trace was being made.

In the records elevation represents irrigation headwards; depression, tailwards. Irrigation will be seen to be somewhat irregular in rate, but to be fairly continuous. The volume passed can be calculated, knowing the dimensions of the capillary and the rest of the apparatus (Wells and Dales, 1951), or may be determined empirically. The average rate was found to be 0.3–0.5 ml./min. for a 2-gram (fresh weight) worm. Wells (1952) found a similar rate for *Sabella pavonina* of comparable weight. By inserting a small bung into the opening of a worm's tube attached to the recording apparatus, extension of the worm and irrigation could be stopped. If the period of closure did not exceed 10–15 minutes (Fig. 3, A : 1; Fig. 3, B : 3) normal activity was resumed after release. If this period was exceeded (20–45-

minute closure) as in Figure 3, A : 2; Figure 3, B : 1, 2, release was followed by very vigorous irrigation, as much as 0.75 ml./min. being passed for an hour or more by a 2-gram worm.

By connecting another piece of tubing to both the open end of a plastic tube in which a worm had been accommodated and attached to a recording apparatus (Fig. 1, B), and to the jet from the float chamber instead of the capillary leak, the circulation could be closed without preventing the worm from irrigating. Under

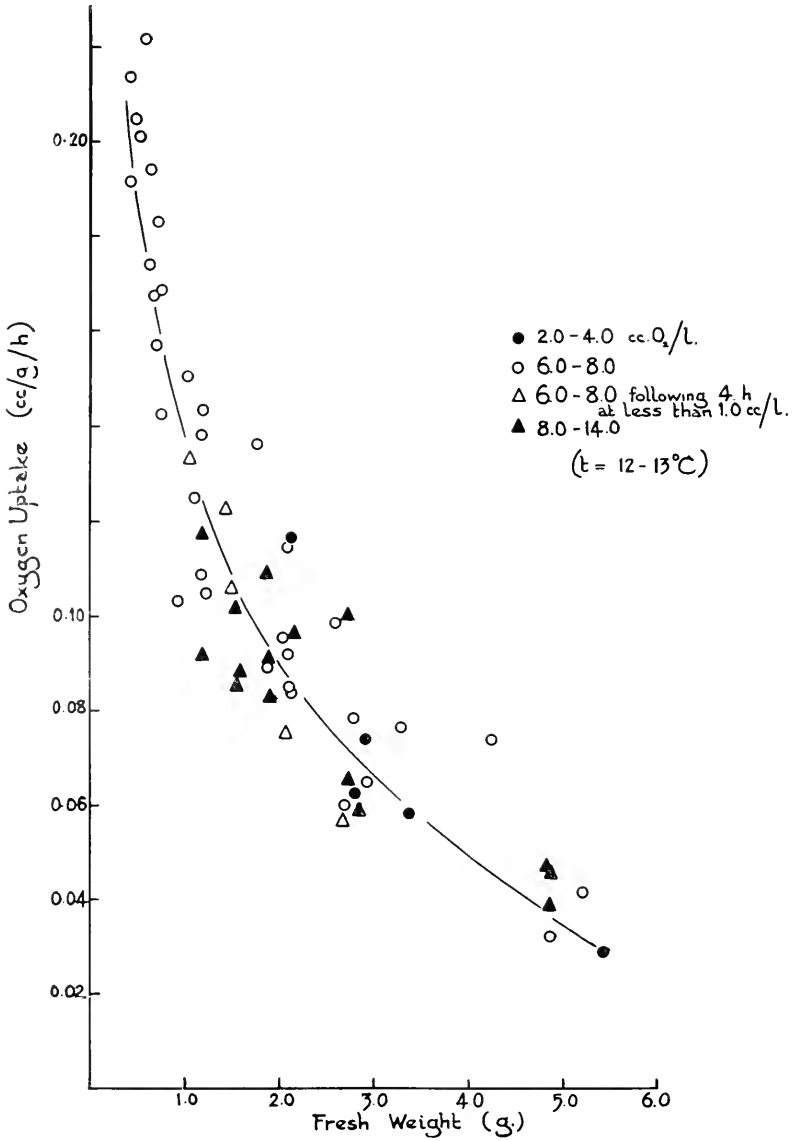

FIGURE 4. Rate of oxygen uptake under different external oxygen concentrations.

normal conditions worms underwent short bursts of "testing" activity, driving first in one direction and then in the other, and on release continued vigorous irrigation for some hours. Wells (1951) found that *Sabella spallanzanii* responded similarly. In Figure 3, B : 3, a burst of testing irrigation in a headward direction was in progress on release, and this direction was maintained for the following two hours, the worm gradually returning to its normal behaviour pattern.

Oxygen Uptake

The rate of oxygen uptake was measured by the modification of Fox and Wingfield (1938) of the well-known Winkler technique. The rates of uptake made under different oxygen concentrations plotted against total fresh weight are presented in Figure 4.

Uptake was measured in closed bottles after a period long enough to ensure accurate estimation, but normally not so long that the quantity of oxygen in the bottle was reduced to not less than 4–5 cc./l. or the quantity of carbon dioxide increased to a level at which the rate might be affected. Bottles of approximately 270 ml. capacity were used and most measurements were made after a 1–4-hour period, according to the size of the animal. Worms thoroughly acclimatised to plastic tubing were used, as measurements so made might be expected to be closest to those under entirely natural conditions; Fox (1938) found that the oxygen uptake in *Sabella spallanzanii* was 20–30% lower in worms freshly deprived of their tubes. Each animal was therefore enclosed in a bottle large enough to ensure normal activity; worms were able to expand the crown, to withdraw and to irrigate. The bottles were occasionally inverted to ensure thorough mixing; most of the worms were acclimatised to being disturbed and their behaviour appeared to be affected only momentarily. All the determinations were made at 12–13° C.

(*a*) *Response to raised or lowered oxygen content of the water*

The normal rate of oxygen uptake could be maintained by the worm when the oxygen content of the water was lowered to 2 cc./l., either gradually by the animal itself over an extended period in a closed bottle, or by bubbling nitrogen through the water before the experiment. When the oxygen content was slowly reduced by the animal itself, the rate of uptake was significantly reduced between 1.3–2.5 cc. O_2/l.

If enclosed in a small chamber so that the rate of pulsation of the branchial vessels could be observed, the normal rate of 9–10 pulsations/min. (13° C.) fell off rapidly below about 2.5 cc. O_2/l., and ceased altogether around 1.3 cc. O_2/l. Fox (1938) found the same effect in *Sabella spallanzanii*.

The rate of oxygen uptake was not affected by raising the oxygen content of the water, similar values being obtained up to 14.0 cc. O_2/l. It is interesting, however, in confirmation of the findings of Fox and Taylor (1955), that worms were not adversely affected by these high concentrations, and survived indefinitely in the laboratory circulation which had a high oxygen content (7–8 cc. O_2/l.) owing partly to the action of the pumps. These sabellids are indeed usually found in habitats with a good circulation of water where the concentration of oxygen is likely to be high. Fox (1932) and Ewer and Fox (1940) have shown that the chlorocruorin of *Sabella spallanzanii* blood is adapted for oxygen transport only at

high outside concentrations, and C. Manwell (private communication) has found the same in *Schizobranchia insignis*.

(b) Uptake of oxygen when extended

When the crown was removed, and after the animal had recovered from the operation for a day or two, the oxygen uptake by the rest of the body was measured; the value obtained was about 25% that of the normal animal.

With normal animals irrigation, and hence normal respiratory exchange across the body wall, could be stopped by plugging the hind end of the tube (Fig. 1, C). Observation suggested that such worms remained extended more continuously, and measurement of oxygen uptake in closed bottles showed that the values obtained were not significantly different from those of normally irrigating worms. In other

TABLE I

Rate of oxygen uptake under normal conditions, when irrigation is prevented (as in Fig. 1, C), and when confined within the tube (as in Fig. 1, D)

Worm number	Total fresh weight	O_2 uptake under normal conditions (cc./g./hr.)	O_2 uptake without irrigation (Fig. 1, C)	O_2 uptake under forced withdrawal (Fig. 1, D)
16	0.460	0.1920	0.1790	0.0782
17	0.520	0.2330	0.2266	0.0819
18	0.600	0.2210	0.2165	0.0759
19	1.205	0.1132	0.1568	0.0463
20	0.565	0.2212	0.2298	0.0696
21	0.765	0.1684	0.1851	0.0855
22	2.100	0.0916	0.1114	0.0316

words, normal oxygen uptake can be maintained by the crown alone (*i.e.*, without uptake through the body wall), though it may have to remain expanded to do so. Wells (1952) found that crownless *Sabella pavonina* perished if unable to irrigate.

(c) Uptake of oxygen when withdrawn

When withdrawn within the tube the crown is not well displayed for respiratory exchange or feeding, as the numerous branched filaments are tightly rolled together. By fitting a narrower tube to the opening (Fig. 1, D) extension could be prevented but irrigation continued. Under these conditions oxygen uptake was reduced, usually to about 40% of the normal value. On release, worms remained extended for some time. Values obtained for seven worms in which measurements of oxygen uptake were made under normal conditions, with irrigation prevented, and when wholly withdrawn, are compared in Table I.

RATE OF FILTERING BY THE CROWN

When the crown is extended water flows through the filaments as a result of ciliary activity. A measure of the volume of water strained through the crown may be obtained by the use of colloidal graphite suspensions, since the particles coming into surface contact are removed from suspension by mucus. Removal of particles is exponential if the system remains constant in volume and the particles

remaining in suspension are evenly distributed (Dales, 1957). Particles may be ingested or rejected, but in either case are removed from suspension. The rate at which unit volume is cleared of particles may be calculated by measuring the decrease in density at known intervals against controls (Jørgensen, 1949). The filtering rate in ten experiments was calculated over a three-hour period using worms of 0.5–2.0 grams fresh weight at 12–13° C. The mean rate of filtering was 70.7 ml./g./hr. This is of the same order of magnitude as has been found in other sabellids (Dales, 1957).

Discussion

All these observations suggest that the life of *Schizobranchia insignis* is very similar to that of *Sabella spallanzanii*. Both species may be found in tubes open at the hind end attached to rocks and wharves. Both irrigate their tubes with equal facility in either direction, and pause in this activity for periods rarely exceeding 10 minutes.

It is difficult to assess the part played by the crown in supplying the respiratory needs of the rest of the body since, as Wells (1951, 1952) has pointed out, the needs of the crown itself are high owing to its activity. The vascular supply to the crown may be of service both in conveying oxygen away and in conveying nutrients to the ciliated epithelium and other tissues of the crown. The ability to continue to live, and to regenerate the crown when this is amputated, provided that the worm is able to irrigate its tube, suggests that the crown is not essential, although under normal circumstances it may well supply part of the body's needs.

Schizobranchia cannot autotomize the crown as *Sabella* does, so that decapitation results in a more serious loss in the total blood volume and perhaps a more unusual derangement of metabolism than in *Sabella*. While some individuals did regenerate their crowns, many died under laboratory conditions; *Sabella* seems better adapted for this contingency. The crown is also relatively larger in *Schizobranchia*, and the reduction in oxygen uptake to 25% of the normal value may well be partly due to the loss of that part of the uptake accounted for by the crown itself. When the worm is retained in the tube (or when, in nature, the worm is wholly withdrawn) the irrigation current alone supplies oxygen to the animal and removes carbon dioxide and other waste products. Under these conditions the oxygen uptake is 40% of that when extended. While the crown is not then expanded and the animal's need for oxygen may be somewhat less, the cilia on the crown do not cease to move, and the muscular contractions causing irrigation of course continue. It could be argued that if crownless worms can continue to live, providing that they are able to irrigate, and that the oxygen uptake of crownless worms is 25% of what it was before decapitation, then the requirement of oxygen for maintaining irrigation may be met by 25% of the normal total oxygen uptake. Other activities may well be interrupted after decapitation, but the major part of the remaining 75% of the normal oxygen uptake may thus be accounted for by the activity of the crown itself. That worms wholly withdrawn (Fig. 1, D) have an oxygen uptake of 40% of the value for expanded though not continuously irrigating worms (Fig. 1, C) suggests that perhaps a value approaching 60% of the total oxygen uptake is due to the activity of the crown alone. While the circulation of the blood from the crown can supply the respiratory needs of the rest of the body if irrigation is not possible, under normal circumstances it need not do so. Uptake

of oxygen will ensure, through the intermediacy of the vascular system, a supply of oxygen to all parts at all times, for activity of the crown and irrigation are independent activities.

Pulsation of the branchial vessels was observed by Fox (1938) and Wells (1951) in *Sabella* to cease after some time when totally enclosed in a small chamber or in a tube, and cessation may be seen also in *Schizobranchia*. As already noted, pulsations occur under normal circumstances at a rate of about 10/min. at 13° C., but these cease altogether when the oxygen content of the water has fallen to about 1.3 cc. O_2/l. Fox (1938) suggested that this effect may be due to accumulation of carbon dioxide, but as Wells (1951) points out, this is unlikely to occur under normal conditions owing to irrigation. On the other hand, if the worm is wholly withdrawn and, while ceasing to irrigate, its uptake of oxygen remains at 40% of its normal value (0.1 cc. O_2/hr. for a 2-gram worm), the oxygen contained in the 1.5 ml. of water within the tube would be used up in 15 minutes. The possibility that the factor which ends a short rest from irrigation might be lack of oxygen or accumulation of carbon dioxide should not, therefore, be dismissed. In *Schizobranchia*, however, pauses were never observed to be as long as this, and in any case when the irrigatory waves cease the oxygen requirement should be less. In addition, there should be sufficient oxygen in the blood to provide for such brief pauses as occur (Ewer and Fox, 1940) and it seems more likely from the work of Wells (1951, 1955) that the resumption of irrigation is spontaneous.

The measurement of filtration rate showed that 70 ml. of water/g./hr. was moved across the filaments by the activity of the crown cilia, while the normal irrigation rate through the tube was about 12 ml./g./hr. While it would be unwise to draw too close a comparison, these figures suggest that the crown is in fact achieving more effectual work in water transport than the body when irrigating, so that it is not surprising to find that the total oxygen uptake is reduced to 40% when the crown is not expanded. The utilization of oxygen from the water passed through the crown may be obtained from the filtration rate (70 ml./g./hr.) and the rate of oxygen uptake (0.05 cc. O_2/g./hr.). Under laboratory conditions (external oxygen content of 7.0 cc. O_2 l.) this can be estimated at about 10%. When the worm is wholly withdrawn the oxygen consumption falls, as we have just noted, to 40% of the normal value or 0.02 cc. O_2/g./hr., which is withdrawn from only 12 ml., giving a utilisation of about 24%. The rather low utilisation by the crown rather suggests that the flow is maintained more for feeding than for respiration.

Wells (1951) suggested that in *Sabella pavonina* feeding was at least a possibility when the crown is withdrawn. While this may be so in *S. pavonina*, which has a singularly delicate and "open" crown, it seems far less likely to occur in *Schizobranchia* in which the crown is more complicated, much branched, and closely furled when contracted.

The results discussed here suggest that *Schizobranchia* is able to maintain its respiratory needs when withdrawn within the tube, and that it emerges to feed in response to some spontaneous mechanism such as Wells (1955) has described in other polychaetes. This ability to meet the demand for oxygen by irrigation when withdrawn within the tube is a factor with obvious survival value. It is interesting that sabellids such as *Myxicola* (Wells, 1952) and *Chone*, which do not irrigate their tubes, have exceptionally well developed giant fibre systems and retraction responses. This should increase their chances of survival, for not only are these

worms dependent on their crowns for respiratory exchange but the crowns must, therefore, be more constantly displayed.

Summary

1. Observations on the life of a sabellid *Schizobranchia insignis* have been made under conditions resembling as far as possible those found in nature.

2. The amount of time spent with the crown expanded and the amount passed wholly withdrawn within the tube have been measured, and the utilisation of oxygen under these two conditions estimated.

3. The volume of water passed through the crown for respiratory and feeding purposes, as well as the volume pumped through the tube, have also been measured, and the part played by each in respiratory exchange discussed. It was found that about 70 ml./hr./g. animal (fresh weight) is passed through the crown by the action of the filamentary cilia, and the volume pumped through the tube is about 12 ml./hr./g.

4. Utilisation of oxygen by the crown is relatively low (10%); utilisation by the whole worm when withdrawn is about 24%, and the large volume strained by the crown is probably related to the food requirements rather than to the respiratory needs of the worm.

5. It is suggested that the oxygen taken up by the crown is largely utilised in its own activity although it can, and does, provide for the needs of the rest of the body during pauses in irrigation when expanded.

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PRELIMINARY INVESTIGATION ON THE PHYSIOLOGY AND ECOLOGY OF LUMINESCENCE IN THE COPEPOD, METRIDIA LUCENS [1]

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Since the advent of the photomultiplier tube with greatly increased sensitivity to low light intensity, it has been possible to measure marine luminescence quantitatively. Luminescent flashes have been found to be far more prevalent at all depths in the sea than had generally been suspected (Clarke and Backus, 1956; Clarke and Breslau, 1959, 1960; Clarke and Hubbard, 1959; Clarke and Wertheim, 1956; Boden and Kampa, 1957, 1958; Kampa and Boden, 1957). Attempts to identify the source of this flashing, using the luminescence camera built by Breslau and Edgerton (1958), suggest that most of the luminescence is produced by planktonic organisms less than a centimeter long (Clarke and Breslau, 1959; Clarke, personal communication). Certain planktonic species whose luminescence has been investigated do not show spontaneous luminescence in the laboratory. Probably some of the luminescence which has been measured at sea may be artificially stimulated by the unavoidable motion of the photometer suspended from a research vessel. However, Kampa and Boden (1957) have concluded that some luminescence appears to be "natural" or "spontaneous."

Bioluminescence in a small planktonic animal has been examined particularly with a view toward evaluating its potential as a source of luminescence in the natural environment and determining the significance of the luminescence for the organism. The calanoid copepod, *Metridia lucens,* was the animal chosen.

This copepod was recognized as luminescent by Boeck (1865) who described the species. Several additional workers have made microscopic or field observations on the luminescent Copepoda (Dahl, 1893, 1894; Kiernik, 1908; Vanhöffen, 1895; Giesbrecht, 1895), but very little experimental work has been done.

In the present work preliminary investigation of certain physical properties of the luminescent emission and of the physiology of the luminescent mechanism has been attempted, in addition to the experiments designed to ascertain what ecological significance luminescence may have for this copepod.

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Materials and Methods

The copepods used in the experiment were obtained in Cape Cod Bay about 3–5 miles northeast of the mouth of the Cape Cod Canal in 20–30 m. of water. Collections were made on two occasions, July 7 and August 16, 1960, with a ¾-meter #00 plankton net towed near the bottom. The *Metridia* were isolated from the catch and maintained in the laboratory approximately 40 animals to 1000 ml. of food culture. Laboratory cultures of the diatom *Thalassiosira fluviatilis* were used as food diluted 1:20 by volume with millipore-filtered sea water from Cape Cod Bay. This gave a concentration of 6000 to 10,000 cells/ml. in the final food culture.

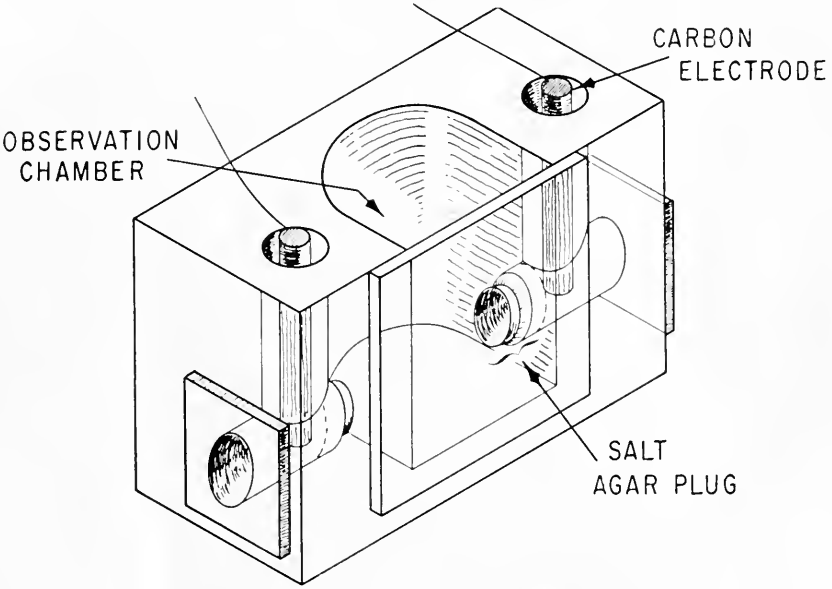

Figure 1. Electrode chamber used for stimulation of *Metridia*. For details see text.

Either streptomycin or penicillin (50 mg./l.) was added to inhibit bacterial growth. For specific experiments, smaller groups of *Metridia* were kept in proportionately smaller volumes of food medium. All groups of animals were kept in a darkened refrigerator at 5–7° C.

Measurements of luminescence were made in a "black box" consisting of a tar-paper covered wooden frame built on top of a table. A large opening on one side of the box covered with a black cloth sleeve and drawstring permitted the investigator's head to remain inside the box for observations or to monitor recording and stimulating instruments outside the box.

The measurements of luminescence were made with the portable bathyphotometer designed and built by Breslau (1959), which employs a RCA 5819 photomultiplier tube with 1200 v. battery power and a transistor amplifier circuit. Experimental material was placed directly in front of the photomultiplier about 18 cm. from the sensitive surface. A Texas Instruments, Inc. single-channel, strip-chart recorder ("Recti/Riter") was used to record intensity (in μw./cm.^{2}) against time during each flash.

Since the *Metridia* do not generally luminesce spontaneously in the laboratory, mechanical or electrical stimulation must be applied to study the characteristics of the flashing. In order to standardize the stimulus delivered to the animals, a simple electrode chamber was constructed as shown in Figure 1. The device was cut out of a piece of lucite and the connecting holes between the two side chambers and the central chamber were filled with 3% agar made with millipore-filtered sea water. For experiments, carbon electrodes wired to a pulse regulator were placed in the side chambers and the whole device was filled with cooled sea water to complete the circuit. *Metridia*, either individually or in groups, were then placed in the central chamber for stimulation.

All stimulation was performed with alternating current controlled through an electronic switch and a continuously adjustable autotransformer, Variac Type W10MT. The switch regulated the duration of pulses to one-tenth of a second and the interval between pulses to two-tenths of a second. Although slightly sensitive to changes in salinity and temperature, the current was regulated accurately to one-tenth of an ampere. The chart speed of the recorder was varied for different experiments. The slower speed (6 in. hr.) was used to record the frequency and intensity of flashes. The faster speeds (6 or 12 in. min.) were used when a measure of total luminescent flux (area under intensity curve) or the duration of a flash was required.

Description and Distribution of Metridia lucens

Metridia lucens is a medium-sized copepod, virtually colorless in the living state. Its size varies between 2.4–3.0 mm. for females and 1.8–2.5 mm. for males.

Although *Metridia lucens* is a common copepod of temperate and boreal water, very little is known regarding its seasonal abundance or life-history. In the Gulf of Maine, Bigelow (1924) noted an increased abundance in the spring and again in September and October. Bigelow (1924) and Clarke (1933, 1934) observed extensive diurnal vertical migrations in this species. In the waters off the coast of Ireland it also seemed to have a period of maximum abundance in May and a smaller period of increase in the fall (Farran, 1920). During the spring it has been reported to be responsible for brilliant phosphorescence on the Irish coast (Farran, 1903, in Bigelow, 1924).

Little is known regarding the internal anatomy of the copepods. Among the calanoids only *Calanus finmarchicus* has been studied in detail (by Lowe, 1935). It is presumed that in the general features of its morphology *Metridia* does not differ greatly from *Calanus* although there doubtless are certain differences in structural detail.

The only light-sensitive organ in most copepods, including *Metridia,* is a single naupliar eye. It seems very doutbful that this organ can have any role in behavior requiring recognition of other organisms because it cannot form images. However, it can presumably detect intensity gradients (as in vertical migration) and possibly the plane of polarization of incident light.

Luminescent glands

The earliest workers recognized that the luminescence produced by *Metridia* was primarily external. Boeck (1865), who described *Metridia lucens,* noted that

the light seemed to be produced in the head region and also from the abdomen. Vanhöffen (1895), working with the larger *M. longa*, observed luminescence distributed over most of the thorax as well as the head. In addition to the external secretion, he also felt that some light was produced internally which indicated the position of the secretory glands.

The authors observed that *Metridia lucens* seemed to produce luminescence, when stimulated electrically, chiefly from the anterior part of the head and from the region of the caudal rami. The separation of these two regions was sufficiently distinct that the light produced persisted sometimes as two discrete points for some seconds.

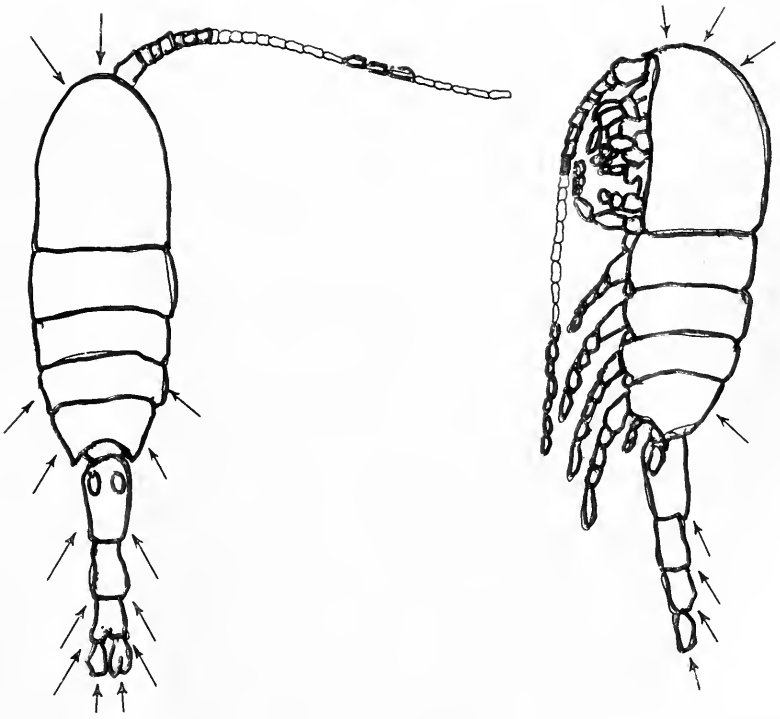

FIGURE 2. *Metridia lucens:* left, a dorsal view; right, a lateral view. Arrows indicate the general regions of the body where luminescent glands were found.

Due to the kindness of Dr. Robert Hessler, some histological preparations of *Metridia lucens*, fixed in Zenker's and stained with hematoxylin and eosin were available for study of the glands and their distribution. Figure 2 shows the regions of the body seen microscopically to produce luminescence in observations on living animals. Glands were located in the histological preparations in most of these places with definite concentrations on the anterior surface of the head and on the posterior portion of the abdomen.

The glands varied in shape somewhat depending on their location in the body. Those in the urosome had a long connecting duct between the glands and the external pore while those in the thorax opened directly to the outside through a

short duct. In several cases masses of dark material which might be the luminescent substance were observed in these ducts.

Sewell (1932, 1947) describes the presence of external pores on the cuticle, presumably associated with glandular structures, in several groups of copepods including *Metridia*. It is not certain, however, that these are the openings to

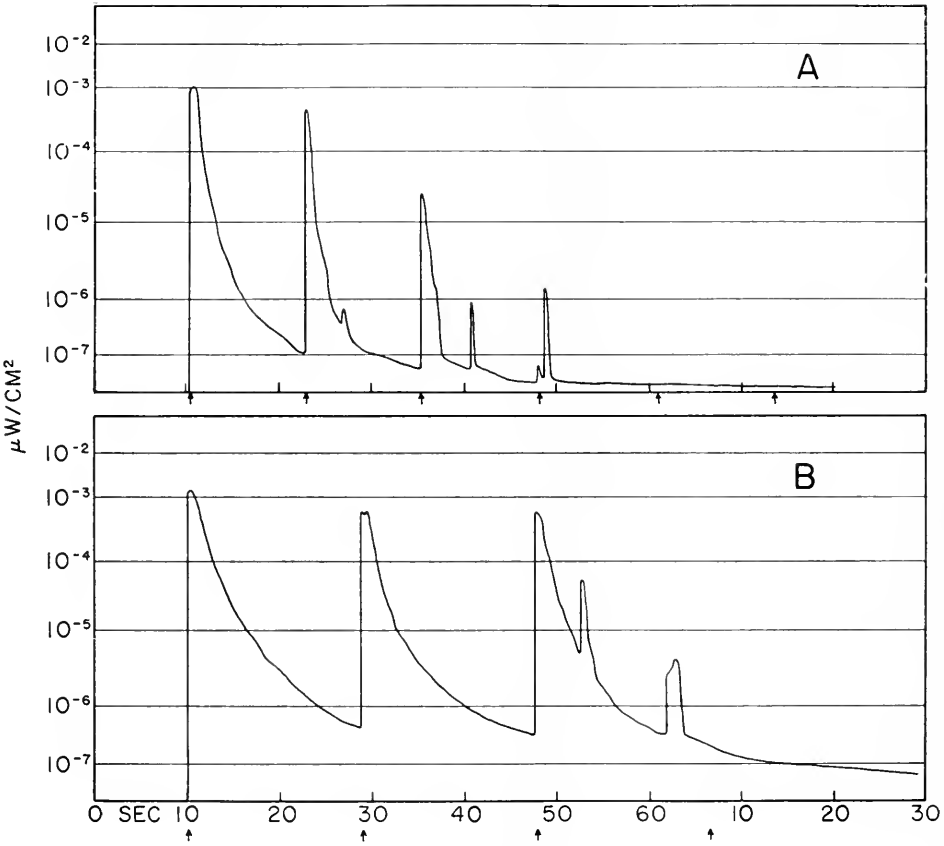

FIGURE 3. Luminescence of single *Metridia* when stimulated in the electrode chamber. Arrows along the time scale indicate instant of stimulation (0.7 amp.). Chart speed is 6 in./min. *A* shows curve for an animal tested six hours after capture; *B* shows curve for an animal tested after being kept in the laboratory for one month.

luminescent glands, particularly since such structures are found in several genera not presently known to be luminescent, notably *Eucalanus* and *Temora*. The distribution of these pores has not been worked out in detail for *Metridia lucens*.

Physical characteristics of the luminescence

The luminescent emission of *Metridia lucens* is generally a bright flash of varying duration. According to Harvey (1952), luminescence in copepods results from the simultaneous discharge of substrate and enzyme into the surrounding

medium; presumably the immediate peak emission occurs at the instant of initial contact between the reacting substances in the presence of oxygen. Generally a gradual decay follows as enzyme and substrate diffuse away into the medium, or perhaps as the substrate is used up.

The absolute intensity of the highest peak of the luminescent emission is in doubt because of the relatively slow response time of the equipment used. Furthermore, the maximum emission intensity varied to some extent for individual animals. However, the maximum intensity measured for *Metridia* was 1.2×10^{-3} μw./cm.^{2}

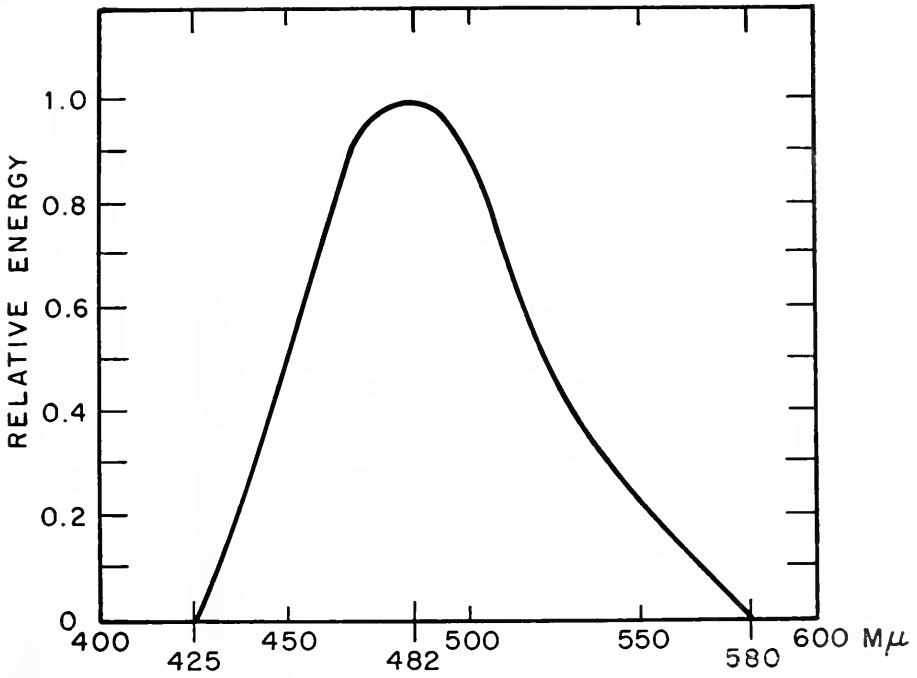

FIGURE 4. Spectrum of luminescent emission at 0° C. from crushed animals.

at the working distance of 18 cm. (Fig. 3). With groups of 5 *Metridia*, there was an additive effect giving maximum intensities of up to 4.5×10^{-3}. The intensity of the response decreased after successive stimuli.

The duration of individual responses varied even more widely than the maximum intensities, and ranged from 3 seconds to 50 seconds for all luminescent responses with intensities between 10^{-3} and 10^{-4} μw./cm^2. There was no apparent relationship between the intensity of a luminescent emission and its duration. For example, two *Metridia*, exactly similar in laboratory history, both gave responses of 3×10^{-4} μw./cm.^{2}, one emission having a duration of 10 seconds, the other a duration of 50 seconds. If responses of a lower maximum intensity than 10^{-4} μw./cm.^{2} are considered, durations as short as 1 second have been measured, particularly at the end of fatigue experiments when the *Metridia* had already responded to 10 or 15 electrical stimuli.

Through the kindness of Dr. W. D. McElroy at the Marine Biological Laboratory, Woods Hole, Massachusetts, it was possible to measure the spectrum of *Metridia's* luminescent emission (Fig. 4). The apparatus used was an Aminco spectrophotofluorometer in circuit with a drum recorder ("x-y" recorder) and an oscilloscope. Because of the rapid decay in intensity of *Metridia's* luminescence, it was necessary to cool a number of the animals in crushed ice in order to slow down the enzyme reaction producing the luminescence. Then, by immediately crushing the animals in a small test tube directly in the spectrophotofluorometer, the luminescence remained at one intensity long enough to record the entire spectrum.

The peak of the spectrum for *Metridia* is around 482 mμ and is therefore similar to that of *Cypridina* and certain other luminescent Crustacea (Nicol, 1960). The curve is slightly skewed toward longer wave-lengths with about half the spectral energy falling in the range between 440 mμ and 525 mμ. The entire spectrum lies between 425 mμ and 580 mμ. This spectrum with its peak at 482 mμ coincides closely with the wave-lengths having maximum transmission through clear, oceanic sea water (Clarke, Chap. 6, 1954).

Experiments on physiology

In order to determine whether laboratory culture had any effect on the luminescence of *Metridia*, freshly captured specimens and some which had been maintained in the laboratory for a month were repeatedly stimulated until failure to respond to two successive stimuli indicated the onset of fatigue. A representative experiment shown in Figure 3 indicates that the maximum intensity and the rate of fatigue were not markedly different for the two specimens. The difference in flash duration is not significant considering the wide range of variation shown by this characteristic.

To study the effect of strength of the stimulating pulse on the luminescence, the current was increased from .3 amp. to .7 amp. which caused a significant increase in the intensity of the luminescence and in the number of responses to stimuli. However, pulses stronger than .7 amp. did not cause further increase in luminescence intensity but seemed to reduce the number of successive responses. Variations in the duration of the pulse over the range tested (.10–1.0 second) had little effect on the intensity or number of successive responses. However, short intervals, *i.e.*, 3 seconds, between pulses induced two or three times as many successive responses as were observed using longer intervals between pulses, *i.e.*, 10–45 seconds.

The effect of previous light- or dark-adaptation was tested with separate groups of animals kept at about 5° C. in the dark, in the light, and in a room exposed to diurnal light changes. The experiment was begun at 1700 on August 3 and the luminescence produced by each group was tested on August 5 and again on August 8 between 1000–1300. No statistically detectable difference was found between the three sets of animals. In another experiment twenty animals kept in a water bath at 5° C., where they were exposed to daily light variation, were tested at night (2330–0030) and during the day (1300–1400). In the case of a few animals the day-time response was somewhat lower than at night but there is no evidence in any of the data for a marked inhibition of luminescence by light or for a daily rhythm.

Having established the fact that the experimental techniques used had no appreciable effect on the luminescent response of *Metridia,* two more physiological experiments were performed. The first was designed to investigate an observation by the authors that animals which fed poorly still luminesced as vigorously as

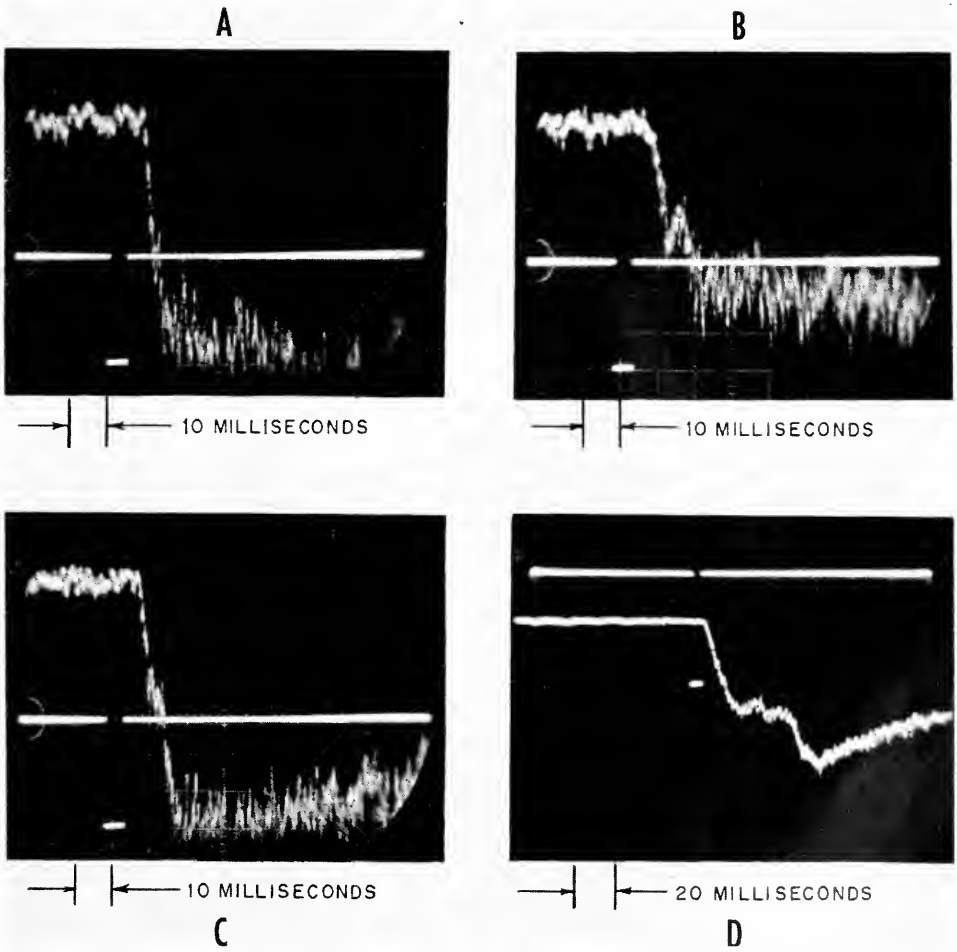

FIGURE 5. Lag time between stimulus and luminescent response. In all four cases the stimulus was 150 v./5 msc. represented by the break in the smooth horizontal trace. Downward deflection of the jagged upper trace (lower trace in *D*) represents luminescence measured by the photomultiplier. The lag times were: *A*, 8 msc.; *B*, 7 msc.; *C*, 8 msc. *D* shows the same type of measurement but includes more of the intensity curve and the stepwise rise to maximum intensity.

animals that fed well. Two groups of animals were set up, one fed on the regular culture medium and the other starved in millipore-filtered sea water. After one week, single stimulus tests were performed. On the basis of the total area under the intensity *vs.* time curve, the results showed no difference between the two

groups. However, when maximum intensity was considered, the results indicated statistically (Wilcoxen Ranked Sum) better luminescence for the starved group.

After the second week, repeated stimulus experiments were conducted on the two groups. Single stimulus data showed no statistical difference in the intensity of the response between the fed and starved groups, nor did the number of successive responses to repeated stimuli show a significant difference. At the end of the third week, however, experiments did demonstrate that the fed *Metridia* had a stronger luminescent response and the same group was able to respond to the electric stimulus a greater number of times than the starved animals.

In another series of experiments the length of time from the beginning of a stimulus to the beginning of a response (the lag time) was measured. For the necessary guidance and equipment to make these measurements, the authors are indebted to Dr. James F. Case at the Marine Biological Laboratory, Woods Hole, Mass. Single animals were tested in a small cell consisting of a 3-cm. piece of glass tubing with agar plugs and silver electrodes at either end. The electrodes were connected to a Grass S4 stimulator and the luminescence was measured with a RCA 931A photomultiplier in circuit with an oscilloscope. An automatic camera photographed the oscilloscope screen to record the results.

Using a stimulating pulse of 150 v. for 5 or 10 msc., the *Metridia* demonstrated a lag time (at room temperature) of 8–10 msc. to the beginning of the luminescent response and a lag time of 15–24 msc. to the maximum intensity of the response. The time to maximum intensity varied widely, depending on whether or not the rise to maximum intensity was direct or in a step-wise fashion, the latter giving lag times as long as 60 msc. (see Fig. 5). By observing the *Metridia* through a microscope during these experiments it was noticed that for a stimulus of 10 msc./150 v. usually both head and tail luminesced while for a stimulus of 5 msc./150 v. only the organs in the head region responded.

Experiments on behavior

Because it has often been suggested that luminescence functions as an escape mechanism for marine animals that luminesce by means of an extracellular discharge, the authors decided to investigate the behavior of *Metridia* in the presence of a predator. A series of experiments was conducted in the dark, in which possible planktonic predators on *Metridia* were placed individually with 10 *Metridia* in 600-ml. beakers. The species tested were: *Paraeuchaeta norvegica* (Copepoda), *Parathemisto* (*Euthemisto*) *gaudichaudii* (Amphipoda), and euphausiids, *Euphausia krohnii, Thysanoessa inermis, Nematoscelis megalops,* and *Meganyctiphanes norvegica.*

Each experiment was continued for at least two days and counts of the number of *Metridia* present were made at intervals of 12 to 16 hours. Only in the case of the two euphausiids, *Thysanoessa* and *Meganyctiphanes,* was there any predation on the *Metridia.* Although not every individual tested fed on *Metridia* with the same rapacity, *Meganyctiphanes* was by far the most successful predator. The best predators among the animals tested were then chosen for further examination.

The predator was placed in a 600-ml. beaker with 10 *Metridia* and this beaker was placed in the black box in front of the photometer. A cool water bath was used to keep the temperature in the experimental vessel between 10°–12° C. The

photometer response was recorded at slow speed (6 in. hr.) so that each flash gave a spike indicating maximum intensity and time of occurrence. Analyzing the results of these experiments was complicated by the fact that both prey (*Metridia*) and predator (*Meganyctiphanes*) were luminescent. However, comparison of the characteristics of *Metridia* and *Meganyctiphanes* luminescence records when the animals were stimulated electrically showed that high intensity responses

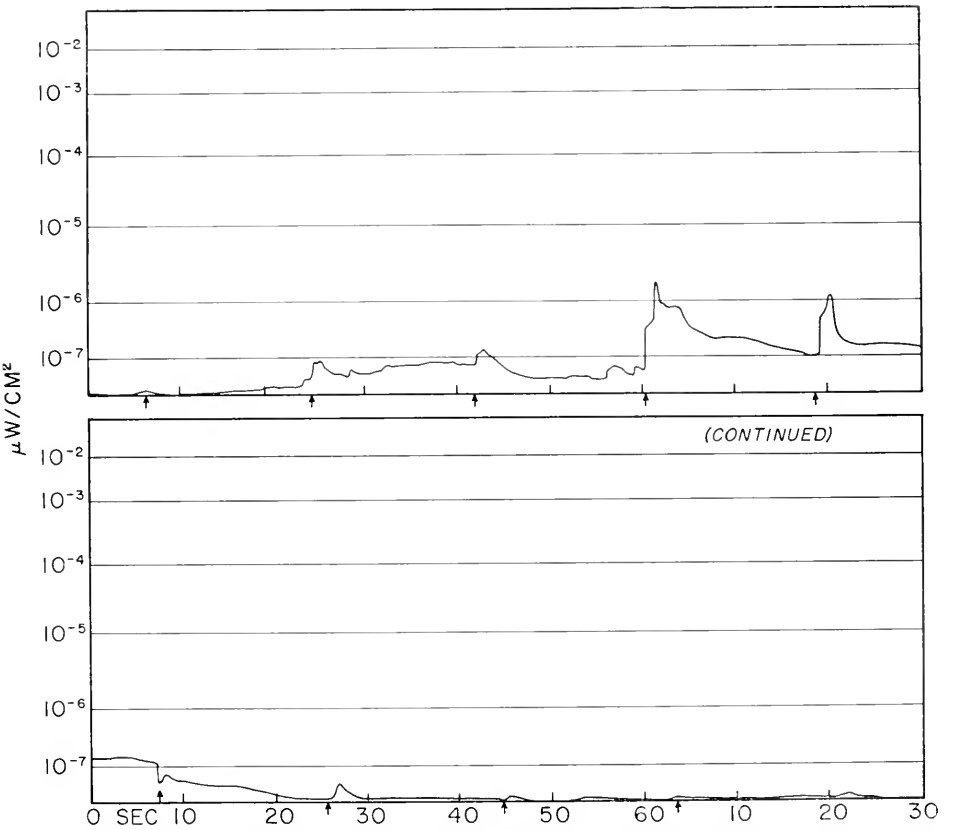

FIGURE 6. Luminescence of a single *Meganyctiphanes norvegica* when stimulated in the electrode chamber. Arrows along time scale indicate instant of stimulation. Chart speed is 6 in./min. The stimulus (0.7 amp.) was as strong as any ever used for *Metridia*. Low intensity of luminescence is notable in comparison to *Metridia's* bright flash.

were almost surely due to *Metridia*. Even using a maximum stimulus (1.1 amp.), *Meganyctiphanes* never produced a response higher than 1×10^{-4} μw./cm.^{2} and it was generally much lower. Furthermore, the luminescent emission of *Meganyctiphanes* was usually a prolonged irregular glow (Fig. 6). The *Metridia*, by contrast, always gave a single flash that appeared as a perpendicular spike on the slow-speed record (see Fig. 7). Using these two criteria, a reasonable interpretation of the records could be made.

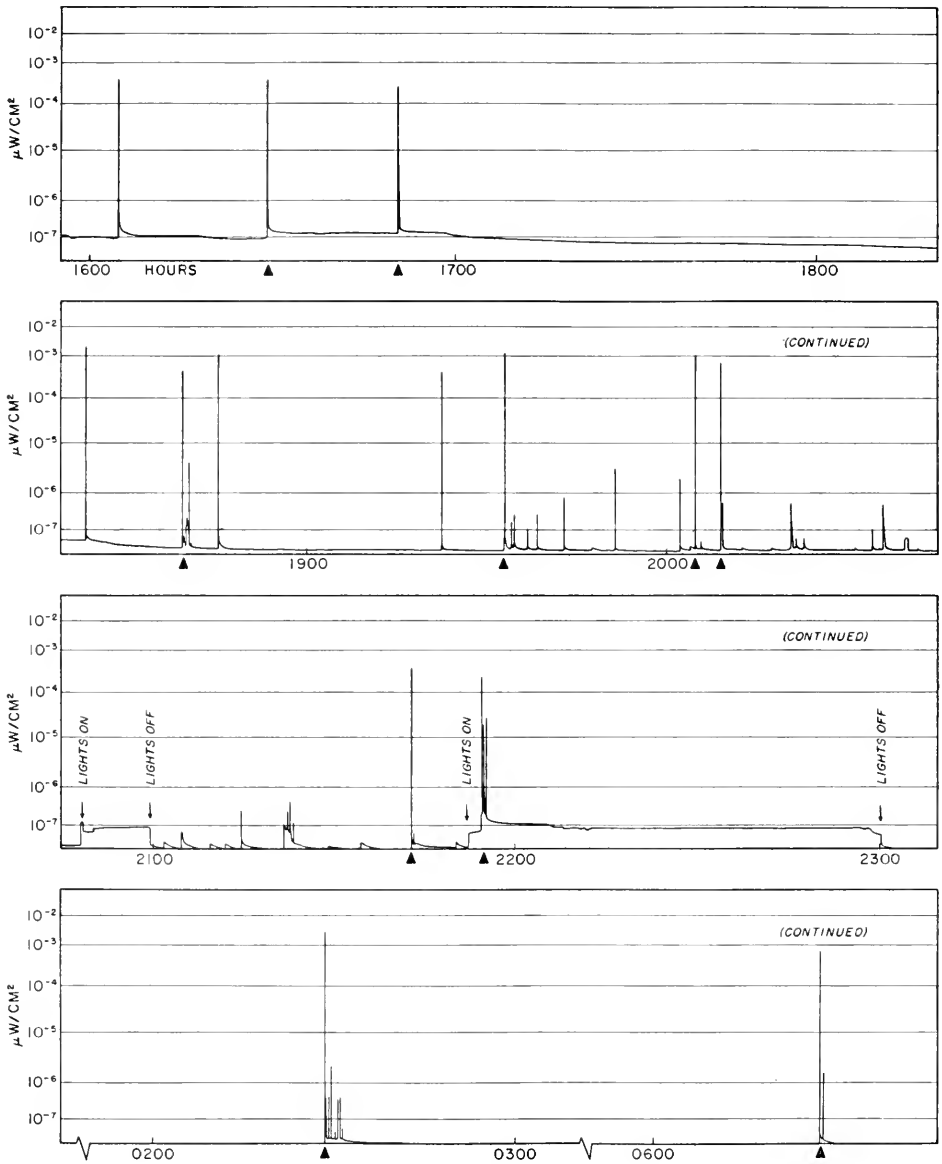

FIGURE 7. Record of behavior experiment 3 (see Table I). At the chart speed of 6 in./hr., luminescent flashes appear as spikes indicating maximum intensity and time of occurrence. Solid triangles indicate successful predation under interpretation outlined in text. The decreasing background intensity between 1600 and 1900 hours is due to the setting sun which reduced the ambient light in the laboratory. The increased background at 2045–2100 and 2150–2300 hours was caused by lights in the laboratory used to monitor the recorder.

A sample record from an experiment with both prey and predator present is show in Figure 7. All the experiments are summarized in Table I. Experiments 6 and 7 in the table show quite clearly that the two species when separated from each other ordinarily do not produce any spontaneous luminescence. Only a single weak flash (4×10^{-7} μw./cm.^{2}), which may have been caused by some accidental mechanical stimulus, was observed for the group of *Metridia* alone. The *Meganyctiphanes* alone produced no luminescence at all. This corroborates Mauchline's (1959) observation that *Meganyctiphanes* does not luminesce spontaneously in the laboratory except during the breeding season (Dec.–Feb.). On the other

TABLE I

Summary of behavior experiments.

The table shows the interrelationship between luminescence and predation in Metridia lucens. *For detailed explanation see text and Figure 8.*

Expt. No.	Date	Total time (hrs.)	Predator		No. of *Metridia*		Number of luminescent responses	
			No.	Species	In expt.	Eaten	Above 10^{-7} μw./cm.^{2}	Above 10^{-4} μw./cm.^{2}
1	8/12–13	15	1	*Meganyctiphanes norvegica*	9	8	30	15
2	8/17–18	8	1	*Meganyctiphanes norvegica*	10	3	17	3
3	9/14–15	16	1	*Meganyctiphanes norvegica*	10	10	33	14
4	9/16–17	13.5	1	*Meganyctiphanes norvegica*	11	5	41	21
5	9/18	10.5	1	*Meganyctiphanes norvegica*	10	4	23	1
6	8/13–14	15.75	—	—	10	0	1	0
7	8/14–15	9.5	1	*Meganyctiphanes norvegica*	0	—	0	0
8	8/11–12	7.25	2	*Parathemisto gaudichaudii*	10	0	1	0
9	8/19–20	15.5	1	*Parathemisto gaudichaudii*	9	0	0	0
10	8/18–19	15.5	2	*Thysanoessa inermis*	10	1	3	0
11	8/24–25	15	1	*Nematoscelis megalops*	10	0	1	0

hand, when the two species were placed in the same container, considerable luminescence was observed and some *Metridia* were eaten (experiments 1–5). Since most of the flashes showed up on the record as single spikes, some with an intensity greater than 10^{-4} μw./cm.^{2} (see Fig. 7), it was concluded that the copepod was primarily responsible for the display.

On the original records (copied in Fig. 7), it was possible to distinguish two kinds of single spikes, ones representing only a single luminescent flash and ones where several tracings were actually superimposed. This latter kind represented several flashes of different intensities which occurred within an interval short enough (30–40 seconds) to prevent their resolution at the slow chart speed. Sometimes this multiple-flash sequence was spread out over a longer period of time and the smaller flashes were resolved on the record (*e.g.* 0230 hours in Fig. 7). The number of multiple-flash sequences was, in almost every case, exactly equal to the number of *Metridia* eaten. These sequences presumably represent a *Metridia's* capture (large flash) and subsequent struggle to escape (small flashes). The

remaining spikes on the record (caused by single flashes) are presumed to represent successful escapes by *Metridia*.

In order to determine if the mere mechanical disturbance of another organism in the container could cause luminescence, groups of *Metridia* were tested with several other species in the container placed in front of the photometer. A large *Parathemisto* (*Euthemisto*) *gaudichaudii*, a vigorously swimming hyperiid amphipod, did not induce any luminescence when placed with *Metridia* nor did it eat any (Table I, experiments 8 and 9). Similar results were obtained with the euphausiid *Nematoscelis megalops* (experiment 11). When *Thysanoessa inermis* was used (experiment 10) a few flashes were produced and a single *Metridia* was eaten during the experiment. A further test of the effect of mechanical stimulation was made by vigorously stirring the water in a beaker containing *Metridia*. Considerable disturbance was necessary before any flashing occurred and even the most energetic agitation elicited a maximum response of only 6×10^{-5} μw./cm.^{2}, less than one tenth of the highest responses shown in Table I and Figure 7.

Direct observation of predation was also attempted in order to determine the nature of the luminescence stimulus. An infra-red-sensitive "sniper-scope" (Edmund Scientific Co.) was used with the infra-red source and a focusing lens placed behind the experimental beaker so that the animals appeared in opaque profile against a light background. The small *Metridia* were not always visible with this optical arrangement but some individuals were seen to be carried toward the euphausiid by the currents set up by the larger animal's pleopods. Sometimes the *Metridia* would dart away before reaching the *Meganyctiphanes*, but at other times the copepod would seem to come in contact with the euphausiid before darting away. On a few occasions the euphausiid started off as though in pursuit, but the actual act of capture was never observed.

These observations are in general agreement with those of Mauchline (1959) who found *Meganyctiphanes* capable of filter-feeding on organic detritus and even sucking into the "food basket" individual copepods (*Paraeuchaeta norvegica*) and *Sagitta* by lateral-ventral movements of the thoracic limbs. The animal can also seize larger objects by raptorial movements of the appendages but in the laboratory "no hunting or stalking of prey takes place" (Mauchline, 1959).

Discussion

Over the years there has been considerable speculation regarding the role of bioluminescence in the life of various marine organisms. In higher marine forms, luminescence has been found associated with either mating behavior, feeding mechanisms, or defense. Among planktonic species, however, there is less agreement as to its functional significance. Besides the three interpretations given above, it has been suggested that this phenomenon may often be coupled with other life processes in lower animals and therefore might have no function of its own (Russell and Yonge, 1928; Harvey, 1929). It has also been suggested that luminescence in planktonic and sessile creatures may serve as a "burglar alarm," thereby revealing a predator to its own enemies along the food chain (Burkenroad, 1943).

From the results of the behavior experiments with *Metridia*, it is apparent that there is some relationship between luminescence and the act of predation. Since the exact nature of the stimulus is still unknown, it is impossible to determine

positively which, if any, of the above hypotheses is applicable. Nevertheless, some of the possibilities may be eliminated.

Any functional use of luminescence involving species recognition, such as mating display or warning systems to other individuals of danger, is doubtful because *Metridia* probably does not have an adequate image-forming eye. Of the remaining speculations presented above, the authors currently feel that the defense mechanism is the one most consistent with the experimental results. However, Burkenroad's hypothesis is not specifically ruled out.

The reasons for favoring the idea of an escape mechanism arise from: (1) certain of the physical and physiological characteristics of *Metridia's* luminescent emission, and (2) a unique pattern of behavior associated with luminescence in this copepod.

The maximum intensity of *Metridia's* luminescence is surprisingly brilliant. At the working distance (18 cm.) used in this study the flash was of the same order of magnitude as that of certain coelenterates and of the crustacean *Euphausia pacifica*, and greater than that of the teleost *Myctophum punctatum*, all measured at 1 cm. (Nicol, 1960). The duration of the flash is long and its spectral composition is similar to the spectrum of the transmission of light through sea water with the maximum of the two curves at nearly the same wave-length. It has also been shown that *Metridia* has an extremely short lag time between stimulus and response. The animal recovers quickly after stimulation and fatigues rather slowly on repeated stimulation, even after several weeks without food, suggesting that the ability to luminesce is important enough to the organism to be maintained under adverse conditions. All these characteristics of *Metridia's* luminescence, both physical and physiological, would certainly be selectively advantageous to the animal if its luminescence functioned as an escape mechanism.

The most significant evidence for the defense mechanism hypothesis, however, comes from observations of the behavior of single *Metridia* stimulated in the electrode chamber. On stimulation a point of luminescence was immediately produced and then in the majority of cases the animal appeared to dart off into the dark, leaving a bright luminescent spot at its original position and sometimes a trail of tiny luminescent specks that soon disappeared. Although the animal itself could not be seen during this reaction, the agitation of the water gave a clue to its behavior and its new position could be verified by passing a second electrical stimulus through the water and observing the new location of the resulting luminescent flash. The original luminescent emission remained a more or less discrete point of light for some seconds after stimulation.

Such a behavior pattern appears to the authors to indicate the manner in which *Metridia* escapes from *Meganyctiphanes*. Although the precise role that luminescence plays in this escape mechanism is still unknown, two speculations are possible. The luminescent emission may startle the attacker, interrupting its feeding procedure, or it may merely function as an attractive decoy. In either case, the *Metridia's* rapid departure from the spot where it had luminesced would complete the escape.

The possibility that luminescence only occurs when the *Metridia* is actually captured is not entirely eliminated. More definitive proof must await the elucidation of the specific stimulus that induces luminescence. Nevertheless the evidence pre-

sented here indicates that luminescence functions on the behavioral level as an escape mechanism for *Metridia*. It would then seem probable that luminescence, which is of such widespread occurrence in the oceans, may well have survival value in defense against predation in some similar manner for many other animals of the plankton.

Summary

1. Skin glands believed to be the source of luminescence were found on the anterior portion of the head, on the last thoracic segment, and on the posterior margins of each segment of the abdomen.

2. The maximum intensity of the luminescent flash was 1.2×10^{-3} μw./cm.^{2} (at 18 cm.). The flash rose rapidly to peak intensity and then decayed slowly. The total duration of the flashes with peaks greater than 10^{-4} μw./cm.^{2} ranged from 3 to 50 seconds.

3. The peak of the luminescence spectrum occcurred at 482 mμ and the curve fell off to one-half the maximum value at 440 mμ and 525 mμ.

4. The ability of *Metridia* to luminesce on stimulation was found to be largely unaffected by prolonged laboratory culture. Starvation had little effect on the luminescence for the first three weeks and there was never any inhibition by previous light- or dark-adaptation.

5. With an increase in the strength of the electric stimulus from 0.3 amp. to 0.7 amp., the intensity of the luminescent flash was found to increase. With pulses stronger than 0.7 amp. no change in intensity was recorded but the number of successive responses to repeated stimuli was reduced. Duration of the pulse had little effect on the intensity or the number of successive responses.

6. *Metridia* showed a lag time of 8–10 msc. to the beginning of the luminescent response. The lag time to the peak of the luminescent response varied from 20 to 60 msc.

7. There was no spontaneous luminescence produced by groups of *Metridia* under conditions of constant darkness. However, the presence of certain planktonic predators, most notably *Meganyctiphanes norvegica*, caused a brilliant display of luminescence. The number of flashes attributable to *Metridia* was always greater than the number of *Metridia* eaten by the predator. There was little evidence that the luminescent euphausiid, *Meganyctiphanes*, flashed spontaneously either in the presence or absence of its prey.

8. Observations on the behavior of *Metridia* during and just after luminescence suggest that the flashing may be involved in an escape mechanism, but the precise effect of the light on the predator has not been determined.

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THE PHYSIOLOGICAL CONTROL OF WATER INGESTION IN THE BLOWFLY[1]

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Because of their small size and terrestrial habitat, insects constantly face a pressing problem in water conservation. Recognition of this fact has stimulated many investigators to study routes and mechanisms of water loss and adaptations for its prevention (*cf.*, Edney, 1957). Some of these studies have dealt with behavioral adaptations, such as humidity preferences, that decrease water loss. With regard to the uptake of water, however, little is known apart from observations of direct water uptake through the integument at very high humidities by some insects. One isolated study of a fly (sp. ?) by Bolwig (1953) showed a negative correlation between response to water by drinking and the vapor pressure of the blood. Otherwise there appears to be no experimental work on the control of drinking by insects (Leclerq, 1946; Edney, 1957). Accordingly, experiments were undertaken to reveal the factors underlying thirst and water ingestion in the blowfly, *Phormia regina* Meigen.

Methods

The blowfly was chosen for study because a considerable body of knowledge relating to its sensory physiology and feeding exists. The flies employed were taken from a culture maintained in these laboratories since 1947. Flies were desiccated or humidified by storage in a sealed vessel containing calcium chloride or water, respectively. Measurement of the water intake of individual flies was carried out by surgically removing the crop after drinking was complete and weighing it. Preferences and volumes consumed of test solutions over periods of 24 hours and longer were measured by the method of Dethier and Rhoades (1954). Injections of fluid into the haemocoel were carried out as before (Evans and Dethier, 1957). Flies were designated water-positive or -negative on the basis of a uniform response to three tests being obtained; a positive response consisted of proboscis extension upon tarsal contact with water. Bleeding was accomplished by cutting off the prothoracic legs close to the thorax and expressing the blood by gentle compression of the thorax.

Results

Sensory control of drinking

A fly that has been deprived of water will respond to it in a variety of ways: it will orient from a distance to a locus of high humidity; it will extend its proboscis

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in response to stimulation by water vapor; it will extend its proboscis in response to water applied to the tarsi or labellum; it will open the labellar lobes and commence sucking in response to water applied to the labellum. After a period of time which is related to the extent of previous water deprivation, drinking will cease and the fly will become refractory to further stimulation by water.

The sense organs on the mouthparts whose stimulation initiates drinking are the same chemoreceptive hairs that respond to sugars (Dethier, 1955). Of the three sense cells that innervate the hair, one is a mechanoreceptor, one responds preferentially to salts, and the other to sugars. Wolbarsht (1957) reported that distilled water applied to certain of the hairs caused a firing of nerve impulses in both chemosensory cells. Each discharged at an initially high rate but adapted rapidly (one had often ceased firing at the end of thirty seconds).

TABLE I

The effect of desiccation on water and sugar consumption by the blowfly. Each value is based upon tests with thirty individual flies. The figures in parentheses represent ranges.

Experiment number	Treatment of flies	Av. wt. (mg.) of fly minus wings	Av. duration (sec.) of sucking of each solution presented successively		
			H_2O	0.1 *M* sucrose	1.0 *M* sucrose
1	Three-day-old flies fed once on 0.1 *M* sucrose, starved 24 hrs., then desiccated 24 hrs.	12.1 (10.2–16.9)	24 (6–52)	46 (23–73)	35 (17–62)
2	Three-day-old flies fed once on 0.1 *M* sucrose, starved 24 hrs., then desiccated 24 hrs.	11.0 (9.7–17.0)	—	—	54 (40–90)
3	Three-day-old flies fed once on 0.1 *M* sucrose, starved 24 hrs., then humidified 24 hrs.	23.4 (19.6–27.7)	0	0	38 (20–60)

As has been reported to be the case for the ingestion of sugar solutions (Dethier *et al.*, 1956), not only the initiation but also the maintenance and termination of water ingestion are dominated by the input of the taste receptors. Water ingestion is driven by the input, and adaptation finally terminates it. This control can be demonstrated by stimulating one side of the labellum until the receptors adapt, whereupon drinking ceases, and then stimulating the remaining receptors, whereupon drinking resumes.

In addition to this primary control of drinking by chemosensory input, other factors are involved in the ingestion of water and nutrients.

Conditions modifying drinking

Three conditions were considered as possibly modifying drinking behavior: namely, starvation and feeding, unacceptable contaminants, and desiccation. Each of these was investigated in turn.

Starvation and feeding. The average life span of *Phormia* in the absence of food is three days; accordingly, experiments on starvation were perforce limited to this period. The daily intake of water of fourteen individual flies was measured. No consistent change in intake was noted over this period. In another experiment designed to control the effects of desiccation, thirty flies were starved 24 hours and then placed in a humidifier for 24 hours. A control sample was placed in a desiccator. The results are summarized in experiments two and three of Table I. The humidified flies did not drink even though they had starved 48 hours. In a more drastic experiment, flies were kept in the humidifier until the last had died of starvation five days later. All remained negative to water till the end. It can be concluded that starvation does not induce drinking as long as water loss is prevented.

TABLE II

The effect of NaCl on water intake by the blowfly. Each value is based on tests with ten individual flies. The figures in parentheses represent ranges.

Materials available to the fly	Mean volume of water consumed in 3 days (μl.)	Mean volume of NaCl consumed in 3 days (μl.)	Mean total fluid intake in 3 days (μl.)
Water (no food)	29 (12–39)	—	29
Water and dry sucrose	16 (12–61)	—	16
0.1 *M* NaCl and dry sucrose	—	23 (9–36)	23
0.5 *M* NaCl and dry sucrose	—	19 (12–38)	19
0.5 *M* NaCl, dry sucrose, and water	12 (5–18)	6 (4–7)	18
1 *M* NaCl and dry sucrose	—	12 (3–24)	12
1 *M* NaCl, dry sucrose, and water	14 (6–18)	4 (0–5)	18
2 *M* NaCl and dry sucrose	—	3 (0–12)	3
2 *M* NaCl, dry sucrose, and water	15 (3–24)	4 (0–7)	19

In order to test whether or not dry food as the only source of nutrition causes an increase in drinking, the daily water intake of individuals of two groups was measured over a three-day period. One group of flies had free access to water but had no food. The other group had free access to water and to a lump of sugar. The results are summarized in the first two lines of Table II. Contrary to expectations, the ingestion of food did not bring about increased drinking even though part of the process of eating solid food involves dissolving it in saliva. Whether the reduction of water intake in the presence of sugar is real is not known.

As a variant of the preceding, the experiments described in Table III can equally well be done on flies that have been made water-positive by storage in a sealed vessel in contact with anhydrous glucose. They have the opportunity to feed continuously, and yet become positive to water after a time. The mechanism is very likely the same as that of storage with calcium chloride, but the latter is a better desiccant.

Contaminants. To test the effect of unacceptable taste stimuli on drinking, several series of experiments involving the addition of sodium chloride to water were undertaken. In one experiment, flies were kept in individual cages equipped with two pipettes, one of which contained water, the other a salt solution. The salt solutions paired with water ranged in concentration from 10^{-5} *M* to 5 *M*.

The volume of each solution imbibed was measured each day. Results are summarized in Figure 1, from which it can be seen that the volume of salt solution drunk decreases as the concentration increases. Concurrently, the quantity of water drunk increases so that the total fluid intake is approximately constant.

Under more rigorous conditions where the fly was provided with a salt solution as its only source of fluid, higher concentrations (as judged by the volume imbibed) were tolerated than when water also was present (Table II). As the

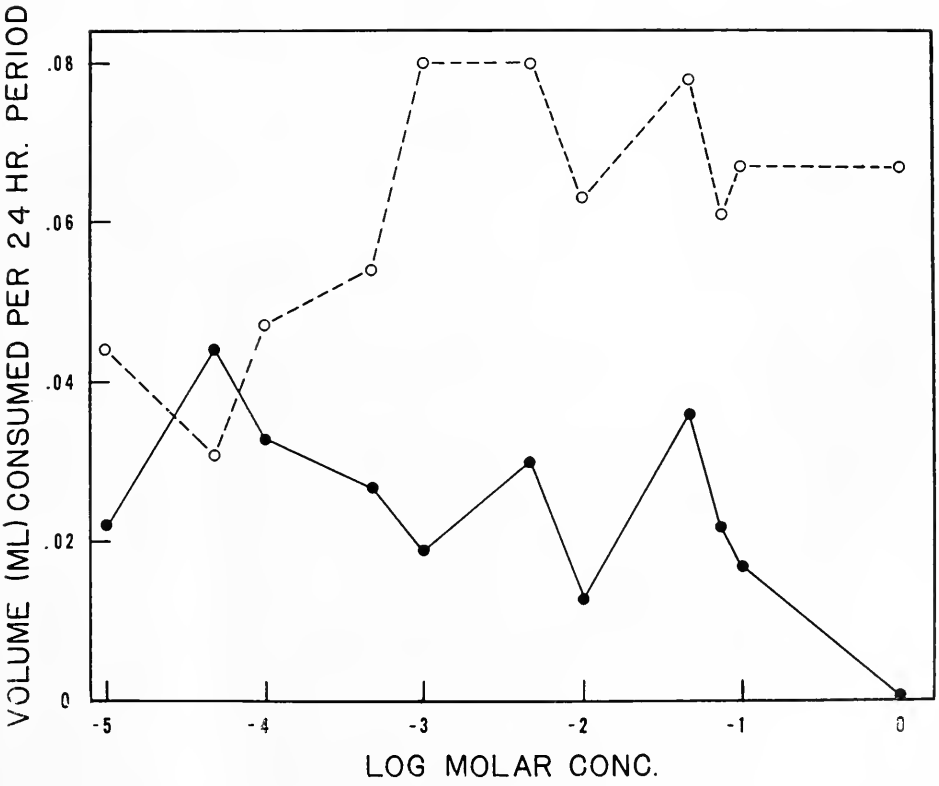

FIGURE 1. Volume of different concentrations of sodium chloride ingested per fly per 24 hours in a two-choice situation. Solid line, sodium chloride. Broken line, water.

concentration of salt was increased, however, the amount of fluid imbibed decreased.

In still another series of experiments, 60 flies were tested for their responses to salt solutions applied to the tarsi, placed in a desiccator with a supply of dry sugar for food, then retested periodically until death. The results obtained with 1.0 *M* NaCl illustrate the trend of events. Before being placed in the desiccator none of the flies gave any response to this solution. As water loss increased, they first would extend the proboscis when the tarsi were stimulated but would not open the lobes of the labellum; later they would open the labellar lobes but not drink; still later they would drink for a few seconds; finally, the drinking time

would increase. In short, as desiccation increased, the rejection threshold of the tarsi to salt rose followed by a rise in the rejection threshold of the mouthparts.

Desiccation. Implicit in all of the foregoing experiments is the idea that water loss powerfully affects drinking. The experiments summarized in Table I demonstrate the effect of desiccation for 24 hours on response to water. They show further that the state of water balance also affects the amount of liquid food ingested. Since desiccated flies take more liquid food than do humidified flies, the response is clearly directed toward the acquisition of water.

TABLE III

The effect of injections on responses of the blowfly to water

Experiment number	Number of flies	Response before treatment	Injected†	Per cent negativ after treatment
1	35	+	2.5 μl. water	6
2	52	+	8 μl. water	85
3	27	+	2 μl. water	7
			2 μl. water	22
			2 μl. water	70*
4	26	+	3 μl. water immediately	58
			at 10 minutes	58
			at 60 minutes	54
5	120	−	3 μl. 2 *M* glucose	82*
6	116	−	7 μl. 2 *M* glucose	96*
7	29	−	2.4 μl. 4× saline	100
8	53	+	3 μl. 4× saline	55
9	69	+	3 μl. 2 *M* glucose	55
10	48	+	3 μl. 2 *M* glucose in saline	56
11	40	+	6 μl. 2 *M* glucose in saline	85
12	92	+	4 μl. mineral oil, moribund at 15 min.	66
13	14	+	Fed 2 *M* glucose 0–60 minutes	100

* Responded subsequently to 0.1 *M* sucrose.

† Water indicates distilled water; the saline was Bodenstein's[10]; 4× indicates saline four times more concentrated; exps. 1–9 from Evans (1961).

The control of water responsiveness

A series of injection experiments was undertaken to assess the effect of blood osmotic concentration on the responsiveness of flies to water (Table III). Injections of water rendered positive flies negative to water (exps. 1–4). The percentage made negative was a function of the volume injected (exps. 1–4). The effects of repeated injections were additive (exp. 3), and the effect was immediate (exp. 4). Next it was found that injections of even huge volumes of highly concentrated solutions did not produce responsiveness to water (exps. 5–7). But these same hypertonic injections could abolish water responsiveness (exps. 8–11), indicating that volume and not osmotic or dilution factors was the significant feature. Even mineral oil, before its toxic effects were apparent, blocked responsiveness to water

(exp. 12). Ingestion of nearly saturated sugar solutions (exp. 13) abolished the water responses.

These results suggested blood volume or pressure as the agent regulating water responsiveness; but since responsiveness had not been induced in any case, the effects could have been unspecific even though responsiveness to sugar was not affected in the few cases tested (exps. 3, 5, 6). Consequently, it was crucial to reduce blood volume and thereby induce water responses. The crude procedure of cutting off the abdomen with crop did not make flies positive; however, bleeding did. If a population of flies was desiccated until some responded to water, bleeding made most of the remainder (45 of 53) responsive to water. Vigorous responses were obtained as quickly as a fly could be tested after the bleeding (a few seconds). Bleeding did not alter the response of already responsive flies (12 of 12). And in the case of flies given water to satiation, bleeding induced water responses in only a few (4 of 13). Apparently there is a threshold such that blood volume must be reduced below some particular level before the treatment is effective.

In another series of experiments, the recurrent nerve was cut in 80 flies which were responsive to water before the operation and an additional 50 flies that were satiated. Sixty per cent of the former became bloated; 50% of the latter.

Removal of the corpora allata in 30 flies produced no abnormal water intake. Removal of the median neurosecretory cells of the brain from 60 flies caused bloating in four.

Where bloating occurred, the behavior pattern was characteristic. Flies immediately began drinking and continued to drink intermittently over a period of one to two hours. Thereupon no further drinking occurred although the proboscis was repeatedly extended.

Discussion

As in the case of the ingestion of sugars (Dethier *et al.*, 1956), the act of drinking appears to be controlled primarily by sensory input. Stimulation of tarsal or labellar taste receptors by water elicits reflexive extension of the proboscis and sucking. When adaptation (central and peripheral) has proceeded to some level, ingestion is terminated; the stimulus becomes ineffective.

The behavioral threshold of the fly to water, however, varies as a function of water balance. Specifically, water responsiveness can be abolished by injections into the haemocoel and can be induced by bleeding. The chemical nature and the concentration of injected material do not matter; the effect is related only to the volume and can be altered by injection or bleeding. Since these treatments are fully effective as quickly as can be tested, neural mediation of the effect is indicated, probably via a mechanoreceptor that signals distension or pressure. Accordingly, the input of the mechanoreceptor must act somewhere to set the level of taste threshold to water.

The behavioral threshold to water is also affected by cutting the recurrent nerve immediately anterior to the hypocerebral ganglion. Flies undergoing this operation became bloated on water. Dethier and Bodenstein (1958) reported that flies in which the recurrent nerve had been cut became bloated on sugar solutions. They interpreted this effect as interference with the elevation of sugar threshold that normally follows sugar ingestion. Evans and Barton Browne (1960) confirmed

the fact of hyperphagia following recurrent nerve section but found that the sugar threshold still rose in the normal fashion. Incidental observations suggested to them that the effect might be due to a hypersensitivity to water. Day (1943) and Thomsen (1952) had observed polydypsia in some flies after removal of the corpus allatum. However, allatectomy frequently involves a variable degree of injury to the recurrent nerve which could account for the low incidence (ca. 10%) of bloating observed by these workers. We have not been able to produce polydypsia as a result of allatectomy. Removal of the medial neurosecretory cells of the brain also sometimes results in bloating, but this does not necessarily imply a hormonal mechanism any more than a neural one since these cells are in neural connection with the recurrent nerve.

Sensory control (*i.e.*, sensory input to drive and adaptation to stop) of drinking still operates in polydypsic flies so that an operated fly becomes bloated through repeated rather than continuous drinking.

It was observed that operated flies kept in contact with water for long periods of time no longer imbibed any even though they continued to respond feebly for more than 24 hours. If such flies were presented with sugar, they resumed vigorous sucking until the crop and abdomen burst. There is obviously in the fly bloated on water a strong pressure opposing further intake. The water stimulus is not intense enough to produce effective sucking, but sugar, a stronger acceptable stimulus, can still produce effective sucking. This suggests that back-pressure was responsible for blocking continued imbibition of water.

Since the behavioral threshold to water is affected by bleeding, by injection, and by cutting the recurrent nerve, the simplest explanation is that the recurrent nerve carries the neurons that signal blood volume or pressure and set water sensitivity. Elucidation of this point will have to await further knowledge of the finer details of structures innervated by the recurrent nerve and of connections with other parts of the nervous system.

At this point evidence obtained earlier regarding the control of ingestion of sugars bears on the possible mechanisms and greatly complicates the interpretation. On the basis of much evidence, Dethier (1955) postulated that each of the two chemosensory neurons in the receptor subserved one of the taste qualities, acceptance and rejection. According to this hypothesis, water and sugar would be ingested because they stimulate the one neuron, and salts would be rejected because they stimulate the other (termed, respectively, *S* and *L* by Hodgson and Roeder, 1956). Subsequent electrophysiological studies have supported this view in general. Sugars do activate primarily the *S* fiber and salts the *L* fiber (Hodgson and Roeder, 1956). Furthermore, Wolbarsht (1958) and Tateda and Morita (1959) have shown that neither fiber exhibits appreciable spontaneous discharge, and Wolbarsht (1958) showed that there is no reason to believe that electrical responses of the two receptor cells influence one another. Consequently, the hypothesis explaining the qualities of taste is compatible with this evidence: discharge of the *S* fiber initiates and drives feeding, and discharge of the *L* fiber inhibits the feeding reflex somewhere beyond the sense cell level.

Taste thresholds to sugars vary with feeding and starvation, and the mechanism has been studied in some detail (Dethier *et al.*, 1956; Evans and Dethier, 1957; Dethier and Bodenstein, 1958; Evans and Barton Browne, 1960). All of the evi-

dence suggests that it is the presence of sugar solution in the foregut (exclusive of the crop) that sets the level of the sugar threshold. The detector in the foregut and the intervening processes have not yet been elucidated, but it was suggested (Evans and Dethier, 1957) that the final effect was to inhibit centrally the effect of *S* fiber discharge. The problem now arises as to how taste thresholds to water and sugar can be independently regulated as the present results show that they are.

Now it should be pointed out that the neural explanation of the two taste qualities is not really so simple as the discussion above and some of the literature suggests. Some data will be cited that show the unexpected complexities that have emerged from electrophysiological studies. Wolbarsht (1957) reported that both *S* and *L* fibers respond to distilled water. In addition to the two chemosensory cells, there is a third sense cell associated with the socket of a chemoreceptor hair (Dethier, 1955) that Wolbarsht (1958) has shown to be a mechanoreceptor activated by motion of the hair. The distal process of the cell does not enter the hair (Dethier and Larsen, personal communication) and therefore chemicals applied to the hair tip would not be expected to stimulate it. It is known that bending of a hair can evoke proboscis extension in a very starved fly (Dethier, 1955). Hodgson and Barton Browne (1960) reported that bending of a hair influences, albeit unpredictably, the electrical response of the *L* and *S* fibers to chemicals. The experiments dealing with ingestion of salt solutions place limitations on hypothetical interpretations of the water threshold mechanism. Since the sensory input due to water can drive the ingestion of more and more concentrated salt solutions as the fly is made more dehydrated, the sensory effects of water are balanced against those of salts, just as are the stimuli, sugar and salt.

In view of the data presented it seems to us that the hypothesis that water and sugar act on the same neuron is no longer tenable. Evidence of the existence of a specific water receptor is now being sought.

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FLIGHT AND SWIMMING REFLEXES IN GIANT WATER BUGS

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Loss of substrate contact or tactile stimulation initiates a "classic" flight reflex in insects (Fraenkel, 1932; Chadwick, 1953). Either one or both factors can operate to elicit the reflex; flight ceases when the legs again make contact with the substrate. When giant water bugs were removed from substrate contact, they did not fly, but instead swam. If they stopped, they would begin again with direct tactile stimulation. In short, they appeared to swim in those situations in which terrestrial insects fly. Although a few of the water bugs eventually flew, they did so only after a considerable period; during this time they were swimming. This study is an attempt to analyze the swimming and flight reflexes of these giant water bugs.

Materials and Methods

Two species of giant water bug were used, *Lethocerus americanus* and *Benacus griseus*. The bugs were captured by light trap (a sheet and a Mercury Vapor bulb, General Electric H100 L4) between April and September, 1960, on the Edwin S. George Reserve, the wildlife reserve of the University of Michigan, Livingston County, Michigan. A total of 60 animals were used; they were kept in the laboratory on a diet of small fish.

Giant water bugs are large (about 4.5 to 6.5 cm. long) dorso-ventrally flattened predaceous insects. The forelegs are raptorial with enlarged femora and bear only a single tarsal claw; the middle and hind legs are adapted for swimming; they are flattened and bear hairs so arranged as to be raised during the power stroke of the leg and depressed during the forward stroke. The swimming legs have the usual two tarsal claws. Respiration is accomplished with two retractable tubes which protrude from the posterior end of the abdomen (Fig. 4).

In the analysis of swimming and flight reflexes, the bugs were suspended from an applicator stick using a mixture of paraffin, beeswax, and resin to attach the stick to the prothorax. They were then placed in the air stream of a wind tunnel and given a stick to hold which served as a contact stimulus for the legs. The wind tunnel was made from wood and light cardboard and included a cardboard honeycomb baffle to cut down turbulence which, as determined by smoke, was slight; the diameter of the tunnel mouth was 10 cm. For wind a fan was used, the speed of which could be controlled by a rheostat. Wind speed was calibrated with a Taylor Briam's Type Anemometer (No. 3132); it ranged up to 7.0 m./sec. In certain experiments small jets of water or air, which were directed by attaching a glass tube to a rubber hose, were used; no attempt was made to measure the velocity of these.

Swimming

Loss of substrate contact almost invariably elicited swimming movements. The rate and duration of these movements varied. The initial rate for 19 bugs in quiet air ranged from 120 to 320 strokes per minute with an average of 206; the duration ranged from 6 seconds to more than 180 seconds with an average of 51 seconds. This swimming response was clearly distinguishable from haphazard movement; the forelegs were carried forward of the head, and in intense swimming they were stretched forward almost full length. The abdomen was raised, and the middle and hind pairs of legs were usually protracted and retracted (see Hughes, 1952, for definitions) simultaneously and not alternately as reported by Lauck (1959) for a different species. Although alternation was never observed, it was noted that the two pairs were sometimes not quite simultaneous. The two legs of each pair operated simultaneously as reported by Lauck.

Swimming could be stopped by giving the bug a stick to hold. Contact with any one tarsus was sufficient; when the bug made contact, the ipselateral leg reached for and grasped the stick. Swimming also ceased with contact on other parts of the leg, *e.g.* tibia and femur, especially if tension was applied; Diakonoff (1936) reports similar results in a flying cockroach. Sometimes, however, the bug dropped the stick or "walked" off it and continued to swim. If the stick was removed carefully, leaving the legs folded under the body, the bug usually remained motionless. Swimming in such a situation could be initiated by gently lowering the legs until they were outstretched. Bugs also stopped swimming on occasion when they presumably saw the stick in front of them, reaching out and seizing it with the forelegs. Touching any part of the forelegs resulted in attempts to grasp the stick.

In experiments testing the effect of increasing wind velocity, the bugs were holding a stick which was removed at each higher velocity; it was returned when the bug stopped swimming. After 30 seconds the velocity was increased by about 1 m./sec. and the process repeated. Rate and duration of swimming increased up to a point and then decreased; this decrease will be discussed in greater detail below. The lowest wind velocity measured, 0.5 m./sec., was sufficient to increase rate and duration in 50% of the bugs; for the remainder higher velocities were needed. Twenty per cent of the bugs reached their maximum rate at 1.6 m./sec.; maxima were attained up to 6.7 m./sec. Maximum durations occurred from 0.5 to 7.0 m./sec., the total range used in these experiments. Except for one bug which gave a brief burst of strokes at around 400/minute, the greatest rate of swimming observed was 320 strokes/minute which was reached by half the animals; they could not be induced to swim faster. If wind was blown on an animal from the side, it often responded with compensatory movements of the legs on the opposite side. Figure 1 shows rate and duration with increasing wind speed for three representative bugs.

If the bug was holding an object, wind alone initiated swimming and consequent dropping of the stick in 25% of the cases. Usually, however, swimming occurred only when wind was combined with loss of substrate contact. Ordinarily loss of contact was the significant stimulus, but often the few bugs that would not swim with just loss of contact could be induced to do so if wind was simultaneously applied. A bug that had been swimming, but had stopped, would start again when wind was applied.

In addition to loss of contact and wind, direct tactile stimulation, *e.g.* of the abdomen, and vibration or movement of the bug while suspended also caused swimming. Any movement, whether up and down or to and fro, and any vibration, caused either by tapping the stick to which the bug was attached or pounding the

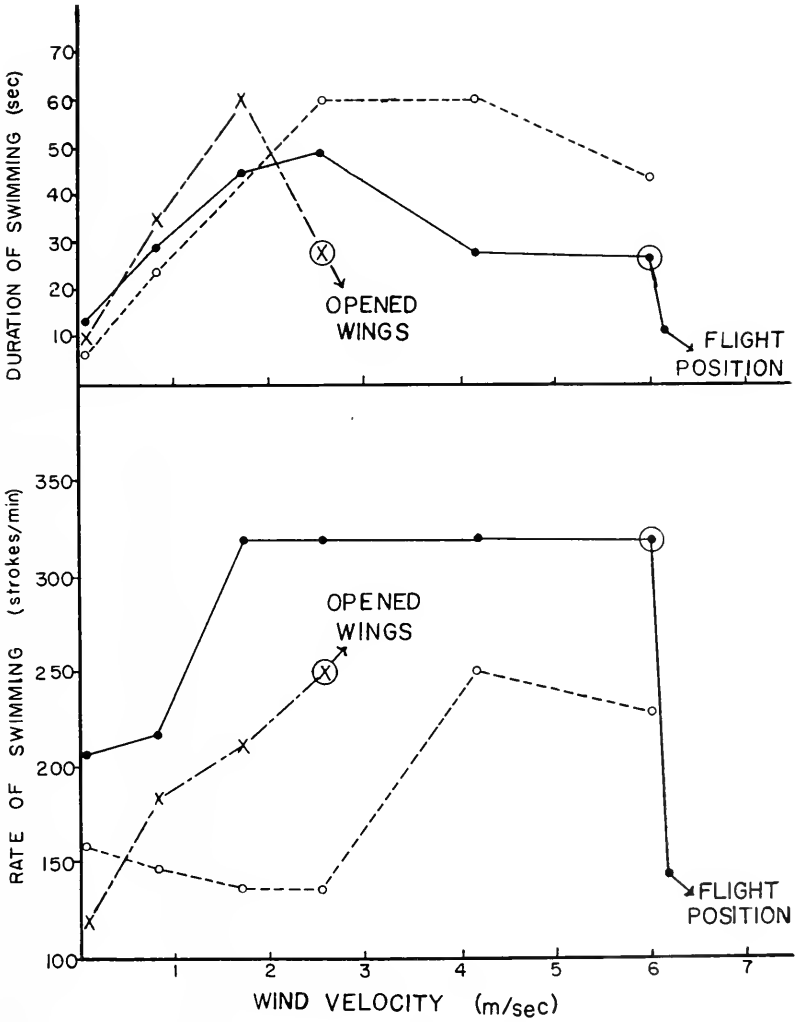

FIGURE 1. Graphs of rate and duration of swimming plotted against wind velocity for three representative bugs. Circled points indicate beginning of first noticeable flight preparation movements; these were not observed in one animal.

table with the fist, elicited the swimming. Fraenkel (1932) reports that flight in *Vespa, Calliphora, Apis, Schistocerca,* etc. resulted from a blow on the abdomen, and Diakonoff (1936) found that cockroaches flew if allowed to fall, a phenomenon he termed a "fall reflex."

Not too surprisingly, suspended bugs also swim when placed in water, although the swimming is very quickly adapting, lasting only a few seconds. Swimming can be further induced by directing a current at the bug, moving the animal through the water, or by taking the animal out of the water, but again the swimming is quickly adapting. By far the most rapid swimming comes when the bug is allowed to touch some object with its forelegs which it then attempts to grasp. This too adapts, but after a longer time. Swimming also follows on occasion when the bug presumably sees an object in front of it. A water jet directed at the bug from one side causes some compensatory movements of the legs on the opposite side. The same results are observed when the bug is rotated through the water in a small circle; this phenomenon was also recorded by Hughes (1958) in *Dytiscus*.

Sense organs mediating swimming

Fraenkel (1932) found that his insects would not stop flying when their tarsi were removed, which led him to believe that a receptor sensitive to contact was located there. Diakonoff (1936), however, was unable to find sensilla on the tarsal claws of the cockroach and found that in addition to the tarsi, stimuli on the tibiae, femora, and even coxae could stop flight. Since water bugs swam on loss of substrate contact, presumably a mechanism similar to that eliciting flight in the above cases is involved. It was found that swimming ceased either with tarsal contact or with stimuli on the tibiae or femora.

Touch receptors seem to be implicated in the instance of more rapid swimming when the bug touches an object with its forelegs. The leading edges of the femora of these bugs are covered with an extensive sensory area, and this area when touched is especially apt to elicit increased swimming. The exact nature of these receptors and others at the same spot affords a promising line of future investigation.

Specific receptors, eliciting swimming in response to either air or water currents, have not been located. With the forehead, eyes, hair beds behind the eyes, hair beds at the bases of the fore femora, and hair beds at the junction of the pro- and mesothorax covered individually or all together with paraffin, the swim reflex did not appear to be hindered in any way. Although the antennae were not removed completely, located as they are in grooves under the eyes, the bugs still began to swim in currents after an operation to destroy the brain, indicating that neither the antennae nor for that matter any other head receptors innervated by the brain are mandatory for the initiation of this swimming. It is suspected that swimming in response to current can be initiated by any of several receptors located on the body. Certainly the body possesses many groups of hairs located at various joints and articulations, and that several of them may be "current receptors" is indicated by the fact that a bug will swim in a current coming from virtually any direction.

When the bugs are in water, however, there do seem to be specific sense organs which initiate swimming. The first hint of such receptors came while a bug whose nervous connectives had been severed between the pro- and mesothoracic ganglia, the cut being made just posterior to the forelegs, was being studied. Such an insect loses all muscle tone posterior to the cut, and the legs hang limply. When this bug was put in water, the legs began to protract and retract slowly and rhythmically with enough force to give the bug some forward momentum. Further observation revealed that this swimming commenced only when the legs had floated

up so as to be extended almost laterally from the body. Swimming was also observed when the bug was held upside down and the legs were in almost the same position as when floating, but this was never more than a few strokes.

In attempts to locate more closely the receptors responsible for this swimming response, the leg segments and joints from all four swimming legs were removed successively with the following results:

(1) Removal of first tarsal segment and joint between the two tarsal segments —bug swam, but kept legs rather sharply bent at tibio-femoral joint.

(2) Removal of second tarsal segment and tibio-tarsal joint—bug swam with shorter and more rapid strokes.

(3) Removal of tibia and tibio-femoral joint—bug swam with short, rapid, and choppy strokes that were not well co-ordinated.

These results seemed to indicate that the receptors responsible for the swimming response were located somewhere proximal to the tibia. Because of the flotation of the legs which seemed to be necessary, the location was suspected to be at either the coxo-trochanteral or femoro-trochanteral joint; the former location appeared to be the more likely. Hair beds are located on the trochanters at this joint just distal to the trochanteral condyles (Fig. 2). When the legs hung down as they did when the bug was suspended, these hair beds were covered by membranous cuticular folds present on the coxae; when the legs floated in water, the hair beds were uncovered.

In bugs with the connectives severed between the pro- and mesothoracic ganglia, the trochanteral hair beds on various legs were burned with a hot needle. If these were destroyed on all four swimming legs, the bugs showed no response when placed in water; if the hair beds on the middle legs were destroyed, the hind legs still swam, with the converse true if the hind leg hair beds were burned. In a bug lacking the hair bed on one middle leg, the other three legs swam in the usual fashion while the operated leg gave strokes on each alternate stroke of the rest; with the hair beds on three legs burned, only the single intact (hind) leg gave swimming strokes, and these were slower than previously. In bugs with the central nervous system intact, when the hair beds were destroyed on all four swimming legs, walking was more or less as usual, but the bugs seemed to have difficulty gaining traction on surfaces where normal animals had no difficulty. In both water and air, swimming strokes were short and jerky; in air, swimming proved also to be more difficult to induce than in normal individuals. These hair beds thus appear to be intimately involved with swimming and co-ordination of leg movements.

The trochanteral hair beds are apparently excited by the cuticular folds which cover them when the legs hang down or are folded beneath the body. As the legs float up when the bug is in water, these folds roll back progressively until the hair beds are uncovered when the legs are extended laterally. Presumably, then, when stimulation of the hair bed by the cuticular fold ceases, the leg begins to swim. Possibly direct contact with water prompts the swimming movements to some extent although this is not the only factor since inverted bugs with severed connectives also swim. Pringle (1938) described three hair plates on the leg of the cockroach, including one at the coxo-trochanteral joint, which he believed were also stimulated by a cuticular fold; the hair plates were incompletely adapting.

Because of the location and action of these sense organs, Pringle considered them "position" receptors. The action of the hair beds on the legs of the giant water bug seem to have an analogous function, *i.e.* registering the position of the legs until they finally reach swimming position, whereupon the swimming reflex is triggered.

This proposed action of the hair beds helps to explain some aspects of the bugs' behavior. As mentioned earlier, a suspended bug tends not to swim when its legs are folded under the body as when grasping a stick. This lack of response would, on the above explanation, be due to the covering of the hair beds by the cuticular folds. In nature the bugs cling to submerged vegetation; if they were torn free, the resultant flotation of the legs would provoke swimming and lead to regaining of foothold.

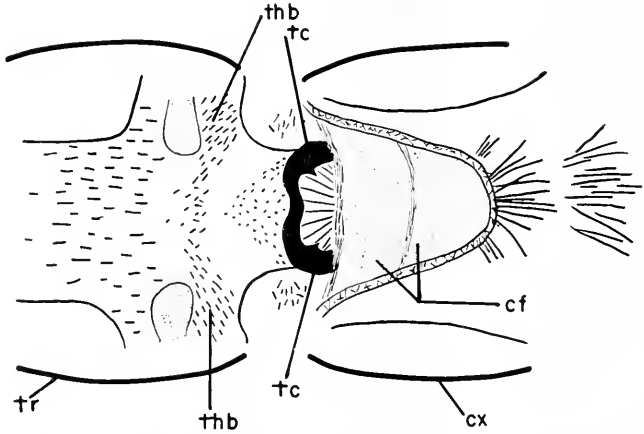

FIGURE 2. Ventral view of the coxo-trochanteral joint. The coxa and trochanter have been depressed dorsally as they would be if the leg were floating to expose the trochanteral condyles and hair beds. When the leg hangs down, the cuticular fold covers these two structures; the fold rolls back as the leg floats up. tr, trochanter; cx, coxa; thb, trochanteral hair bed; tc, trochanteral condyles; cf, cuticular fold.

Vision also seems to affect swimming. If a suspended bug is rotated through the water in a tight circle, the inside legs show compensatory movements that oppose the direction of rotation. In a bug with its eyes covered, the compensatory movements are so reduced as to be almost negligible. Hughes (1958) found reduction in the compensatory movements of a rotated *Dytiscus* when the eyes were covered.

FLIGHT

Pre-flight behavior in giant water bugs follows a fairly elaborate and somewhat varied pattern. The first sign is usually scraping of the hind legs over the wings and depression of the abdomen. There then follows twitching of the legs, which in the more advanced stages can be quite violent; this twitching is often accompanied by "shrugging" movements in which the pterothorax and abdomen are moved rapidly anterior-posterior at the articulation between the pro- and mesothorax. The wings can, at least from the author's observations, be opened at any stage of these preparations.

This rather extensive pre-flight behavior is apparently necessary because of a ball and socket mechanism which locks the wings to the pterothorax (Lauck, 1959); this mechanism is illustrated in Figure 3. The ball protrudes posteriorly from the dorsal margin of the mesepimeron and inserts into the socket on the costal margin of the hemelytron; the mesal border of the clavus matches the wing grooves on the postnotum. In order to open the wings, the bug must first release the ball and socket mechanism, which is probably accomplished, according to Lauck, by a combination of contractions of the third axillary muscle and the tergo-sternal

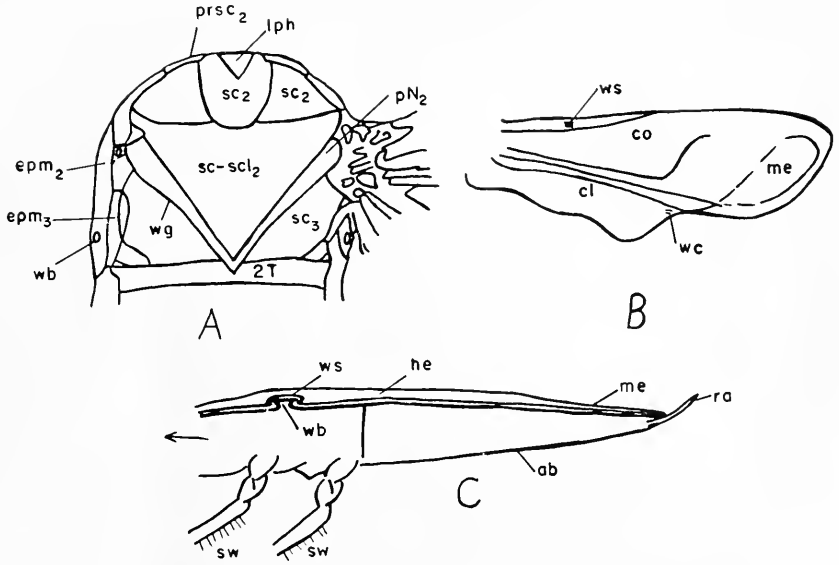

FIGURE 3. *Lethocerus:* views of pterothorax and hemelytron to show position of wing ball (wb) and wing socket (ws). The posterior margin of the clavus (cl) fits along the wing groove (wg). A: Pterothorax with wings on left side removed. prsc, prescutum; ph, phragma; sc, scutum; sc-scl, scuto-scutellum; pN, postnotum; epm, epimeron; wb, wing ball; wg, wing groove; T, tergite of abdomen. B: Ventral aspect of left hemelytron. ws, wing socket; co, corium; cl, clavus; me, membrane; wc, wing clip. C: Diagram showing wing locking mechanism. he, hemelytron; ra, respiratory apparatus; ab, abdomen; sw, swimming leg. Arrow points anteriorly. A and B redrawn from Lauck (1959) by permission of the publishers. Not drawn to same scale.

muscles which levate the wings. The various violent leg twitchings, depressions of the abdomen, and oscillations of the body characteristic of the pre-flight behavior are apparently the result of attempts by the animal to get the wings unlocked.

There is, however, another possible reason for the pre-flight movements. Krogh and Zeuthen (1941) note that lamellicorn beetles "pump" before flight; they measured the rise in temperature of the muscles during "pumping" and found that not until the temperature was at least 32° C. would the beetles fly. The flight temperature varied from 32° to 37°. Poor fliers like the beetles needed higher body temperatures to fly than did sphingid moths which are quite active fliers. Since giant water bugs are relatively poor fliers, it is possible that the pre-flight movements raise the body temperature enough to fly.

In spite of the extensive pre-flight behavior in most animals used, only a few actually flew; of 44 suspended bugs, four flew while four more opened their wings, but did not fly. Several others showed a tendency to assume the flight position, but never reached the stage of opening the wings. The flight position is shown in Figure 4. The swimming legs are carried folded flat against the underside of the body, although not in this illustration; the abdomen is depressed; and the respiratory apparatus is fully extended and held erect. Those bugs that did fly were, with one exception, suspended for five minutes or longer and most of the time in winds of greater than 6 m./sec. Weis-Fogh (1956) found that in locusts wind speeds of greater than 2 m./sec. were necessary to initiate flight.

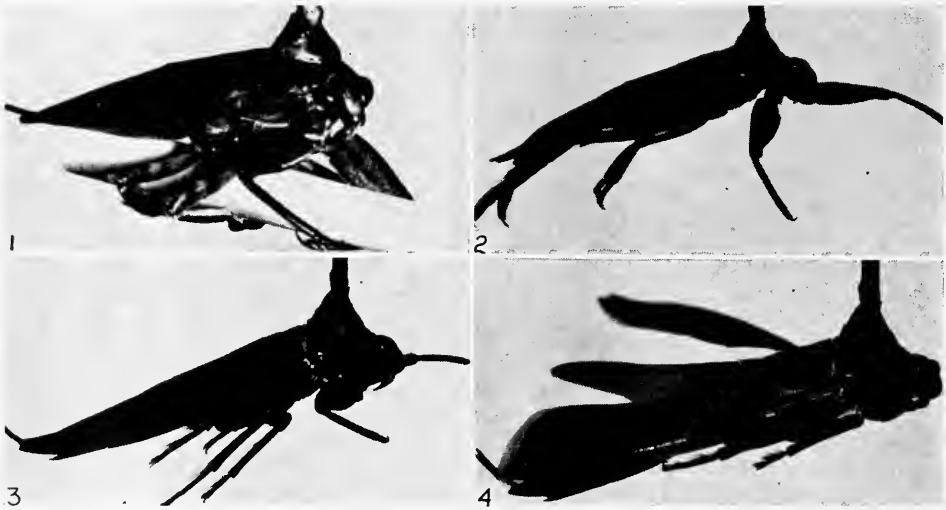

FIGURE 4. (1) Suspended bug holding drinking straw as substrate contact. (2) Swimming bug; swimming legs are approximately at the end of the backstroke. (3) Bug in flight position; note position of swimming legs and respiratory apparatus compared with (2). (4) Bug with wings open; again note position of swimming legs and respiratory apparatus.

Once a bug had flown, the threshold for further flight or wing opening was lowered considerably. Flight could be stopped by bringing the bug into contact with the substrate and could usually be initiated again if the animal was suspended. If flight was not induced by suspension, it could then be initiated by putting the bug into a wind. The contact-loss of contact mechanisms is presumably similar to those mentioned above when discussing swimming.

The stimulation of flight by wind is of some interest. It was found that a jet of compressed air delivered through a bit of glass tubing was most effective in promoting flight or wing opening (in bugs that had already flown or opened their wings) when it was aimed directly at the bugs' heads from in front. In these bugs the wings invariably opened while the air jet was blowing on the head and would close when it was removed. If the area of the head above the beak and between the eyes was covered with paraffin, the response disappeared; it reappeared when the paraffin was removed. This was true for all 8 of the bugs tested. Partial

covering of the forehead with paraffin did not abolish the response; so long as part of it was exposed, the response was maintained. Examination of a bug's head under the dissection microscope revealed that the area being considered was covered with fine hairs, virtually invisible to the naked eye, which are presumably responsible for the initiation of wing opening or flight when stimulated by air currents. Weis-Fogh (1956) found 5 paired groups of wind-sensitive hairs on the head of the locust which were sufficient for both the initiation and maintenance of flight, but were not necessary for either. Aside from the hair beds, flight in the locust could be initiated by loss of tarsal contact, which was also found to be true with giant water bugs, and could be maintained by wind on the moving wings, which was not observed in this study. In both bugs and locusts the direction of the wind impinging on the hair beds was not particularly important.

Interaction of Flight and Swimming

There seems to be little doubt that the initial response of these insects to loss of substrate contact is swimming. As previously mentioned, with increased wind speed both rate and duration of swimming increased up to a point, which varied from one bug to another, and then decreased. At first it was thought that this was due to fatigue or adaptation, but careful observation of the bugs' behavior revealed that the most likely possibility was the inhibition of swimming by the pre-flight activities even in those bugs, the most usual, in which neither flight nor wing opening ever occurred. In the latter cases, however, the bugs often did assume flight position with the legs, abdomen, and respiratory apparatus (Fig. 4). Reduction of swimming also occurred when the bugs were given successive bursts of wind at a constant speed (5.9 m./sec.), although it was not so marked.

The Central Nervous System

The anatomy of the giant water bug central nervous system reflects the general anatomy and habits of the bug. The sub-oesophageal and prothoracic ganglia are fused into one ganglion located between and slightly anterior to the bases of the coxae of the raptorial prothoracic legs which are innervated from this ganglion. The meso- and metathoracic ganglia are also fused into a common structure located between the bases of the mesothoracic legs. This ganglion innervates all four swimming legs and the wings. The brain and circumoesophageal connectives appear to be grossly similar to those of other insects.

A bug with its brain destroyed (using a hot needle) moved about apparently quite normally. Closer observation, however, revealed certain rather distinctive abnormalities. For instance, when walking about, a brainless bug tended to lose its balance and fall over on its back when stepping over small objects; once on its back it had considerable difficulty righting itself, often being unable to do so. An intact animal would, when placed on its back, bridge up with its forelegs and give a hard kick with the middle and hind legs on one side pivoting over on the tip of the abdomen; a brainless bug, on the other hand, was unable to bridge as high with the forelegs or to use the swimming legs effectively to flip over. When placed in wind, the brainless bugs differed from the normal in two ways. First, they would swim for much longer periods, usually showing no signs of slowing

down; and second, they accepted a stick and thus ceased swimming much more readily. Roeder (1937) and Roeder *et al.* (1960) note that the praying mantis also exhibits hyperactivity with the brain destroyed, walking until exhaustion. Bugs with only half the brain destroyed carried out the classic maneuver of circling to the intact side. Severing the connectives just behind the forelegs resulted in loss of tone in the swimming legs, but the legs continued to swim when the animal was placed in water, as noted above; the forelegs often twitched for a time after the cut was made.

Discussion

The fact that when the trochanteral hair bed on one mesothoracic leg was destroyed, that leg swam on the alternate strokes of its counterpart seems to indicate transfer of impulses from one side of the mesothoracic ganglion to the other. Rowe (1960) has shown electrically that such intraganglionic transfer occurs, while several authors (*e.g.* Diakonoff, 1936; Ten Cate, 1941; and Hughes, 1957) have behavioral evidence for it. Destroying the hair beds on both of a pair of swimming legs resulted in loss of activity of that pair while the other two continued to swim. Thus, as was the case with Pringle (1940), the author was unable to demonstrate transfer of a reflex from one thoracic ganglion to another even though the meso- and metathoracic ganglia are, in this case, fused.

Roeder (1937; see also Roeder, 1953) proposed a model for the operation of the insect central nervous system; in this model the brain exercises inhibitory control over locomotion, in view of the locomotor hyperactivity of brainless insects. Since giant water bugs are also hyperactive when brainless, they appear consistent with Roeder's model. Bugs whose connectives had been severed posterior to the fused sub-oesophageal and prothoracic ganglia lost all muscle tone in the swimming legs, but because of their fusion, it was not possible to separate the two ganglia functionally. There is some evidence from studies on cockroaches (Diakonoff, 1936; Ten Cate, 1941; Chadwick, 1953) that the prothoracic ganglion may be essential for normal co-ordination.

If Hemiptera are secondarily aquatic, then the swimming reflex of aquatic forms like the giant water bugs may be considered a modification of the flight reflex of exclusively terrestrial insects. The reflexes, under natural conditions, would be triggered by similar sets of circumstances. A floating water bug, for instance, is free of substrate contact, and a swimming reflex might result, particularly since the usual habit of the bug is to cling to floating vegetation. A falling terrestrial insect, on the other hand, is also free of substrate contact and generally flies. The two situations of floating and being air-borne are essentially the same, and the reflexes of a particular insect, be they swimming or flying, are modifications to suit the particular medium.

The escape responses are similarly modified. Strong tactile stimulation, especially of the abdomen (Fraenkel, 1932), causes terrestrial insects to leap off the substrate and fly. In the aquatic bugs tactile stimuli or vibrations result in violent swimming whether the animal is in water or suspended in air.

But if the swimming reflex is a modification of the flight reflex, why then do the water bugs sometimes fly? There appear to be two major possibilities. First, the body posterior to the articulation of the pro- and mesothorax of a bug

suspended in air hangs down at a rather sharp angle; in water this part of the body is buoyed up. Diakonoff (1936) reports that movement at the pro-mesothoracic articulation of the cockroach results in a "fall reflex" that elicits flight and is apparently due to stimulation of the numerous receptors at the articulation. A similar mechanism may stimulate flight in giant water bugs. Second, when the bugs are suspended in wind, the hair beds on the head, which have been shown to be receptors concerned with flight, are stimulated. This stimulation, if strong enough or if summation occurred, would presumably overcome the swimming reflex and elicit flight.

One would predict, on the assumption that swimming with lack of substrate contact is a modification of a flight reflex, that it would be a fairly general adaptation among aquatic insects. This prediction appears to be largely true. Hughes (personal communication) has observed the swimming reflex in *Hydrophilus* and *Dytiscus*, and the author has found it in gyrinids, hydrophilids, dytiscids, corixids, and the genus *Belostoma*, as well as in the giant water bugs discussed here. Further investigations of the phenomenon in these groups are now in progress.

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Summary

1. Giant water bugs swim when suspended free of the substrate. This situation contrasts with that of terrestrial insects which fly when freely suspended. Swimming can be stopped by returning contact to the bugs.

2. Suspended bugs respond to wind with a general increase in rate and duration of swimming, followed by a decrease in both.

3. When bugs are in water, swimming is stimulated by a hair bed located on the trochanter at the coxo-trochanteral joint. These hair beds seem to be stimulated by cuticular folds which cover them when the legs hang down, but roll back and leave them uncovered when the legs float, resulting in swimming.

4. Flight or wing opening occurred with 8 of 44 suspended bugs. A hair bed on the head functions in both the maintenance and initiation of flight in response to wind.

5. The bugs possess an elaborate pre-flight behavior which is apparently necessary to unlock a ball and socket mechanism attaching the wings to the pterothorax. This pre-flight behavior inhibits swimming and causes the decline in rate and duration mentioned in (2) above.

6. In the central nervous system the sub-oesophageal and prothoracic ganglia are fused, as are the meso- and methathoracic ganglia. There is behavioral evi-

dence for transmission of impulses across a ganglion, but not from one ganglion to another, even though the ganglia are fused.

7. There is evidence that the swimming reflex is a general phenomenon; apparently it is an aquatic modification of the flight reflex.

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ION REGULATION IN TETRAHYMENA [1]

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Fresh water animals maintain their cells hyperosmotic to their environment (Prosser *et al.*, 1950). In higher animals this is accomplished by specialized organs or tissues (*e.g.* the vertebrate kidney, frog skin, and the anal papillae of dipteran larvae) which adjust the osmotic level of body fluids to a level isosmotic with the cells.

Lower invertebrates (protozoa, sponges and coelenterates) have no osmotically regulated body fluids. Therefore all cells in fresh-water representatives of these groups have the problem of continuous water influx, and must have osmoregulatory ability (Kitching, 1954).

A high potassium content relative to their medium is characteristic of all living cells that have been investigated. Most cells are richer in potassium than sodium, and there is often less sodium in cells than in the medium. Evidence has been offered that in such fresh-water animals as *Hydra* and *Spirostomum,* inorganic ions are readily available for exchange with the environment (Lilly, 1955; Carter, 1957). Table I lists potassium and sodium levels in several fresh-water invertebrates. Therefore regulation of body volume in such forms involves regulation of ions as well as water.

This paper reports an investigation of ionic regulation in *Tetrahymena pyriformis,* a fresh-water ciliate. A remarkable ability to maintain a high potassium concentration as well as a lower sodium concentration in very dilute medium was found. Evidence for a sodium extrusion mechanism was also found. These findings will be discussed in terms of a model system for ion regulation in *Tetrahymena,* and in terms of relevance to similar problems in other animals.

Methods and Materials

Tetrahymena pyriformis, strain W, was grown axenically in 2% proteose-peptone medium (hereafter called normal medium), the ion content of which is indicated in Table II. One-liter Roux culture bottles containing 500 ml. of medium were innoculated with 5 ml. of a culture in log phase of growth. After four days' growth at 22–25° C., the cells were concentrated approximately ten-fold by gentle centrifugation. After experimental treatment, which involved either dilution of cell suspension in normal medium with distilled water or increasing medium

[1] The methods reported in this paper were worked out initially by the junior author with the assistance of Miss Marina Dan. The results and hypotheses are the work of the senior author who gratefully acknowledges the help and criticism of Dr. H. B. Steinbach during the course of this study. The work has been supported by National Science Foundation grant G–4526 and U. S. Public Health Service grant RG–6879.

[2] Trainee in United States Public Health Service Training Grant Program 2G–150.

TABLE I

K and Na (or osmolar) concentrations of some fresh water invertebrates. (See Willmer (1958) for a table of osmolar concentrations of some protozoans determined by a variety of methods.)

Organism	Concentration	Method of determination	Literature source
Acanthamoeba (distilled water washed)	K—26.9 (meq./l. cells) Na—14.3	elemental analysis	Klein (1959)
Spirostomum	K—7 (meq./l. cell water) Na—1	equilibration with isotopic tracer	Carter (1957)
Tetrahymena (in 2% proteose-peptone)	K—31.7 (meq./l. cells) Na—12.7	elemental analysis	Dunham and Child (present report)
Spongilla (summer)	osmolarity equivalent to 27 meq. NaCl/l. cell water	vapor pressure determination	Zeuthen (1939)
Pelmatohydra (whole animal)	K—14.4 (meq./l. cell water) Na—2.7	equilibration with isotopic tracer	Lilly (1955)
Tubifex (whole animal)	K—27.0 (meq./kg. wet Na—23.4 weight)	elemental analysis	Dunham (unpublished)
Anodonta (muscle)	K—10.6 (meq./kg. wet Na—5.2 weight)	elemental analysis	Hayes and Pelluet (1947)

concentration by adding NaCl or KCl, cell suspensions were reconcentrated when necessary so that 10 ml. gave a packed cell volume of about 0.2 ml., as determined by centrifugation at a relative centrifugal force of 1600 to constant volume (10 minutes in 10-ml. Kolmer tubes).

Dry weight of cells was determined by drying to constant weight at 60° C. Cell counts were made in a hemocytometer.

Cells were extracted for ion analyses by suspending them in dilute acetic acid (1 drop glacial acetic acid in 10 ml. water), heating near boiling for 5 minutes and allowing them to stand for one hour. K and Na analyses were made with a Coleman model 21 flame photometer. Preliminary Cl analyses were made with an Aminco-Cotlove chloride titrator. Analysis of nitric acid digests of residues after extraction indicated that more than 98% of intracellular K, Na and Cl was extracted. Intracellular cation concentrations are expressed in meq./l. cells, after appropriate correction for extracellular space as determined by use of radioactive iodinated serum albumin (Risa) added immediately prior to centrifugation. Total exchangeability and kinetics of intracellular K and Na were determined using trace

TABLE II

K and Na concentrations of Tetrahymena *in 2% proteose-peptone (normal) medium. K, Na, and Cl concentrations of normal medium. Standard errors and number of determinations are given.*

Cells	meq./l. cells
K	31.65±0.43 (44)
Na	12.68±1.35 (44)
Medium	***mM***
K	4.75±0.13 (60)
Na	36.5±0.13 (70)
Cl	28.7±1.02 (6)

amounts of the appropriate radioisotope, K^{42} or Na^{24}, obtained from Oak Ridge National Laboratories as chlorides in HCl solution, and neutralized with NaOH before use. Counts per minute of wet samples were determined with an end-window counter or a NaI-Tl crystal scintillation well counter. Per cent exchanges of intracellular K and Na were calculated from the specific activities of the cells and of the medium.

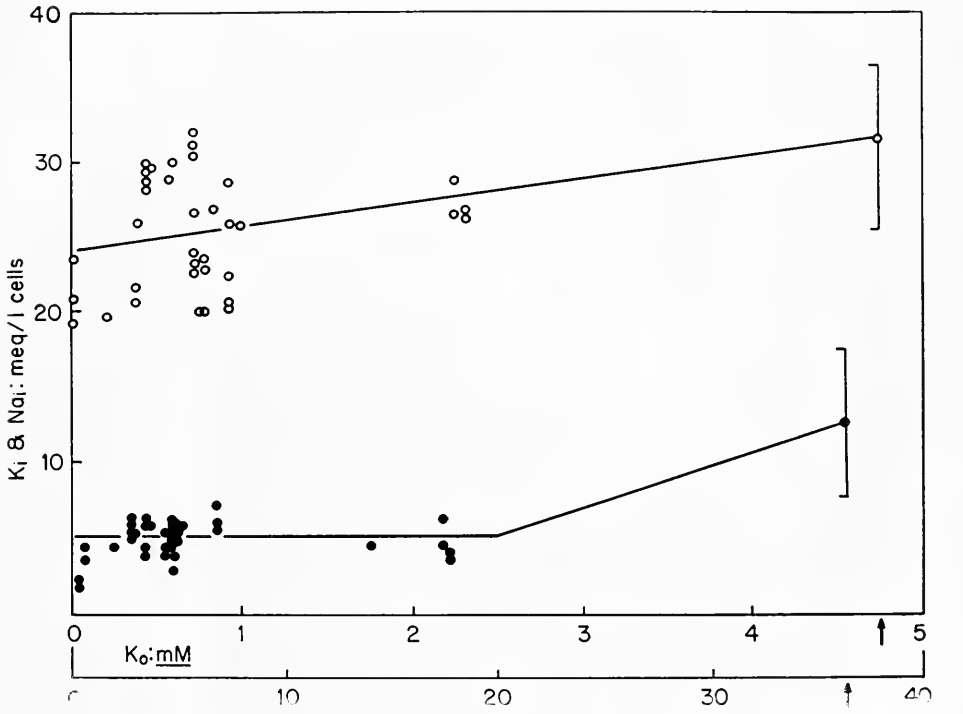

FIGURE 1. K and Na content of *Tetrahymena* in normal and diluted media. Ordinate: cellular concentrations of K and Na (meq./l. cells); abscissas: concentrations of K and Na in the medium (*mM*). Open circles: K_i; solid circles: Na_i. Points for K_i and Na_i in normal medium (see arrows) are averages of 44 determinations; vertical lines delimit the total range of the determinations in normal medium.

RESULTS

Table II shows the K and Na content of cells in normal medium, and the K, Na and Cl content of normal medium. The volume and weight of an average cell in normal medium were 1.83×10^{-5} μl. and 1.97×10^{-2} μg., respectively, as determined from cell counts and packed cell volumes (corrected for Risa space). Dry weight of cells in normal medium was determined to be 19.4% of wet weight, so cells are 80.6% water. The percentage of Risa space in a volume of packed cells in normal medium was 15% (eight determinations ranging from 14% to 18%, SE = 0.57). This value was not significantly different for cells equilibrated with medium diluted eight-fold.

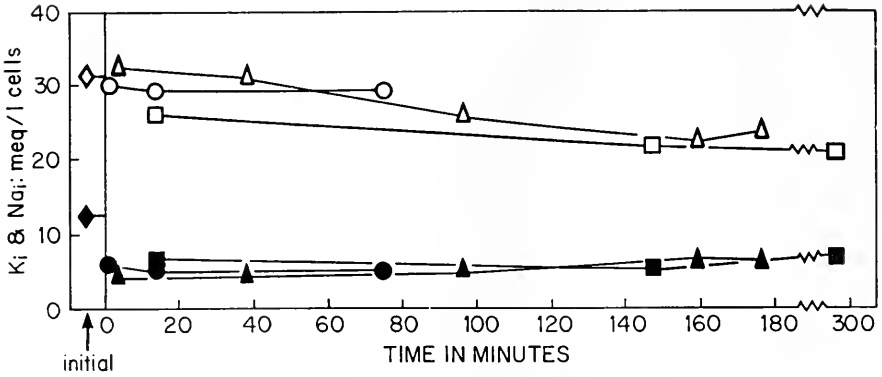

FIGURE 2. Changes in cellular K and Na in *Tetrahymena* after dilution of normal medium. Ordinate: cellular K and Na concentrations (meq./l. cells); abscissa: time (minutes). Open symbols: K_i; solid symbols: Na_i. Initial K_i and Na_i (diamonds) are averages of values in normal medium. Dilutions were made at zero time. The extents of the dilutions of normal medium in the three experiments were as follows: triangles, 6-fold dilution; circles, 8-fold dilution; squares, 13-fold dilution.

In order to demonstrate how intracellular K and Na are maintained over a range of medium concentrations, cells were allowed to equilibrate for at least 30 minutes in various dilutions of normal medium from two-fold to over 100-fold, the extreme dilution involving several distilled water washes. Figure 1 shows

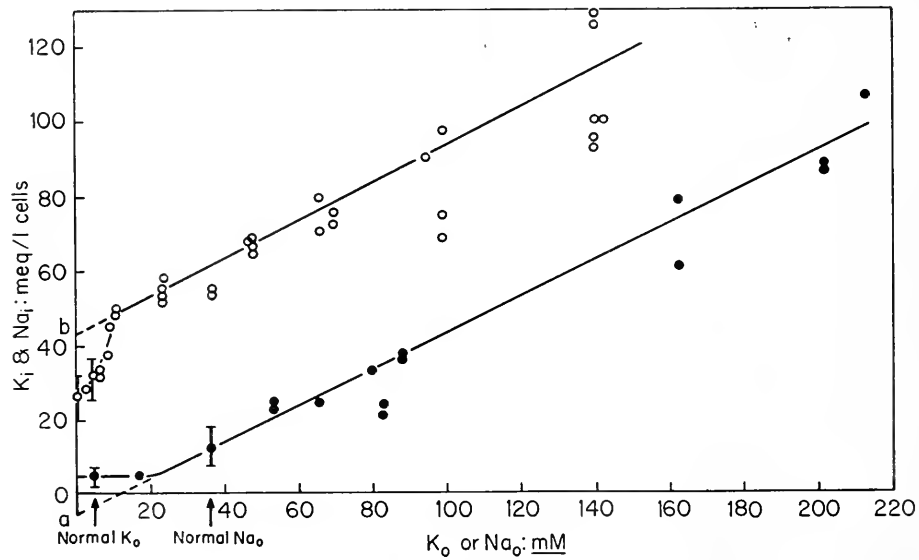

FIGURE 3. K and Na content of *Tetrahymena* in media concentrated with KCl or NaCl. Ordinate: cellular concentrations of K and Na (meq./l. cells); abscissas: concentrations of K and Na in the media. Open circles: K_i; solid circles: Na_i. *a* and *b*: ordinate intercepts of the linearly increasing portions of the Na and K curves, respectively. Points for K_i and Na_i in normal and diluted media are taken from Figure 1.

final intracellular concentrations of K and Na (K_i and Na_i) plotted against medium concentration (K_o and Na_o). K_i and Na_i are quite constant over the range of medium dilutions investigated: average $K_i = 25.4$ meq./l. cells and average $Na_i = 5.0$ meq./l. cells; $K_i/Na_i = 5.1$. Figure 2 shows the results of three experiments in which changes of K_i and Na_i were followed after six-fold and greater dilutions of normal medium. Changes in Na_i take place within the first 30 seconds, after which Na_i is constant. K_i decreases very slowly after medium dilution, so it is difficult to assign an equilibrium level. Therefore it was arbitrarily decided that K_i values in cells in dilute medium more than 30 minutes would be reported with the reservation that time for equilibration may involve a matter of days. (In one experiment, K_i in cells in medium diluted 20-fold for two days was about half normal K_i.) Rates of decrease of K_i in cells in dilute medium were never greater than 10% per hour, and generally were much slower.

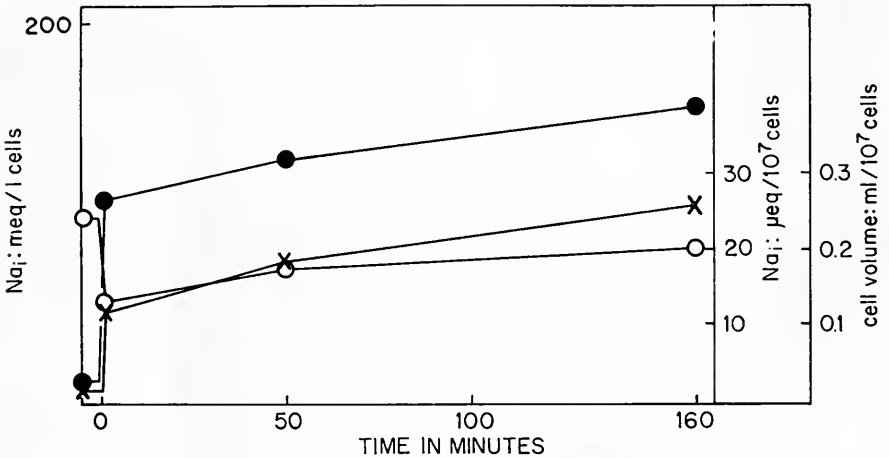

FIGURE 4. Changes in volume and Na content of *Tetrahymena* after increasing the NaCl concentration of normal medium. Ordinates: cell volume (open circles); Na content per unit number of cells (crosses); Na content per unit volume of cells (solid circles). Abscissa: time (minutes). NaCl concentration of normal medium was increased by 176 *mM* at zero time. K_i per unit number of cells at 160 minutes was not significantly different from initial K_i.

Cells were equilibrated for 30–120 minutes in media made more concentrated than normal in either K or Na (added as chlorides). Figure 3 shows K_i and Na_i values from these experiments plotted against K_o and Na_o, respectively. Both curves are linear above certain medium cation concentrations, with values of 0.51 for the slope of K_i/K_o above 11 *mM* K_o and 0.48 for the slope of Na_i/Na_o above 20 *mM* Na_o. Below 20 *mM* Na_o, Na_i is constant at 5.0 meq./l., whereas below 11 *mM* K_o, the K curve is roughly sigmoid.

The kinetics of net influx of cation and the concomitant water movements were investigated by subjecting cells to sudden large increases in K_o or Na_o. Changes in packed cell volume, cation concentration per unit volume of cells, and cation concentration per unit number of cells were followed. Figures 4 and 5 show the results of subjecting cells to increases above normal of 176 *mM* NaCl and 137 *mM* KCl, respectively. In both cases there is a large initial influx of the

elevated cation (shown by increase in μeq./10^7 cells) and large efflux of water (45% cell shrinkage in each case) in the first 1.5 minutes. K_i per cell increases initially about 1.75 times, and subsequently increases slowly to 4.5 times the initial level by 200 minutes, accompanied by water re-entry. Na_i per cell increases initially to 6 times initial level, and subsequently slowly increases to 13.7 times initial level at 160 minutes, with water re-entry. So equilibration in both cases is fast, but much faster in high Na medium. When Na_i increased, final K_i per cell was not significantly different from the initial value, and likewise for Na_i per cell after 200 minutes when K_i increased. This lack of reciprocal changes means that increases in K_i and Na_i are accompanied by proportionate increases in

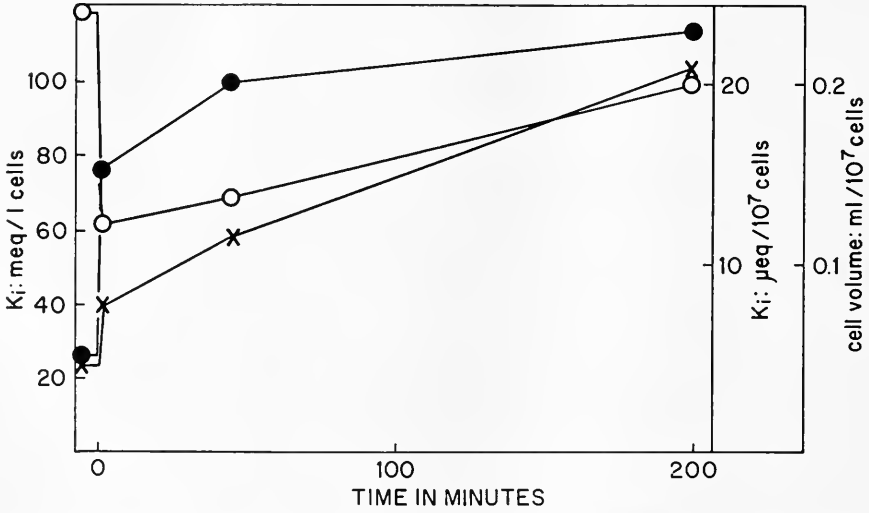

FIGURE 5. Changes in cell volume and K content of *Tetrahymena* after increasing KCl concentration of normal medium. Ordinates: cell volume (open circles); K content per unit number of cells (crosses); K content per unit volume of cells, solid circles. Abscissa: time (minutes). KCl concentration of normal medium was increased by 137 *mM* at zero time. Na_i per unit number of cells at 200 minutes was not significantly different from initial Na_i.

some anion, or decreases in some other cation, if electroneutrality is preserved within the cells. (In a few experiments in which both K_o and Na_o were increased, both K_i and Na_i increased in the same way as when studied singly, as described.)

Preliminary analyses of Cl content of cells show that Cl does not balance increases in K_i or Na_i. Figure 6 shows Cl_i values in cells in normal, dilute, and high NaCl and KCl media. Cl_i is quite constant in media ranging from a 2-fold dilution (14 *mM* Cl_o) up to 123 *mM* Cl_o (99 *mM* K_o) and 73 *mM* Cl_o (83.5 *mM* Na_o): for 18 determinations, Cl_i averaged 6.4 meq./l. cells, ranging from 0 to 13.2 meq./l. One set of determinations at 150 *mM* Cl_o (163 *mM* Na_o) showed Cl_i as high as 37 meq./l., but Na_i was 78.5 meq./l. in this case. For this one deviant value, only one-half of Na_i could be balanced by Cl, all other determinations showing no relationship at all between Cl_i and K_i or Na_i.

Preliminary experiments were done on the washout of high K or high Na content of cells transferred to normal medium after one hour's equilibration in

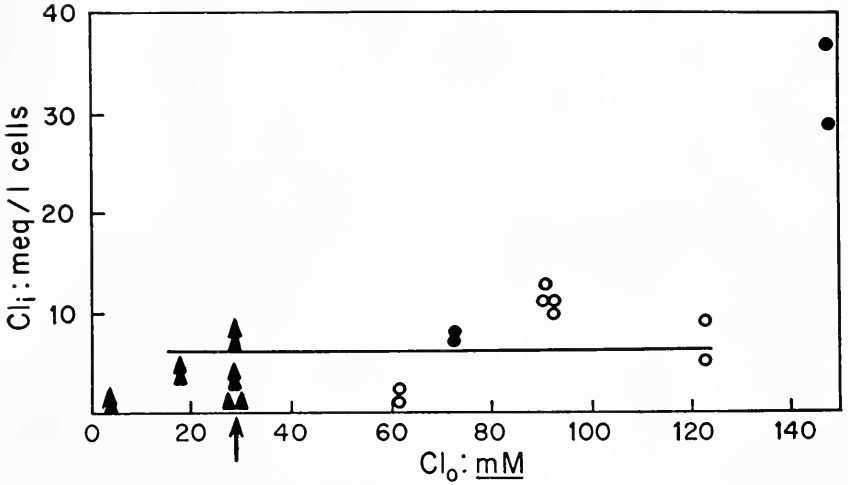

FIGURE 6. Cl content of *Tetrahymena* in normal, diluted, and high NaCl and KCl media. Ordinate: cellular concentrations of Cl (meq./l. cells); abscissa: Cl concentration of the media. Triangles: Cl_i in normal and diluted media; open circles: Cl_i in high KCl media; solid circles: Cl_i in high NaCl media. Arrow indicates Cl concentration of normal medium. Horizontal line indicates average of all Cl_i values in medium Cl concentrates ranging from 17 *mM* to 123 *mM*.

high K or Na medium. Cells were equilibrated in normal medium with the KCl concentration increased by 65 *mM*. Ten minutes after washing with normal medium, K_i had decreased from 64 meq./l. to 57 meq./l., and after 45 minutes to 48 meq./l. Initial K_i was 33 meq./l. Therefore a portion of elevated cellular K washed out much more slowly than it can be increased. This observation is consistent with the slow decrease of K_i from cells after dilution of normal medium. Cells were also equilibrated for one hour in normal medium plus 170 *mM* NaCl, then washed with normal medium. Ten minutes after washing, Na_i was only slightly above normal Na_i, indicating that Na_i is easily washed out of cells, as a portion of Na_i is after dilution of normal medium.

TABLE III

Kinetics and extent of exchange of cellular K and Na with normal and diluted media containing trace amounts of K^{42} or Na^{24}. Each horizontal row of data represents at least two experiments with replicate determinations.

	Medium conc. (*mM*)	Total cell conc. (meq./l. cells)	Time for maximum exchange (minutes)	Time for ½ maximum exchange (minutes)	Exchangeable cell content (meq./l. cells)	Unexchangeable cell content (meq./l. cells)	Exchangeable fraction
K, normal medium	4.75	31.6	180	30	29.2	2.4	92.5%
K, diluted medium	0.83	27.4	180	30	25.0	2.4	91.0%
Na, normal medium	36.5	12.7	120	<1	11.2	1.5	88%
Na, diluted medium	3.0	5.5	120	3	3.25	2.25	59%

Table III summarizes the results obtained when cells were exposed for 5 or more hours to normal and diluted media containing trace amounts of K^{42} or Na^{24}. Cellular K and Na are largely available for exchange with medium K and Na, and exchange is rapid, particularly the exchange of Na. However, small amounts of both K and Na do not exchange in a 5-hour period, although the exchange reaches a maximum level by three hours for K_i and two hours for Na_i. The amounts of unexchangeable K_i and Na_i do not change significantly with medium dilution.

Discussion

That *Tetrahymena* is capable of maintaining a high cellular K content relative to the K content of normal and diluted media is evident from Figure 1 and Table II. Ratios of K_i/K_o are of the order of 100 and higher in very dilute medium. *Tetrahymena* also retains a small amount of Na in very dilute medium.

Sizable portions of cellular K and Na are exchangeable with the medium, as shown in Table III. Therefore K and Na are not retained in the cells by an impermeable membrane. Table III also shows, however, that small and constant amounts of both K and Na are unexchangeable. Since net changes are also evident, exchange diffusion cannot be responsible for the ready exchange of isotopes.

A system of internal binding sites with specific affinity for K is suggested to explain active K maintenance by *Tetrahymena*. Na may be retained by a similar mechanism. In addition, a Na extrusion mechanism is proposed.

Na in *Tetrahymena* is best explained in terms of a formal model involving compartmentalization of Na_i. Two components of Na_i are constant, *i.e.* do not vary with Na_o. One, 1.9 meq./l., is unexchangeable with the medium. The second, 3.1 meq./l., is held constant, but is rapidly exchangeable. Forty-eight per cent of cell volume is free "Na space," and is available to a mobile Na component which is freely diffusible, and proportionate to Na_o. However, this mobile component is maintained 20 meq./l. of water less than Na_o by Na extrusion. Below Na_o there is no mobile Na component and Na_i is constant at 5 meq./l., but at 20 *mM* Na_o the Na extrusion mechanism is operating maximally and mobile Na_i begins increasing with Na_o. Since the other two Na components are constant, total Na_i increase is linear and represents only the mobile component. There are no net exchanges between any of the compartments. These compartments can be visualized as physiological entities only, since no morphological significance can be attached to them. This model is suggested by the following points of evidence:

(1) Na extrusion is indicated first, by the constant level of Na_i up to 20 *mM* Na_o, and second, by the negative intercept on the ordinate axis of the linearly increasing portion of the Na_i/Na_o curve (indicated in Figure 3). (Permeability of the cells to Na precludes passive exclusion of Na.)

(2) The mobility of Na_i above the constant 5 meq./l. is apparent from the rapid equilibration of cells with high Na medium, and the rapid washout of Na from cells both when normal medium is diluted and when high Na cells are washed in normal medium.

(3) The linearity of the Na_i/Na_o curve above 20 *mM* Na_o and the mobility of Na_i above 5 meq./l. allow one to conclude that the slope of the Na curve, 0.48, represents the fraction of cell volume occupied by the mobile Na component, or "Na space."

(4) The magnitude of the gradient effected by maximum Na extrusion is proportional to the magnitude of the negative intercept (about 5 meq./l. cells) corrected for the constant amount of retained Na_i (5 meq./l. cells), or 10 meq./l. cells. Since the Na space is 48%, the difference in Na concentration between medium and cell water is $10/0.48 = 20.8$ meq./l. water. This value should and does correspond closely to the medium Na concentration at which the Na extrusion mechanism becomes saturated. This saturation concentration, the "threshold" of Na_i increase, is analogous to the threshold of glucose excretion in renal tubules at the concentration of maximum glucose reabsorption (Shannon and Fisher, 1938), which was also interpreted as representing saturation of an accumulating mechanism.

(5) The evidence for the unexchangeable and exchangeable but constant components of cellular Na has already been presented.

The nature of the preservation of electroneutrality in the cells upon Na or K entry is not at all clear. There is definitely no reciprocal relationship between Na and K. There is little intracellular Cl, even upon large increases in the medium of either NaCl or KCl. These results also obviate explaining active retention of K in terms of a Donnan equilibrium resulting from Na extrusion, unless Cl is also specifically excluded from the cells, which seems unlikely at present. If a Donnan situation obtained, the relationship $K_i/K_o = Cl_o/Cl_i$ would be expected to hold in any medium, and it obviously does not. With increasing medium KCl, K_i/K_o decreases to less than 1, while Cl_o/Cl_i increases to as high as 22. The nature of the Cl exclusion is suggested here to be electrostatic rather than a matter of specificity or impermeability. Possibly cellular K and Na are associated with fixed anionic groups more or less strongly, depending on the ion and the medium concentration of the ion. Steinbach (1947) suggested that K is always associated with organic components which occupy spaces unavailable to Cl. This still does not explain the preservation of electroneutrality. This problem is under investigation.

The K_i/K_o gradients in normal and diluted media are definite evidence for specific K retention. The rapid and nearly complete exchange of K_i and the rapid net increase in K_i with K_o make membrane impermeability or any membrane involvement unlikely explanations for K retention.

In media with K_o higher than normal, the slope of the K_i/K_o curve is steep up to 11 *mM* K_o, above which K_i increases less sharply with K_o, and in a linear fashion. The slope of this portion of the curve is 0.51, close to the slope of the Na_i/Na_o curve (0.48), suggesting that this increasing K_i is occupying a cellular space identical with the "Na space."

However, a portion of this increased cellular K does not readily wash out of the cells, and is not nearly as mobile as high cellular Na. Cells equilibrate less rapidly with high K medium than with high Na medium. Increased K_i is not accompanied by reciprocal Na changes or by Cl_i increase, as noted above. These considerations suggest that the increase in K_i involves association with previously "empty" K binding sites in the "Na space." (Steinbach (1940) reported K increase without Na decrease or Cl increase in *Phascolosoma* muscle. He suggested (1947) that vertebrate skeletal muscle behaves as though there were a limited number of groups capable of binding K which are normally saturated, whereas heart and invertebrate muscle are normally not saturated with K.) The slower rise of K_i

with K_o greater than 11 *mM* and the linearity of this rise suggest that the actively maintained, or bound, K is at a maximum above 11 *mM* K_o. Then the intercept of the linear portion of the K_i/K_o curve at the ordinate axis, 43 meq./l. (shown in Figure 3), should be the level of maximum actively maintained K. In cells equilibrated in high K medium, and washed in normal medium, K_i fell to 48 meq./l. after 45 minutes, a concentration comparable to the ordinate intercept of the linear K_i/K_o. This constitutes additional evidence for the maximum saturation of bound K. Initially it would appear that K maintenance in *Tetrahymena,* because of the sigmoid shape of the K_i/K_o curve below 11 *mM* K_o, does not fit elementary Langmuir adsorption theory (applicable to Michaelis enzyme kinetics), which it should if a system of binding sites with a saturation level is invoked. However, it is likely that this is not the true shape of the curve. The K washout experiment described above shows that K_i, although it can be rapidly increased, can be only slowly washed out. Since the initial K_o in the experiments involving changing K_o was always that of normal medium, an inflection in the K_i/K_o curve is expected at the K_o of normal medium, and it is observed (see Figure 2). Therefore the explanation of the sigmoid shape of the curve lies not in the nature of the K retention mechanism, but in the experimental procedure. Then the real relationship between actively maintained K and K_o should fit a Langmuir adsorption isotherm, and a binding sites mechanism for K retention is consistent with the data.

No comparable obscuring factor exists in the case of Na_i since Na apparently washes in and out of cells with equal facility in the range of medium concentrations investigated. The Na retention system is apparently saturated at a very low Na_o.

The data suggest compartmentalization of K as well as Na: unexchangeable K (2.4 meq./l.), exchangeable but bound K (maximum about 43 meq./l.), and freely diffusing K, with no threshold K_o. (See Cowie, Roberts and Roberts, 1949, for a discussion of compartmentalization of K in *E. coli.*)

The hypothesized ion regulatory machinery of *Tetrahymena,* shown to be consistent with the data, consists, first, of a system of internal binding sites which specifically accumulate and retain K; second, a system for retention of a constant, low level of Na, and third, a Na extrusion mechanism. Cl plays little role in ion balance in *Tetrahymena.* Cellular K and Na are separable into three components: unexchangeable, exchangeable but bound, and mobile components.

The Na extrusion mechanism may facilitate water removal, and therefore may be associated with the contractile vacuole. The water economy of *Tetrahymena* may be analogous to that of other fresh-water animals, in that *Tetrahymena* may not be capable of secreting pure water, but water removal may be facilitated by ion secretion (cf. Prosser *et al.,* 1950).

The relationship of Na retention to Na extrusion and/or K retention, cannot be decided from the data. Retained Na may represent lack of complete specificity of the K binding sites, but in this case a reciprocal relationship between actively retained K and Na would be expected. The retained Na might be a reservoir necessary for vacuolar function. This possibility is consistent with the rapid Na turnover indicated by rapid exchange, but cannot be easily resolved with the constancy of the retained Na relative to Na_o. A third possibility is a specific protoplasmic requirement for a low, constant Na level, for which there is no

evidence here and little precedent. (*Hydra* (Lenhoff and Bovaird, 1960) and *Chilomonas* (Pace, 1941) have possible specific Na requirements.)

The specificity of K retention in *Tetrahymena* indicates a protoplasmic K requirement. (Kidder *et al.* (1951) demonstrated a nutritional requirement for K in *Tetrahymena.*) The similarity of levels of K in *Tetrahymena* and the other animals listed in Table I suggests a minimum requisite protoplasmic level of K. It is often held that high cellular K is only a reflection of a Donnan equilibrium resulting from Na extrusion (see Hodgkin, 1951). Carter (1957) attributes K maintenance in *Spirostomum* to Na exclusion. This has been shown here not to be so in *Tetrahymena,* and Robertson (1957) has shown that in a number of animals, including some marine invertebrates, a portion of cellular K cannot be accounted for by a Donnan equilibrium, but must be due to specific K retention. Steinbach (1947) suggests that cellular K is regulated relative to a constant protoplasmic composition rather than to serve an osmoregulatory function. No doubt in marine animals and vertebrates, with their relatively high ionic content, some cellular K is held non-specifically to preserve electroneutrality. Cellular K levels vary considerably, particularly among fresh-water animals, and probably reflect the ability to regulate body fluids, and to an extent the activity of the animal. Therefore in vertebrate cells, there may be a K component reflecting a Donnan equilibrium plus a component serving a specific protoplasmic role. Since evolution of basic cellular mechanisms is generally conservative, a similarity between K retention mechanisms in *Tetrahymena* and other animals is an attractive possibility. *Tetrahymena* affords a system for studying this mechanism without high ion concentrations and large Donnan effects, which would obscure specific K retention in other animals.

Akita (1941) reported data on Na, K and Cl contents of *Paramecium* which were comparable to the data presented above on *Tetrahymena.*

Summary

1. The K and Na content of *Tetrahymena pyriformis* has been determined, and the mechanisms of ionic regulation were investigated.

2. The main findings were: K and a small amount of Na are maintained in very dilute medium. Cellular K and Na are readily exchangeable with K and Na of the medium. However, small, constant amounts of each are unexchangeable. Cells rapidly equilibrate with media high in K or Na. High K washes out of cells slowly, whereas Na enters and washes out of cells with equal facility. There is no reciprocal relationship between cellular K and Na. *Tetrahymena* contains little Cl. Increases in cellular K or Na are not accompanied by increases in Cl.

3. The results are interpretable according to the following proposals: K is specifically accumulated and retained by a system of internal binding sites with a saturation level. Na is probably retained by a separate mechanism. There is also a Na extrusion mechanism which has no relationship with K or Na retention. Cellular K and Na are compartmentalized into three components: unexchangeable, exchangeable but bound, and freely diffusible components.

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FURTHER STUDIES ON ALLOCENTROTUS FRAGILIS, A DEEP-SEA ECHINOID [1]

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In a previous paper some data on the natural history and breeding of a deep-sea echinoid, *Allocentrotus fragilis*, were presented (Boolootian *et al.*, 1959). Further studies are reported here, primarily to define more clearly the breeding season of the species, as well as to get further information on its nutrition.

Breeding Season

The breeding season in the previous study appeared to coincide with winter (December to March) but remained uncertain because storms on Monterey Bay during the critical period interfered with collecting at the time the boat was available. For the present study the urchins were collected from the same beds and by the same methods as previously, and the gonad index (the ratio of gonad volume to wet weight times 100) was used to estimate the breeding condition of the specimen as before. The breeding activity has now been followed for almost three years, and although the data for each year are incomplete, pieced together for the entire period in Figure 1, they give support to the notion that a single breeding season occurs in this species. Perhaps several periods of spawning and redevelopment of eggs occur in a given individual of this species but monthly sampling does not give information on this point. However, the appearance of germinal vesicles in spawned-out ovaries suggests just this. It proved impossible to keep specimens in healthy condition in the laboratory for more than about a month, although it was noticed that the red-spot "disease" was much less frequent in animals kept in aquaria in the dark (Araki, personal communication). It may prove possible to keep the animals in the laboratory for a longer time once the most favorable conditions are discovered.

More decisive evidence for an annual cycle is obtained from a study of teased pieces of the gonads and from attempts to fertilize the eggs. Such a study indicates that although the gonad index may be high and sperms may appear in September, October and November, the eggs are almost all in the germinal vesicle stage, each with a large nucleus. Such eggs do not mature after shedding and in no case are they fertilized on addition of active and presumably mature sperm. In December most of the females had ripe eggs and only occasional germinal vesicles were seen among them. The eggs fertilized and developed into normal plutei. The same was found to be true during February and March. Some of the females examined

[1] I am indebted to Messrs. George Araki, Peter Glynn and Joseph Balusteri for collecting the *Allocentrotus;* to Messrs. John Lawrence and James Stanley for help with some of the chemical determinations; and to Mr. Albert Towle for help with some of the respirometric determinations.

had few eggs but those eggs which remained in the ovaries fertilized and developed normally, suggesting that they were only a remnant, the bulk of the gametes having been released. During the period immediately following the breeding season, few females had eggs and germinal vesicles again became apparent in some. Thereafter, all the teased gonads examined microscopically appeared indeterminate as to sex. Apparently the tissue had entered a resting stage. The gonads remain indeter-

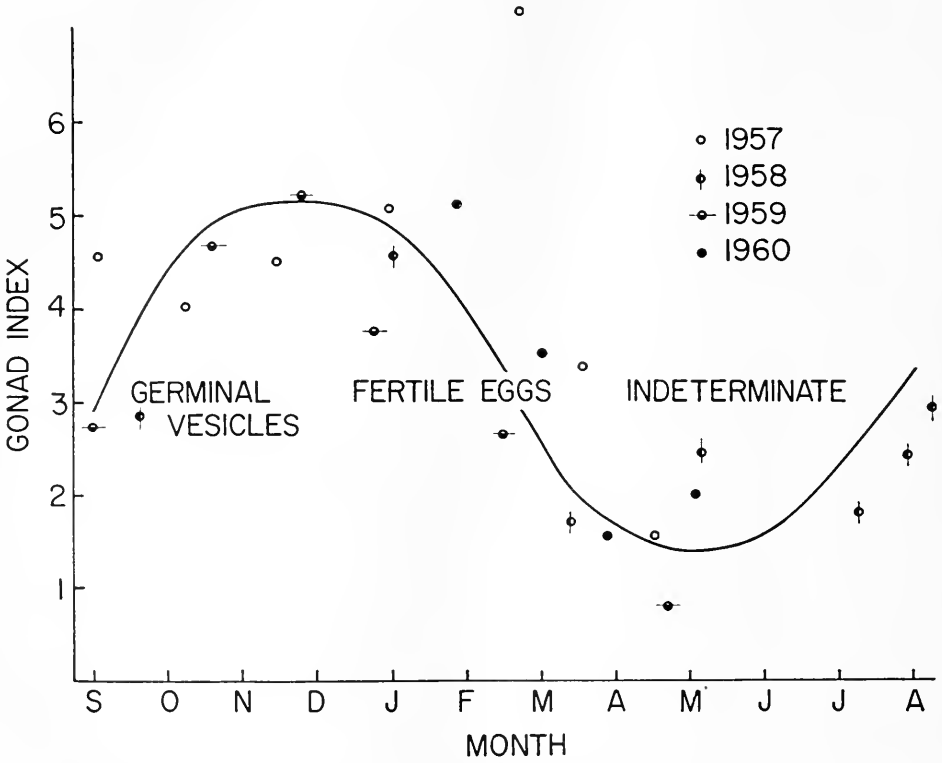

FIGURE 1. Reproductive cycle of *Allocentrotus fragilis* as measured by the gonad index (size of gonad relative to body weight) and presence and ripeness of the gametes. The gametes were studied closely only during 1959–60.

minate for several months but at the end of summer sperms can be seen and small germinal vesicles again make their appearance, long before the gametes form. Active sperms are present over a much wider span of time than mature eggs.

NUTRITION

Since the intestines of *Allocentrotus* brought in from the field are sometimes devoid of food, and at other times have very little, it would appear that the urchins may go for long periods of time without food. This seems likely since defecation may continue for a week or more in the laboratory, indicating slow digestion of an ample meal. In the previous study, on only one occasion were we fortunate enough

to obtain specimens richly charged with food when a diatom bloom occurred in the area. In the present study many collections yielded animals relatively full of diatoms, presumably because of similar blooms.

On November 7, 1960, a collection was made nearer the edge of the urchin bed in an attempt to get smaller specimens for a study of respiration. The intestines of these specimens were filled with bites out of large algae: green, red and brown. The fragments of algae were irregular and much larger than the balls of diatoms illustrated in the previous paper. In the foregut the algae were undigested and had little of the gelatinous material around them. In the hindgut more fully digested algae were enclosed in the mass of gelatinous material within which were

TABLE I

Chemical composition of gonads and gametes of Allocentrotus fragilis *in per cent of dry weight*

Tissue	Condition	Lipid	Non-protein N	Protein	Glycogen
Testis	gravid	14.54 15.34 13.08	3.26 3.62 4.24	28.70 30.25 31.93	0.36 — —
Testis	starved animal	18.6 17.7	— —	— —	— —
Testis	spent	12.36	3.66	36.13	—
Sperm	—	3.60	4.22	38.7	—
Gonad	indeterminate	14.50 17.85	3.65 3.66	23.88 27.05	0.83 —
Ovary	gravid	17.79 14.61 15.01	3.40 4.40 2.94	34.09 32.47 27.39	0.69 — —
Ovary	starved animal	20.8 20.0	— —	— —	— —
Ovary	spent	12.83	3.17	20.87	—
Eggs	—	18.07	2.44	28.68	—

many bacteria, much as previously described in *Strongylocentrotus purpuratus* (Lasker and Giese, 1954). Fecal pellets collected from animals which had been in the laboratory aquaria overnight retained their shape and looked more like the rounded pellets previously described from *A. fragilis*. The algal fragments in many of them were almost completely digested, only colorless pieces remaining. In only one previous collection of *Allocentrotus* had individuals with pieces of larger algae (*Cladophora*) in the gut been obtained, all the others having diatoms and fragments of various minute materials present at the bottom of the sea. The November 7th collection followed rough seas which may have torn algae from the rich offshore beds nearby, making them available to the urchins.

When *Allocentrotus,* kept in the laboratory without food for a month, were

dissected, they were found to be free of intestinal contents. Since in some collections urchins were observed free of food, it is likely that in their natural environment they are occasionally unable to get food for a comparable period of time. A store of nutrients is therefore necessary to maintain the urchins between the sporadic droppings of material from the surface waters. The sea urchin has three major organs in which storage might occur: gonads, intestine and body wall. The latter (hereafter called the test) consists of the test proper, the epidermis, the tissues of the coelomic lining and the water vascular system attached to it. Biochemical analyses [2]

TABLE II

Chemical composition of intestine, intestinal contents, and shell of sea urchins in per cent of dry weight

Tissue	Condition	Lipid	Non-protein N	Protein
		Allocentrotus fragilis		
Intestine	well-fed	28.88	2.17	35.38
		26.81	3.13	31.22
		23.24	2.77	38.84
	starved	20.6	—	—
Contents	fresh meal	8.28	1.66	22.83
	feces (rectal)	1.87	0.33	7.15
Test	well-fed	1.79	0.22	5.66
	starved	0.9	—	—
		0.8	—	—
Diatoms*	entire	8	—	28.1
		Strongylocentrotus purpuratus		
Foregut	well-fed	12.28	3.84	39.33
Hindgut	well-fed	12.52	3.30	33.94
Contents	fresh meal	3.88	0.34	10.70
		Strongylocentrotus franciscanus		
Gut	well-fed	22.2	—	—
		18.7	—	—

* From Pease, 1932; 63.2% carbohydrate present in diatoms.

indicated that the fragile urchin, like the purple sea urchin (Giese *et al.*, 1958), stores considerable lipid and a small amount of glycogen in its tissues, as seen in the data in Tables I and II. Protein, the main structural constituent of protoplasm, is also present in considerable quantity, as expected in any tissue. It would appear that the main reserve food is lipid, glycogen being a minor reserve. Since relatively little sugar appears in the body fluid, the latter finding is perhaps not surprising.

[2] The methods employed were like those described elsewhere (Giese *et al.*, 1958). Many of the measurements were done in triplicate; later only duplicates were run since the repeats were so much alike.

The data in Table I show that considerable lipid is present in the gonads of *Allocentrotus,* a bit more in the gravid than in the spent ones. There is more lipid in the eggs than in the ovary but relatively little in the sperms taken alone. Without comment, the data in Table I do not express fully the meaning of the changes in the organic content of the gonads of the animals during the breeding season. The gonad index varies by a factor of at least 5 (Fig. 1), the maximal variability being 9-fold. Therefore, the lipid content per unit dry weight of a gonad is not a true measure of the reserves, since the shrunken gonad of a spent or indeterminate gonad may be only one-fifth the size of the gravid gonad. The total lipid present in the gonad of a gravid animal would be at least 5 times as great as in a spent animal.

TABLE III

*Wet and dry weight of tissues and tests**

Tissue	Wet wt.	Dry wt.	% solid	% water	GI**	% body fluid
			Allocentrotus fragilis			
Whole	35.6	3.8	10.6	89.4	2.06	58
Whole	66.9	6.25	9.4	90.6		
Whole	96.6	11.38	11.8	88.2	2.63	62
Gonad (♂)	2.54	0.51	20	80		
Gut	2.1	0.66	31.3	68.7		
Lantern	1.75	1.06	60	40		
Test	22.7	7.45	32.8	67.2		
			Strongylocentrotus purpuratus			
Whole	98.3	32.55	33.2	66.8	8.6	22.3
Whole	64.6	24.5	38	62	10.83	30.3
Gonad (♀)	7	1.45	20.6	79.4		
Gut	2.64	0.95	36	64		
Lantern	2.2	1.6	73	27		
Test	3.2	20	61	39		

* Some data for *Strongylocentrotus* are given for comparison to *Allocentrotus*. Note the more massive skeleton in *Strongylocentrotus*, its lesser water content, and the lesser amount of body fluid.

** GI refers to gonad index (defined in the introduction).

Lipid is stored in quantity in the intestine and body wall. Analyses indicate that as much as 29% of the dry weight of the gut may consist of lipid, but only about 2% of dry weight of the test and its tissues consists of lipid.

While the per cent lipid content of the test appears small, it must be remembered that the test forms a considerable part of the entire dry weight of the urchin (Table III). An urchin which when wet weighs 96.6 grams, weighs only 11.38 grams when dry, including the body fluid salts, indicating that 88.2% of its wet weight is water. The dried gonads weigh 0.51 gram and the intestines, washed free of gut contents and dried, about 0.66 gram. The dry test and lantern weigh 9.5 grams. According to the data in Table II about 1/20 of the test (5.66%) is protein, which is probably an approximate measure of the amount of tissue present. Therefore,

about 5.66% × 9.5 grams, or 0.54 gram of tissue, is probably present in the test. The amount of tissue in the test is thus probably equal to, or greater than that in the gut (no account was taken of the other organic constituents in the above calculations). It is possible that some protein forms a network in the test, in the interstices of which the salts are deposited, since the echinoderm test is supposedly a mesodermal structure like vertebrate bone (Hyman, 1955).

To compare the relative stores of lipids in gonad, test tissue, and gut, it is only necessary to multiply the amount of each tissue by its content of lipid. In this respect it appears that 9.5 grams × 1.79% or 170 mg. are stored in the test and lantern, 0.5 grams × 15.5% or 77 mg. are stored in the gonad, and 0.66 grams × 26.8% or 176 mg. are stored in the gut of an animal which weighs 96.6 grams wet weight. This shows that in regard to its storage of lipid, the test and intestine may be of equal importance in an animal of intermediate gonad index (2.63) such as the one tested here. For an animal with an index of 5 the amount of lipid stored in the gonad would be increased by a factor of almost 2, making it about equal to the gut or test. For an animal of low index (1.5 to 0.8) the amount of lipid stored in the gonad would be reduced by a factor of 1/2 or 1/3, and the stores in the intestine and the tissues of the body wall would then be of even greater importance.

Since so much lipid is stored in the intestinal walls, it seems most likely that the food is either the source of the lipid or that the lipid is manufactured from carbohydrates or protein in the diet. The gut contents vary in lipid, a relatively fresh meal of diatoms containing 8.28% of lipid, whereas a well-digested mass of material contains only 1.87% of lipid (Table I). Pease (1932) lists the lipid content of diatoms as 8% of the dry weight.[3] It therefore seems likely that the lipid probably is obtained from the diatoms and other food eaten by the urchin. The lipid is probably digested and stored, some of it in the gut, some in the other tissues.

In this respect it is interesting that another animal feeding upon diatoms, the sipunculid worm *Phascolosoma agassizi,* also has large stores of lipid in its gut, about 25% of the dry weight (A. Towle, personal communication). On the other hand, the data in Table II show that the purple sea urchin, *Strongylocentrotus purpuratus,* which feeds upon larger algae, stores only half as much lipid in its gut as *Allocentrotus.* Nonetheless, lipid is prominent and a fairly large amount is present in the intestinal pellets, 3.88% of the dry weight in a fresh meal in the intestine. It is surprising in this regard that *S. franciscanus,* which has a diet much like *S. purpuratus,* has much more lipid stored in its intestine (Table II). Algae may at times also accumulate considerable lipid (Milner, 1953; Fogg and Collyer, 1954). Perhaps *S. franciscanus* has a diet richer in lipids than *S. purpuratus.*

How the nutrient gets from the gut to the other tissues is not known at present. Lipid may pass out as small droplets of fatty material like the chylomicrons of mammals or it might be carried out and distributed by wandering cells. Lipid is present in the body fluid but the exact amount has not been determined for lack of adequate methods.

[3] Diatoms in culture do not always have this much lipid. According to Barker (1935) diatoms in a culture in the laboratory first synthesize carbohydrates during photosynthesis, the ratio of oxygen production to carbon dioxide consumption being unity. However, as the diatoms age they accumulate oil which is visible in droplets.

Upon starvation, stored nutrients are utilized. This was most evident in the shrinkage of the gonads of starved animals. Seven *Allocentrotus* starved for a month in an aquarium supplied with running sea water showed somewhat shrunken gonads—at least the index for the animals at the time of collection on November 6 was 3.2—a month later one would expect it to have risen to about 4.7; instead the gonad index for the starved animals was 2.5 on December 7. Similar shrinkage had previously been noted for the purple shore urchin (Lasker and Giese, 1954). It is probable that the nutrients stored in the gonads had been resorbed. While it seemed likely that the lipids were preferentially utilized, biochemical analysis revealed that per unit weight lipid increased in amount in the gonad, although it decreased both in total quantity in the animal and per unit weight in the body wall and the intestine. While the increase in lipid content per unit weight in the gonad may appear paradoxical, it is to be expected if one or more of the other nutrients in the gonad are utilized at a more rapid rate than the lipids. Since the total bulk of the gonad shrinks and the only other major organic material present in the gonad

TABLE IV

Respiration of tissues of Allocentrotus*

Tissue	Q_{O_2} μl./mg./hr.	R.Q.	Water content per cent	Number of experiments
Test	0.076	0.58	65.6	2
Testis	0.570	0.92	80.4	3
Ovary	0.088	0.65	80.8	8
Gut	0.383	0.57	79.2	10

* Note the high respiratory rate for the testis and gut as compared to the ovary and test.

is protein, proteins are probably being selectively metabolized in the gonads during the period of starvation. Wilber (1947) has described similar results after starvation of *Phascolosoma gouldii*.

RESPIRATION

A few studies were made of the respiration of *Allocentrotus* tissues, primarily with a view of determining how it compared with other marine animals. The respiratory quotient was determined to ascertain, if possible, what types of foods were being used by the urchins.

The data are given in Table IV. It is at once apparent that the rate of tissue respiration is comparable, per unit wet weight, to that for other sea animals (Nicol, 1960, p. 152). Of greater interest is the respiratory quotient characterizing the respiration of the sea urchin. If lipid is of importance in the economy of the sea urchin, a respiratory quotient of about 0.7 might be expected. If the urchin uses carbohydrates or mixtures of these with proteins and lipids, the respiratory quotient should be higher, approaching 1.0 when only carbohydrate is utilized.

Determination of the respiratory quotient of entire animals proves difficult because at the end of an experiment it is necessary to liberate the carbon dioxide which is trapped in the buffering system of the sea water bathing the urchins. Since to do this the sea urchins must be removed from the vessel, the extra manipulations

may permit the sea water to equilibrate with the air. Furthermore, the urchins shed some spines, pedicellariae or other skeletal pieces containing lime salts. Consequently, it is necessary to filter such water through bolting cloth to get rid of the calcareous materials. This involves still another step during which equilibration of the bathing water can occur with air, further vitiating the correction. The R.Q. for an entire animal small enough to fit into a Warburg flask was 0.7, suggesting lipid utilization. However, if any carbon dioxide had accumulated in the sea water during respiration, the manipulations preceding addition of acid and measurement might have liberated it, favoring a lower R.Q. value. Therefore, the data cannot be considered satisfactory since the method is unsatisfactory.

To by-pass this difficulty the gonad and gut tissues of the urchins were removed from animals, washed in sterile sea water, and the pressure changes measured manometrically in the presence of KOH in one series, and in the absence of KOH in another. In the latter case 3 N sulfuric acid was contained in the side arms, and at the end of the experiment the acid was added to liberate the excess carbon dioxide contained in the sea water surrounding the tissues. The data for respiration of the tissues in Table IV are therefore more satisfactory than those for the entire animal. Excepting the testes, the R.Q. for the tissues is between 0.6 and 0.7, definitely suggesting lipid metabolism. Since the experimental deficiencies mentioned for the studies on the entire animal do not apply, the data on tissues are more convincing than those for the entire animal. Presumably the respiration of the entire animal is the sum of the respiration of its various parts (Field *et al.*, 1939). Consequently, one might suppose that the data for the tissues are applicable to the entire sea urchin. The high R.Q. for the testes could result from utilization of carbohydrate along with some other nutrients.

Discussion

The present study suggests that lipid may play a significant role in the economy of *Allocentrotus*. The sea urchin has a considerable supply of lipid in its usual diet of diatoms. It stores considerable lipid in its intestine and gonad and some even in the tissues adherent to the skeleton. Furthermore, the stores of lipids decrease in amount when the sea urchin is starved for a month. The amount of lipid is thus closely related to the nutritive state of the animal. This has proven to be the case in other echinoderms from this area (Giese, 1959) and in other regions as well (unpublished).

The small amount of glycogen found in tissues of *Allocentrotus* suggests that either some other kind of carbohydrate is stored in this urchin or else that carbohydrate plays a minor role here. Glucose does not increase the respiration of gut or gonadal tissue here, just as it failed to do in tissues of a purple sea urchin.

The respiratory quotient for the tissues studied—gut, test and ovary—is about 0.6 to 0.7. This indicates that some lipid is being used for respiration; that is, it is being metabolized. Addition of glucose does not change the R.Q. It thus appears possible that lipid is being used preferentially for metabolism although it is more likely that added sugar fails to stimulate respiration because it fails to enter the tissues.

In view of the apparent reliance of *Allocentrotus* on lipids, its occasional eating of large algae—red, brown and green—which have little lipid, but much polysac-

charide, is interesting. Either *Allocentrotus* uses only the readily digestible materials in the algae or it has enzymes to utilize some polysaccharides, as does the purple sea urchin (Huang and Giese, 1958; Eppley and Lasker, 1959). The algae in the fecal pellets of *Allocentrotus* feeding on large algae were rather completely digested, suggesting that more than the lipids are being utilized. It would be interesting to know whether the urchin itself has such enzymes, and secondly, whether the monosaccharides obtained are stored as polysaccharides in the urchin or are converted to lipid.

The observation that *Allocentrotus* takes in large algae indicates that it is more resourceful than had been previously considered (Boolootian *et al.,* 1959). In the laboratory it failed to eat the large algae but apparently it does so when it gets them in nature.

While *Allocentrotus* can withstand starvation at least a month, and probably longer, judging from the healthy appearance of the specimens starved (in the dark) for over a month, in nature this is probably not the rule since dissected animals usually had at least a few pellets in the gut. Specimens choked with pellets of algae at the time they were collected had nothing whatsoever in the gut.

No clear-cut correlation between the breeding season and the availability of food has been observed in the monthly collections. An annual reproductive cycle is suggested by the present study but the stimulus to the development of the gonads still remains elusive.

Summary

1. Additional data on the size of the gonads relative to the body and the presence and ripeness of gametes and their maturity were gathered for a year on the deep-sea echinoid, *Allocentrotus fragilis.*

2. The data indicate that the breeding season is an annual cycle with a maximum gonadal size in January and February, accompanied by the presence of mature eggs and sperm.

3. The sea urchins in one collection were found to have fed on large algae, the pellets resembling those of the intertidal sea urchin, *Strongylocentrotus purpuratus.*

4. A considerable quantity of lipid is found to be stored in the wall of the gut, less in the gonad, and still less in the body wall (per unit dry weight). Total amount of stored lipid is largest in the gut, next in the test, and least in the gonad.

5. The usual diatom diet of the sea urchin contains much fat; the algae tested in one series contain considerably less.

6. The respiratory quotient of the gut, ovary and test of the sea urchin was found to be about 0.6 to 0.7, suggesting utilization of lipids. The R.Q. for the testis was 0.92.

7. Some comparisons are made between *Allocentrotus* and the purple intertidal urchin, *Strongylocentrotus purpuratus.*

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A DUAL EFFECT OF CARBON DIOXIDE ON INSECTS POISONED BY OXYGEN [1]

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The presence of small amounts of carbon dioxide during exposure to high pressure of oxygen accelerates the appearance of convulsions and death in oxygen poisoning of vertebrates (Hill, 1933; Shaw *et al.,* 1934). Several explanations have been offered to account for this phenomenon. For example, it was early proposed that during exposure to oxygen, carbon dioxide accumulates in the tissues, and that carbon dioxide is the effective agent in poisoning (Gesell, 1923). Other workers claimed that carbon dioxide contributes only secondarily to the lethal effect of oxygen itself (Shaw *et al.,* 1934), and the effect of carbon dioxide on oxygen poisoning of vertebrates is no doubt a complex one (Lambertsen *et al.,* 1953). Williams and Beecher (1944), in a study of oxygen poisoning in Drosophila, found that this sensitizing effect of carbon dioxide on oxygen poisoning was not restricted to vertebrates. They reported that the presence of small amounts of carbon dioxide increased the sensitivity of adult *Drosophila azteca* to 10 atmospheres (atms.) of oxygen. In view of these findings, we did not anticipate a curious result that we encountered while investigating oxygen poisoning in the parasitic wasp, *Mormoniella vitripennis:* carbon dioxide appeared to protect these wasps from oxygen poisoning (Goldsmith, 1955). This discovery, which found no parallel in the literature on oxygen poisoning, prompted a detailed study of the effects of carbon dioxide on oxygen poisoning of adult and developing insects.

Materials and Methods

1. *Experimental animals*

The chalcid wasp, *Marmoniella vitripennis* Walker, was used for these experiments. Procedures for rearing and handling this insect have already been described, along with detailed accounts of its life history and postembryonic development (Tiegs, 1922; Schneiderman and Horwitz, 1958; Goldsmith and Schneiderman, 1960). The present experiments utilized animals at three stages of development: (1) "pink stage" developing adults (24 hours after ecdysis from the final larval cuticle); (2) "black stage" developing adults (12 to 24 hours prior to adult emergence); and (3) adults (various ages, both males and females). Details of the method used to select insects of a uniform age and stage of development are given in a previous paper (Goldsmith and Schneiderman, 1960).

In most experiments with adult insects, 10 males or 10 females were placed in a one-dram shell vial, loosely plugged with cotton; a single vial was compressed

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in each pressure chamber. The compression chambers were constructed of transparent polymethylmethacrylate (lucite), and the activity of adult wasps could be observed during exposure. Observations were begun when full pressure was reached and continued at ten-minute intervals until decompression.

Characteristically, normal wasps quickly right themselves if they fall or are knocked over. During the exposure to various gas mixtures, the times at which the first and last adults in a group became unable to right themselves, as well as the times at which the first and last adult ceased moving, were noted. In experiments in which the recovery of adults was to be studied, the chambers containing the experimental insects were decompressed 10 minutes after all the adults ceased moving. The activity of the wasps was observed immediately and then at intervals of 12 to 24 hours for several days. The reappearance of the righting behavior and the ability to walk were convenient measures of the extent of recovery. In observations during the recovery period, the wasps were knocked on their backs and the number which righted themselves as quickly as the control wasps was recorded. Most of the adults that could right themselves attempted to walk, but not all regained the agility of normal adults. Wasps that had recovered enough to run normally could, when tapped to the bottom of the glass vial, walk or run up the vertical side (30 mm.) within one minute. This last test was used as a convenient measure of full recovery. The activity and co-ordination of wasps which walked normally was not otherwise distinguishable from the control wasps which had not been exposed.

The responses of the sexes were similar, and results for males and females were averaged together. The data from each treatment were compared with those from other treatments in the same experiment. Each experiment was repeated at least once and usually several times.

2. *Compression and decompression*

Most experiments were carried out at 10 atms. of oxygen at 20° C., the same conditions used by Williams and Beecher (1944). The compression chambers enclosing the experimental wasps were equilibrated at the desired temperature for one-half to one hour prior to compression. In experiments with carbon dioxide, the desired pressure of carbon dioxide was superimposed on the atmosphere of air present in the compression chamber following equilibration at the experimental temperature. The amount of carbon dioxide added was monitored on a sensitive Bourdon-type gauge which had a capacity of one atmosphere (gauge pressure). After the addition of carbon dioxide, the compression chamber was sealed and the 1-atm. gauge was replaced by a gauge with a capacity of 20 atms. Following this, ten atms. of oxygen were added to the chamber within one minute. The procedure of adding the desired gases to the atmosphere of air already present in the tank insured that the insects were never subjected to oxygen tensions which were below normal. Throughout this paper all pressures are reported as gauge pressures.

Decompression was performed step-wise over a period of five minutes. Further details of these compression and decompression procedures have been given in an earlier paper (Goldsmith and Schneiderman, 1960).

Results

1. *Effects of carbon dioxide at normal oxygen tension*

Before appraising the influence of carbon dioxide on oxygen poisoning, it was necessary to assess the effects of carbon dioxide in the presence of a normal oxygen tension (0.2 atm.). The desired pressure of carbon dioxide was added to the atmosphere of air initially present in the compression chamber. Studies with nitrogen and helium have shown that small increases in ambient pressure, as would result from the added carbon dioxide, do not affect the insect's behavior. (Goldsmith, 1955).

As is well known carbon dioxide is commonly used as an anesthetic for insects. Ordinarily the movement and co-ordination of adult wasps were not conspicuously affected by one hour of exposure to 0.1 or 0.2 atm. of carbon dioxide. Occasionally, the wasps appeared less active in 0.2 atm. of carbon dioxide than in air, but they always maintained a standing posture, and they resumed normal activity as soon as they were returned to air. In 0.5 atm. of carbon dioxide, the wasps ceased moving after approximately 10 minutes.

The effectiveness of carbon dioxide was only slightly increased when 10 atms. of nitrogen were superimposed upon the carbon dioxide. Adults compressed with 10 atms. of nitrogen plus 0.2 atm. of carbon dioxide were only slightly less active than normal. However, when 10 atms. of nitrogen were superimposed upon 0.5 atm. of carbon dioxide, the insects ceased to move almost immediately. Since one atmosphere of air was present in all experiments, these results cannot be attributed to lack of oxygen. After four hours, the insects in 0.5 atm. of carbon dioxide and 10 atms. of nitrogen were decompressed. These wasps rapidly regained normal activity as did wasps decompressed after four hours in 0.5 atm. of carbon dioxide. From these results, it appears that in *Mormoniella* carbon dioxide at 0.1 atm. has no detectable effect, at 0.2 atm., a very slight effect, and at 0.5 atm., it is an effective anesthetic which has no detectable after-effects.

Table I

Duration of exposure to cause loss of spontaneous movements in adult Mormoniella exposed to various gas mixtures

Exp't No.	Number of wasps	Age of wasps (days)	Time at which first insect lost spontaneous movement to time at which entire population succumbed (minutes)		
			10 atms. O_2	10 atms. O_2 and 0.2 atm. CO_2	10 atms. O_2 and 0.5 atm. CO_2
26	200	0–1	70–80	55–65	not determined
33	95	0–1	40–50	35–45	0*
31	155	1–2	40–50	40–50	not determined
25	60	1–2	50–60	50–60	0
23	50	1–2	40–50	35–45	not determined
24	60	0–2	40–50	40–50	not determined
32	75	4–5	40–50	30–40	0

* Activity disappeared immediately.

2. Effect of carbon dioxide on the onset of oxygen poisoning

To appraise the effects of carbon dioxide on the onset of oxygen poisoning in adult wasps, insects in 10 atms. oxygen and 10 atms. oxygen plus 0.2 or 0.5 atm. carbon dioxide were observed at 10-minute intervals. Table I shows that complete loss of movement in all individuals consistently occurred within 10 minutes after the first wasp succumbed. In a few experiments (numbers 26 and 32), adults in oxygen plus 0.2 atm. of carbon dioxide appeared to lose spontaneous movement somewhat sooner than wasps in oxygen. This effect, however, was neither as marked nor as consistent (*cf.*, numbers 24, 25, 31) as that described by Williams

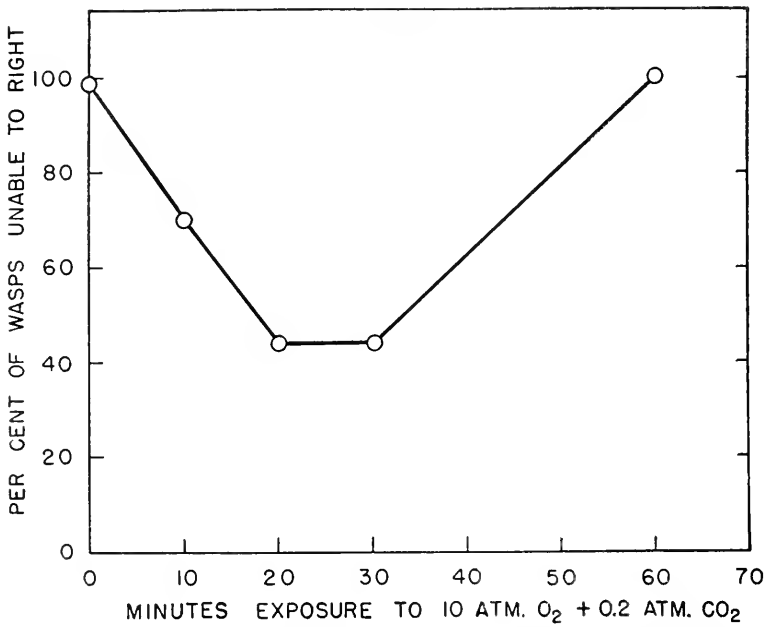

FIGURE 1. The loss of normal activity (as shown by loss of the righting reflex) during exposure to oxygen. Observations were made at 10-minute intervals on 80 adult wasps compressed in 0.2 atm. of carbon dioxide plus 10 atms. of oxygen. In this experiment, the activity of almost all the wasps was depressed initially, but there was a considerable return of activity before the paralytic symptoms of oxygen poisoning commenced (30 minutes).

and Beecher (1944) for 10- to 11-day-old *Drosophila*. They found that adults lost spontaneous activity almost twice as fast in oxygen mixed with 0.16 atm. carbon dioxide as in oxygen alone.

Invariably 0.5 atm. carbon dioxide plus 10 atms. oxygen immediately paralyzed adults, and they remained motionless for the duration of exposure. This was to be expected, since in control experiments 0.5 atm. carbon dioxide and 10 atms. nitrogen also had an immediate anesthetic effect.

In some experiments wasps in 0.2 atm. of carbon dioxide plus 10 atms. of oxygen ceased moving almost immediately; however, wasps that were affected in

this way often recovered while still under compression.[2] This can be seen in the experiment in Figure 1. Immediately after compression, all but 1% of the wasps had lost their righting behavior. After 10 minutes about 30% had regained the ability to right themselves, and after 20 minutes more than 50% could right themselves. Thereafter anesthesia set in again, and after 60 minutes of compression none were able to right themselves.

This initial loss of activity and subsequent recovery was not invariable. In eight different experiments involving a total of 500 adults, 37% of the insects exhibited this initial paralysis. In three of these experiments none of the adults was initially affected, while in one experiment 99% became motionless immediately. It seems likely that this variability in the effect of 0.2 atm. carbon dioxide administered with 10 atms. oxygen can be attributed to variation in the sensitivity of different groups of wasps and to the fact that 0.2 atm. was approximately the threshold pressure of carbon dioxide for producing an immediate loss of co-ordination in 10 atms. oxygen. In the presence of 10 atms. oxygen, the initial depression of activity was never observed at pressures of carbon dioxide less than 0.2 atm., but at higher pressure (0.25, 0.5 atm.) of carbon dioxide, activity ceased almost immediately. Apparently in some cases, an insect's activity can be immediately, albeit temporarily, depressed by a mixture of carbon dioxide and oxygen at a carbon dioxide concentration which by itself is not anesthetic (0.2 atm.).

3. *Effect of the presence of carbon dioxide during oxygen poisoning on recovery*

Although the previous experiments revealed that under some conditions simultaneous exposure to carbon dioxide and oxygen resulted in almost immediate, but temporary, loss of co-ordination, the presence of carbon dioxide also prevented permanent injury by oxygen. Thus, wasps exposed to oxygen plus carbon dioxide showed striking recovery from the effects of oxygen poisoning.

Groups of wasps were kept compressed until 10 minutes after all visible movements had ceased. They were then decompressed, and their behavior observed for several days. Maximum recovery occurred within 48 hours. In a typical experiment (Fig. 2, Curve 1) at the time of maximum recovery, only 33% of the wasps that had been exposed to oxygen behaved normally. By contrast, most of those that had been exposed to oxygen and carbon dioxide recovered; in fact, all of those exposed to oxygen plus 0.2 or 0.5 atm. of carbon dioxide (Fig. 2, Curve 1) regained normal activity. Clearly, carbon dioxide has a marked protective effect.

The protective action of carbon dioxide was even more conspicuous when wasps were kept compressed for periods one and one-half times and twice that required to render them motionless (*i.e.*, up to 2½ hours). The results are recorded in Figure 2 (Curves 1.5 and 2). When the wasps were returned to air, none exposed to oxygen for 2½ hours survived; however, 27% of the wasps exposed for 2½ hours to oxygen and 0.2 atm. carbon dioxide, and 53% of the wasps exposed for the same period to oxygen plus 0.5 atm. carbon dioxide recovered their righting ability. The presence of small amounts of nitrogen, instead of the carbon dioxide,

[2] In these cases, the time given in Table I does not refer to this initial temporary paralysis, but to the time at which spontaneous movement ceased permanently under compression.

during the period of oxygen poisoning had no noticeable effect on the subsequent recovery of oxygen-poisoned insects.

4. *Effect of carbon dioxide during oxygen poisoning on the development of "pink" and "black stage" wasps*

Having demonstrated that carbon dixoide protects adult wasps from oxygen poisoning, our attention turned to developing wasps. Wasps in the "pink stage" of adult development were subjected to oxygen or oxygen and carbon dioxide for several hours, then decompressed, and their subsequent development recorded. An animal which attains the "black stage" has completed all the externally visible signs of development. During the period of four days which intervenes between the

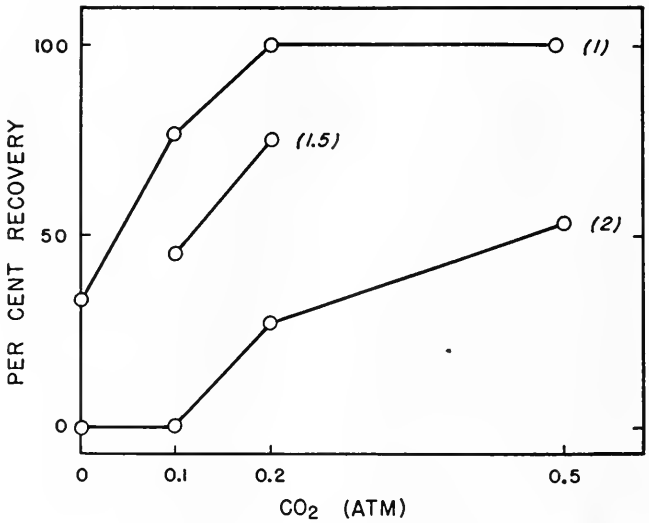

FIGURE 2. The effect of carbon dioxide on the recovery from oxygen poisoning (10 atms. at 20° C.). The ordinate gives the percentage of wasps exposed which had recovered the ability to right themselves 48 hours after decompression. Each curve shows the effect of a different duration of exposure. The basic exposure (1) continued for 10 minutes after spontaneous movements ceased, a total of 75 minutes. Other wasps were given 1.5 times and twice the basic exposure (Curves 1.5 and 2). Results obtained from 150 wasps.

"pink" and "black stages," external development is mainly concerned with epidermal pigmentation (Goldsmith and Schneiderman, 1960). Figure 3 reveals that all but about 5% of the wasps exposed to oxygen for 12 hours at the "pink stage" became black but only 13% of the exposed wasps were ever able to emerge fully; the rest remained completely or partially within the pupal cuticle. Although epidermal pigmentation was not affected, a system necessary for emergence was. These results agree with earlier experiments (Goldsmith and Schneiderman, 1960). By contrast, when carbon dioxide was present during exposure to oxygen, five times as many wasps (65%) emerged fully. Here again carbon dioxide exerted a protective action.

In another series of experiments, wasps were exposed to oxygen and carbon

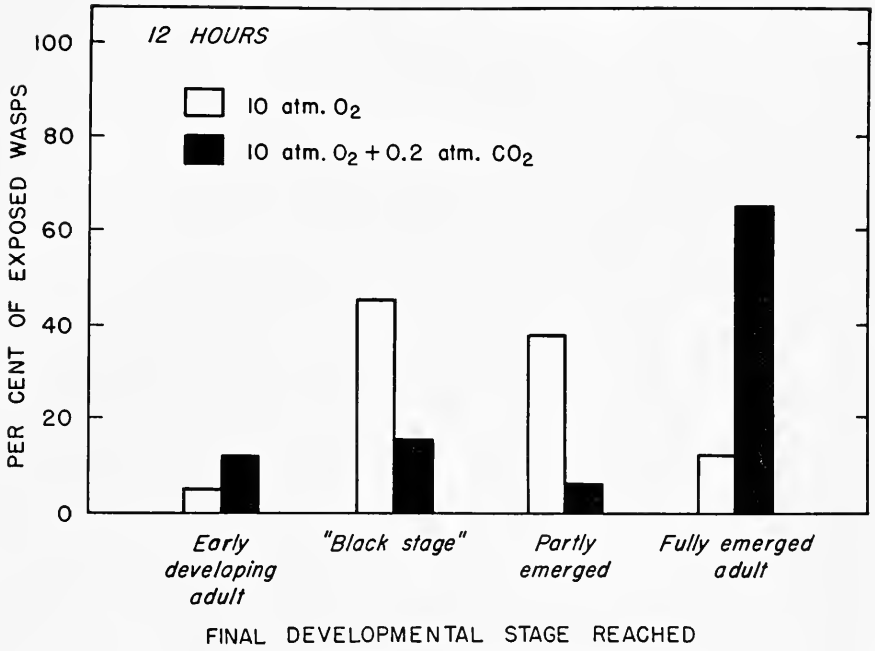

FIGURE 3. The final stage of development attained by "pink stage" developing adults after they were removed from 12 hours of 10 atms. of oxygen (open bars) or 12 hours of 10 atms. of oxygen plus 0.2 atm. of carbon dioxide (solid bars). Forty wasps were exposed to oxygen and 40 to oxygen and carbon dioxide.

dioxide during the "black stage" and again their ability to emerge was recorded. After 6 hours' exposure to oxygen, none of these wasps emerged completely and only 15% were active enough to free themselves partially from their pupal cuticle. The wasps that had been exposed to carbon dioxide plus oxygen were less severely affected; 5% emerged normally and 45% managed to emerge partially (Fig. 4).

5. *Effect of carbon dioxide on oxygen poisoning in Drosophila*

Experiments were also performed on a modest number of adults of *Drosophila melanogaster* (128 adults, 0 to 2 days old). When carbon dioxide was present during the exposure to oxygen, the flies lost their ability to make co-ordinated movements far sooner than in oxygen. Thus, it usually required 40 to 60 minutes of exposure to 10 atms. oxygen at 20° C. to abolish movement. By contrast, in seven separate compressions, all flies compressed with 0.2 atm. carbon dioxide plus 10 atms. oxygen ceased moving during the first 30 minutes of exposure. Such inactive flies did not recover while still compressed. Thus carbon dioxide administered with oxygen causes an initial loss of activity in *Drosophila* which is more marked than that observed in *Mormoniella*.

We may ask if the flies which became motionless with half an hour's or less exposure to carbon dioxide and 10 atms. oxygen were as severely affected as those subjected to oxygen until they were motionless. It is clear that they were

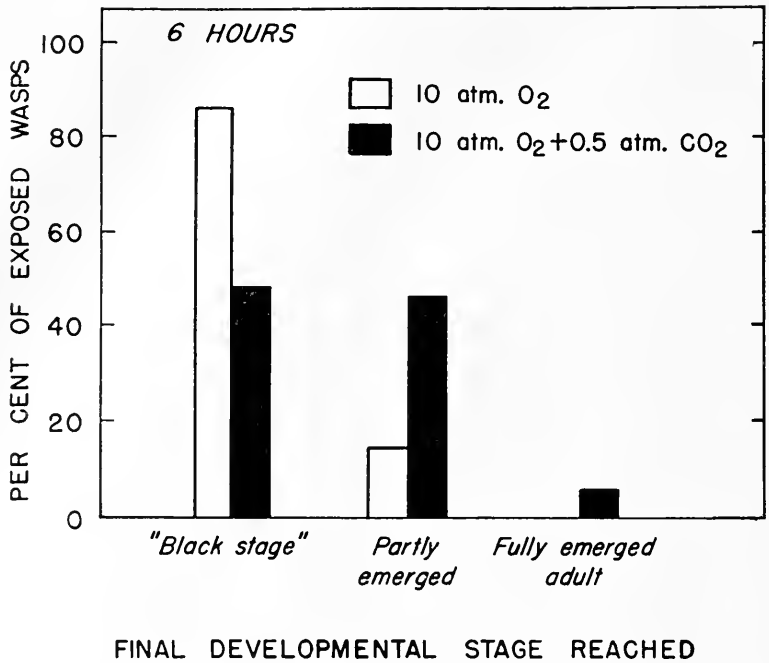

FIGURE 4. The emergence of "black stage" developing adults after they were removed from 6 hours of 10 atms. of oxygen (open bars) or 6 hours of 10 atms. of oxygen plus 0.5 atm. of carbon dioxide (solid bars). Forty wasps were used in each exposure.

not. When decompressed at the time they first ceased moving (*i.e.,* 10–30 minutes in oxygen and carbon dioxide, 40–60 minutes in oxygen), none of the former exhibited permanent injury; whereas, all but about 10% of the latter remained inactive (Table II).

Flies in oxygen and carbon dioxide were also subjected to the same length exposures that caused flies in oxygen without carbon dioxide to cease moving.

TABLE II

Survival of Drosophila melanogaster after oxygen poisoning

Exp't No.	Exposure (minutes)	10 atms. O_2		10 atms. O_2 and 0.2 atm. CO_2	
		Activity prior to removal	% Uninjured or recovered*	Activity prior to removal	% Uninjured or recovered*
21	10	Normal	100	None	100
40	30	Normal	100	None	100
21	40	None	0	Not determined	
43	50	None	20	None	50
40	60	None	10	None	40

* Per cent of adults uninjured is given for flies whose activity was normal on removal. Per cent of adults which recovered fully is given for flies who had lost all activity during exposure.

Under these conditions, it is evident that, as with *Mormoniella,* the presence of carbon dioxide during compression with oxygen promotes recovery of the flies. Thus while on the average only 10% recovered after an hour's exposure to oxygen, 40% recovered when 0.2 atm. of carbon dioxide was simultaneously present.

Discussion

From observations on adult *Mormoniella* and *Drosophila,* it seems clear that the presence of carbon dioxide during oxygen poisoning has at least two effects. First, it may hasten loss of activity without producing permanent toxic effects. Second, it promotes recovery and prevents permanent injury from oxygen poisoning. Oxygen poisoning in adult insects can be conveniently divided into two phases. In the first or reversible phase, the effects of poisoning are evident soon after compression, but if the insects are decompressed promptly there is no permanent damage. If decompression is delayed, the second or irreversible phase ensues; coordination becomes increasingly impaired, and the effects of poisoning persist indefinitely after decompression (*cf.* Williams and Beecher, 1944). Two interpretations of the effect of carbon dioxide in promoting the initial loss of activity are possible. Perhaps the presence of small amounts of carbon dioxide hastens the appearance of the reversible phase of oxygen poisoning or, alternatively, it may be that high pressures, particularly of oxygen, in some way potentiate carbon dioxide anesthesia. The present experiments do not allow us to choose between these two possibilities.

The protective action of carbon dioxide is exhibited with developing insects as well as adults. It has previously been shown that if the proper exposure is selected, wasps exposed to oxygen in the "pink stage" will develop to the "black stage" but fail to emerge. This inability to emerge results from failure of muscle development caused by oxygen poisoning (Goldsmith and Schneiderman, 1960). In the present study, many more wasps emerged after exposure to oxygen and carbon dioxide than after exposure to oxygen alone. Thus, "pink stage" wasps exposed to carbon dioxide and oxygen must have developed muscles.

In their study, Williams and Beecher (1944) concluded that the presence of carbon dioxide sensitized insects to oxygen poisoning. They examined the effects of carbon dioxide on 53 adults of *Drosophila azteca.* They found 10- to 11-day-old flies had lost all their activity when they were decompressed after 21 minutes in 10 atms. of oxygen at 20° C. The rate of poisoning (on the basis of the activity of the adults) was a linear function of the carbon dioxide tension (Williams and Beecher, 1944; Fig. 5). They reported that *Drosophila* in 0.16 atm. carbon dioxide plus 10 atms. oxygen ceased moving after 10 minutes. It is important to note that in this particular experiment they did not directly observe the flies during the compression period and did not look for recovery of the motionless flies after decompression. On the basis of our results, we suggest that the flies which Williams and Beecher exposed for brief periods to oxygen and carbon dioxide were not permanently injured, while at least some of the flies subjected to 20 minutes in oxygen alone probably suffered permanent damage. According to this view, there are no real differences between the results of the present experiment and those of Williams and Beecher. The conclusion of the present study is that although carbon dioxide may exert either a direct anesthetic effect in the presence of high pressures of oxygen or facilitate the appearance of the reversible phase

of oxygen poisoning, the most striking effect of carbon dioxide is its effectiveness in preventing permanent damage from oxygen poisoning. It is provocative to speculate that the effectiveness of carbon dioxide in protecting insects from oxygen poisoning may be the result of its anesthetic properties, which could render the particular system that is sensitive to oxygen less susceptible to injury. Whether other narcotic agents similarly render protection from oxygen toxicity remains to be seen.

We wish to thank Professor C. M. Williams for his help in discussing this work, and in reading the manuscript.

Summary

1. The activity of adults of *Mormoniella vitripennis* and *Drosophila melanogaster* during and after compression in 10 atms. of oxygen plus small amounts of carbon dioxide (0.1, 0.2, and 0.5 atm.) was studied. Addition of carbon dioxide at pressures above 0.2 atm. to the atmosphere of air in the compression chamber anesthetized *Mormoniella* adults; on decompression all adults rapidly regained normal activity. The effects of carbon dioxide administered along with 10 atms. of nitrogen were similar except that with 0.5 atm. carbon dioxide, anesthesia occurred more rapidly.

2. Although in some experiments with adults of *Mormoniella* the presence of carbon dioxide during exposure to oxygen accelerated the onset of paralysis, carbon dioxide actually protected the adults from permanent injury caused by exposure to oxygen. After 2½ hours at 10 atms. about half of the adult wasps which had been in oxygen plus 0.5 atm. carbon dioxide completely recovered while none which had been in oxygen without carbon dioxide survived. The number of wasps which recovered increased as the amount of carbon dioxide present during oxygen exposure increased from 0.1 to 0.5 atm.

3. Although the presence of carbon dioxide did not totally prevent oxygen poisoning, permanent injury to "black stage" and "pink stage" developing adults as well as adult *Mormoniella* was significantly reduced.

4. In the presence of 10 atms. oxygen, adult *Drosophila* became motionless more quickly when 0.2 atm. of carbon dioxide was also present. These motionless flies recovered fully when decompressed. Furthermore, significantly fewer flies recovered following paralyzing exposures to oxygen without carbon dioxide.

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METABOLIC ANTAGONISTS AND PROLONGED SURVIVAL OF SCALE HOMOGRAFTS IN FUNDULUS HETEROCLITUS[1]

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The immunological competence of an animal depends upon its ability to synthesize antibodies, the specificity of which appears to be regulated by nucleic acids. The exact mechanism whereby antigenic information is translated into antibody specificity in the course of protein synthesis, however, remains more speculated about than understood. In order to investigate this, attempts have been made to interrupt the hypothetical pathway along which such information might be expected to be transferred. Accordingly, the syntheses of nucleic acids and proteins have been interfered with by means of various antimetabolites, and the subsequent capacity for antibody formation studied.

As an experimental system, the homograft reaction against foreign scale transplants in *Fundulus heteroclitus* has been adopted. The usefulness of this technique in analyzing the homograft reaction has been demonstrated by Hildemann (1956, 1957a, 1957b, 1958) and Hildemann and Haas (1960). These investigators recognized the rejection of foreign scale grafts as a temperature-dependent inflammatory response leading to the destruction of the graft in 4.3 days at 32° C. to 40.5 days at 10° C. A further innovation was recently reported by Triplett and Barrymore (1960) who utilized the time of disintegration of melanophores on transplanted scales as the criterion for estimating the time of onset of the homograft reaction. According to this method, foreign scale grafts survived an average of 5.8 days at 20° C. and 7.0 days at 17° C. This technique of observing the duration of melanophore survival after grafting has made it possible to judge easily, quickly, and accurately the time required for the host to react against the presence of immunogenically foreign cells. The relatively abrupt nature of the overt response represents a distinct advantage over the more subjective methods of estimating homograft survival times in higher vertebrates.

There is a measure of uncertainty concerning the exact nature of the homograft response, particularly as regards the cellular vs. humoral location of factors alleged to be responsible for reaction against foreign tissues (*cf.* Brent, Brown and Medawar, 1959; Lawrence, 1960; Gorer, 1960). The demonstration by Triplett and Barrymore (1960) that homograft sensitivity can be transferred via intraovarian fluid from pregnant females to their embryos argues in favor of the possible existence of circulating antibodies, at least in fish. Since the present account, unhappily, cannot further resolve this problem, the author elects to assume that

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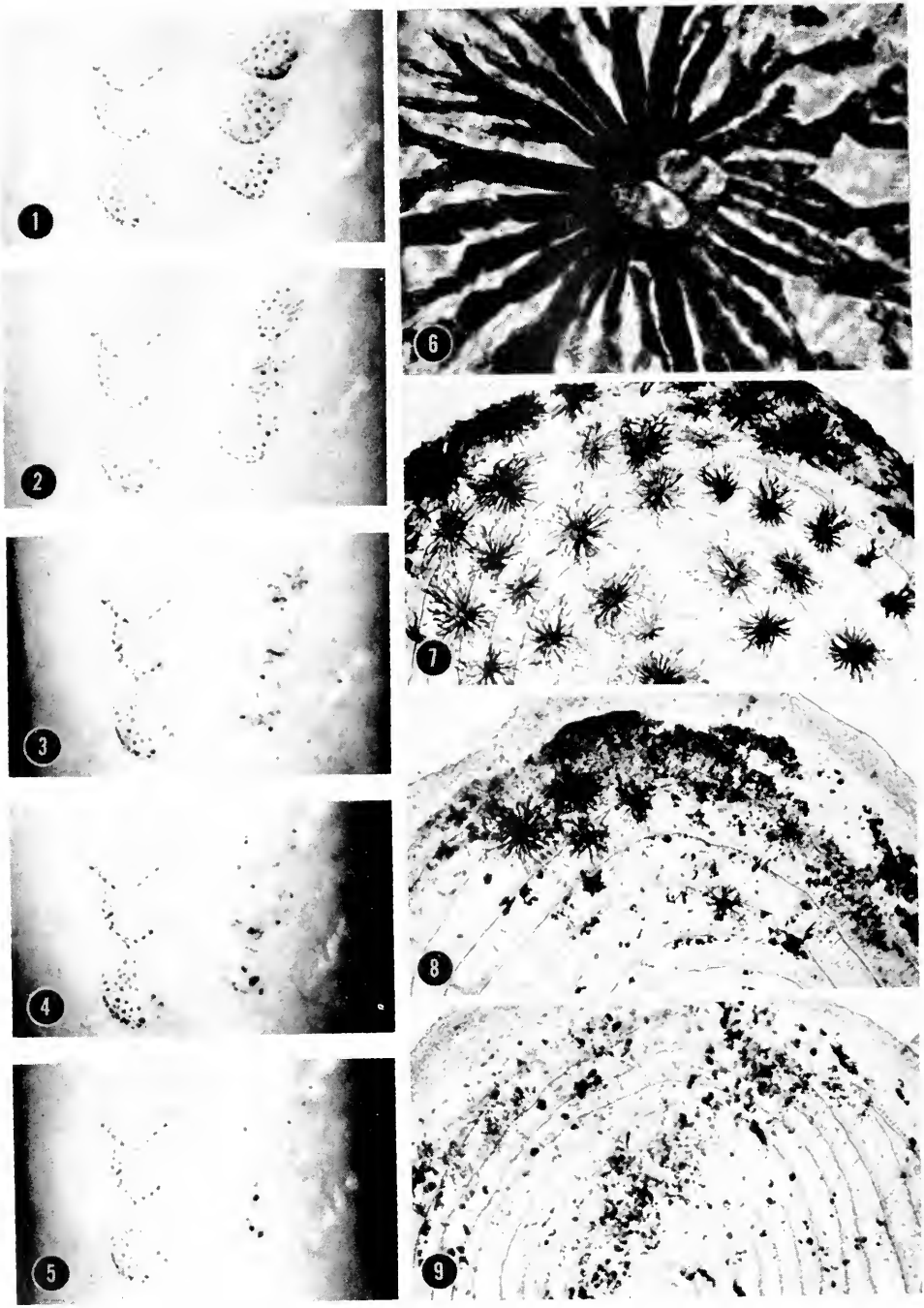
the rejection of homografts is an immunological phenomenon attributable to antibodies, without reference to their disposition or mode of action, but very much concerned with their genesis. As assayed by the prolonged survival of scale homografts on treated hosts, it has been found in the present experiments that under conditions of restricted protein or nucleic acid synthesis, antibody production has been inhibited.

Materials and Methods

Experiments were performed on male *Fundulus heteroclitus* weighing an average of 6 to 8 grams each. Fish were maintained in running salt water aquaria at a temperature of $28 \pm 1°$ C. except where otherwise stated. All operations were performed on fish under chloretone (1 : 1000) anesthesia. Scale transplantations were achieved by inserting scales in the dermal scale pockets from which the original scales had been plucked. If care is taken to graft scales of the same size as those being replaced, the transplants are rarely lost. All transplants were made to the unpigmented ventral region of the fish. In each animal three autografts and three homografts were inserted in parallel rows on either side of the linea alba. Pigmented scales derived from the posterior region of the fish proved to be of a size commensurate with those of the ventral transplantation site. Each experiment comprised four fish in which homografts were made reciprocally between pairs.

Circulation in the scale graft was re-established on the day following operation, at which time there was usually observed a slight nonspecific degeneration of a few pigment cells in both autografts and homografts (attributable to injury attending the transplantation procedures). Animals bearing scale grafts were examined daily under the dissecting microscope to determine the condition of melanophores in the transplants. In control fish, foreign melanophore breakdown was invariably complete on the third day after grafting at 28° C. (Figs. 1–5). The reliability of this was such that the survival of pigment cells only one day beyond this time constituted an unequivocal and statistically significant indication of the efficacy of the treatment being tested. The number of days designated as the survival time refers to the day on which complete or very extensive destruction of pigment cells was observed to occur. As a record of the experiments, photomicrographs were taken through the dissecting microscope of scale transplants at critical times.

In addition to the effects of temperature change, splenectomy, hypophysectomy, and trypan blue, the following substances, occasionally in varying doses, were administered to hosts by daily intraperitoneal injection in 0.1 ml. distilled water. Materials which interfere with nucleic acid synthesis were 5-fluorouracil, 5-fluorodeoxyuridine (FUDR) and 6-mercaptopurine (6-MP). Adrenal cortical hormones included cortisone acetate, 6-fluorohydrocortisone acetate and delta-1-hydrocortisone sodium succinate (Delta-Cortef: The Upjohn Co.). Two antibiotics were tested: chloramphenicol sodium succinate (Chloromycetin: Parke, Davis & Co.) and tetracycline hydrochloride (Achromycin: Lederle). The amino acid analogues used were β-2-thienylserine and DL-β-phenylserine (serine analogues), DL-α-CH_3 phenylalanine, β-2-thienylalanine and DL-β-phenyllactic acid (phenylalanine analogues), and ethionine (methionine analogue).



FIGURES 1–5. Daily photographic sequence of autograft (left) and homograft (right) scales on the first through fifth days after transplantation at 28° C. The row of three homo-

Results

Temperature effect

Groups of fish each bearing autografts and homografts were maintained at temperatures of 7°, 14°, 21°, and 28° C. All except the 7° C. group, which was kept in a refrigerator, were in circulating sea water. The scale grafts were inspected daily and photographed at frequent intervals to determine as precisely as possible the time of melanophore fragmentation. The lower the temperature the greater variation there was in the end point. At 28° C., incipient breakdown of pigment cells was detectable two days after operation, but not until the third day had all melanophores been destroyed. At this temperature, the reaction is relatively abrupt. Homotransplants of fish at 21° C. underwent pigment cell disintegration on the fifth and sixth days after grafting. Those maintained at 14° C. required 14 to 16 days to break down. At 7° C. the pigment cells of the homografts remained intact for 26 days at which time it was necessary to terminate the experiment. In all groups of fish, the autograft scales remained healthy indefinitely.

Splenectomy

In four fish, the spleens were removed via a ventral incision on the day prior to scale grafting. Four controls were subjected to sham operations. In all fish, controls and experimentals, the homografts broke down on the third day. The results are consistent with those of Vogel (1940) who noted that splenectomy failed to protect skin homografts in *Rana pipiens* from destruction.

Hypophysectomy

Animals were deprived of their pituitaries, or subjected to sham operations, two days before scales were grafted. This operation did not enhance the survival of homografts. In 12 hypophysectomized fish and 13 controls, the homografts exhibited breakdown of pigment cells on the third day.

Trypan blue

Four experimental animals received scale grafts on the day of the first intraperitoneal injection of 0.1 ml. of 1% trypan blue in distilled water. Injections were repeated daily. Control fish similarly grafted were injected intraperitoneally with 0.1 ml. distilled water daily. On the third day there was complete breakdown of pigment cells in the homografts of both control and experimental groups, despite the fact that the treated fish had become intensely stained with dye.

Nucleic acid antagonists

The injection of substances which inhibit nucleic acid synthesis proved to be very successful in protecting the homograft scales from the antibody response of

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graft scales exhibited pigment cell breakdown on the third day, with subsequent disappearance of the pigment granules. Autografts remained intact throughout. 10 ×.

Figure 6. A normal, expanded, scale melanophore showing typical binucleate condition. 1000 ×.

Figures 7–9. Appearance of scales before, during and after onset of homograft reaction. 100 ×.

the host. In two separate experimental series in which a total of eight fish were given 1 mg. of 5-fluorouracil each daily, starting on the day of operation, the homografts showed no signs of pigment cell breakdown as long as the fish survived. This dose, although effective, was at the same time toxic and resulted in the deaths of animals after two to six days. Nevertheless, four fish still alive on the fifth day possessed intact scale homografts, and it would appear that their transplants would have survived longer had the hosts lived. Similar experimental series on eight fish injected with 2 mg. of 5-fluorodeoxyuridine (FUDR) likewise resulted in protection of the homografts for up to eight days, by which time five of the animals had died as a result of the toxic effects of the drug. Injection of 2 mg. of 6-mercaptopurine into each of four fish bearing homograft scales afforded protection for four days at which time the injections were discontinued. Thereafter, melanophores were gradually destroyed until only one intact homograft scale remained on the seventh day, when the experiment was terminated. From these results it is clear that near-lethal doses of these drugs effectively prolong the survival time of homografts. In the cases of all three substances, when the doses were reduced to 1/100 of the above levels, no protection whatever was observed.

Adrenal cortical hormones

Daily intraperitoneal injection of a 1 mg. suspension of cortisone acetate to grafted fish starting on the day of operation had no beneficial effect on the survival of the homografts. On the third day there was complete breakdown of all scale pigment cells, probably due to inadequate doses of cortisone. More potent preparations of cortical hormones, however, proved to be more effective. Injections of 2 mg. of 6-fluorohydrocortisone actetate on the day of transplantation and on the two days thereafter resulted in the survival of homografts for four days, at which time about half of all the pigment cells were undergoing fragmentation. This nevertheless marks a definite delay in the destruction of the grafts. In a third experiment, 2 mg. of delta-1-hydrocortisone sodium succinate were injected daily through the second day after operation. In these fish, no pigment cell breakdown was observed on the third day, an incipient destruction was noted on the fourth, and, after five days, disintegration was well progressed in all except one animal in which the homografts remained intact. This compound, therefore, exerted a distinct protective effect.

Antibiotics

Chloramphenicol sodium succinate was tested at two dose levels. Daily injection of 1 mg. per animal intraperitoneally had no detectable effect on the survival time of the homografts. Similar injections of 10 mg. chloramphenicol, however, resulted in survival of homograft pigment cells beyond the third day. On the fourth day there was breakdown of melanophores in the grafts of one fish, and on the next day extensive, but still not complete, disintegration of foreign pigment cells had occurred. All were destroyed by the sixth day.

Tetracycline hydrochloride was likewise tested at two different dosages. Injection of 0.1 mg. per fish gave no protective effect. Administration of 1 mg. of tetracycline per day through the third day resulted in prolonged survival of homograft

pigment cells. In three fish the grafts were destroyed on the fourth day, and in three others they broke down on the fifth day. In two animals the pigment cells of homograft scales were still intact on the seventh day when the experiment was terminated. These antibiotics, therefore, interfere with the immunological response of the host against foreign grafts.

Amino acid analogues

Six different analogues of amino acids were tested for possible interferences with the homograft reaction. In general, they proved to be rather toxic and not very effective in protecting the foreign scale grafts from destruction by the host. In all cases, at least two dose levels were tried, the larger usually representing the limits of solubility in 0.1 ml. distilled water. Two analogues of serine were

TABLE I

Summary of effects of antimetabolites, administered to hosts, on survival of scale homografts at 28° C.

Substance injected (IP)	Dose (mg./fish/day)	Survival time (days)
Controls	—	3
5-fluorouracil	0.01	3
	1.0*	5+
5-fluorodeoxyuridine	0.02	3
	2.0*	6–8
6-mercaptopurine	0.2	3
	2.0	5–7
Cortisone	1.0	3
6-fluorohydrocortisone	2.0	4–5
Delta-1-hydrocortisone	2.0	5
Chloramphenicol	1.0	3
	10.0	4–6
Tetracycline	0.1	3
	1.0	4–7
β-2-thienylserine	1.0	3
	5.0	3–4
DL-β-phenylserine	1.0	3
	5.0*	3–4
DL-α-CH_3 phenylserine	1.0	3–4
	3.3	3–4
β-2-thienylalanine	1.0	3
	5.0	4
DL-β-phenyllactic acid	1.0	3
	2.5*	3
	5.0*	4
	10.0*	—
Ethionine	1.0*	3–4
	3.3*	5

* Lethal dose.

used. β-2-thienylserine and DL-β-phenylserine were injected in doses of 1 mg. and 5 mg. into groups of four fish. The smaller dose in all cases was ineffectual; the larger dose resulted in survival of homograft pigment cells for four days. None of these doses was lethal except in one fish which died on the third day following injections with 5 mg. of DL-β-phenylserine. Analogues of phenylalanine included DL-α-CH_3 phenylalanine which was administered in doses of 1 mg. and 3.3 mg. per day to groups of four fish. In all cases, foreign pigment cell breakdown was initiated on the third day but was not complete until the fourth. There was no detectable difference between the effects of the two doses utilized, nor did either dose prove to be lethal. β-2-thienylalanine was injected in doses of 1 mg. and 5 mg. daily. Only the larger dose prolonged the survival of scale homografts (to the fourth day). DL-β-phenyllactic acid, also an analogue of phenylalanine, was given in four different doses to four groups of fish. Doses of 1 mg. and 2.5 mg. failed to protect the homografts from destruction on the third day. A single injection of 5 mg. to another group of fish on the day of transplantation killed two fish the next day, but enabled the homografts of the remaining two animals to survive to the fourth day before being destroyed. Administration of 10 mg. of this compound proved lethal to all fish within one day. A final analogue, ethionine, was tested at levels of 1 mg. and 3.3 mg. The lesser dose permitted homografts to survive until the fourth day; 3.3 mg. per day through the second day after transplantation resulted in survival of homografts for two days beyond the controls. The latter dose, however, was lethal to three out of four fish by the fourth day after operation, at which time the foreign pigment cells were still intact. The one fish still alive on the fifth day exhibited complete breakdown of its homograft melanophores at that time. Ethionine therefore was considerably more effective in enhancing the survival of homografts than were the other five amino acid analogues tested.

Discussion

The immunological reaction leading to homograft destruction has been divided into three phases (Billingham, Brent and Medawar, 1956), involving the release of graft antigens (afferent phase), the production of antibodies (central phase), and the reaction of antibodies with the graft (efferent phase). Although interruption at any one of these levels would insure homograft survival, it is the central phase which is most amenable to experimentation. The process of antibody production may in turn be partitioned into subsidiary processes, leading from the initiating influence of the antigen on antibody-producing cells (induction or adaptation phase) to the eventual fabrication of antigen-specific antibodies (production phase). The method by which antibodies are formed is essentially a problem of protein synthesis with the added prerequisite that the protein antibody be capable of reacting specifically with the antigen originally responsible for initiating its formation. The synthesis of such specific proteins necessarily involves the participation of a system by which the nature of the specificity can be communicated from the antigen to the molecular architecture of the antibody concomitant with its synthesis. There is reason to believe that nucleic acids constitute such a communication system. This is substantiated by the dependent relationships of protein synthesis on RNA and of specific RNA synthesis on DNA. If this system is to remain sufficiently labile to adapt to new modes of protein synthesis (*e.g.*, specific

antigen-stimulated antibody production) it is necessary to assume that new specific types of RNA molecules can be synthesized on demand. This requirement may be taken to indicate that RNA synthesis is necessary for the production of specific proteins. Schweet and Owen (1957) have postulated that antigen reacts with DNA which in turn makes specific RNA, and that the RNA acts as template in giving rise to specific antibodies.

It is not surprising, therefore, that analogues of purines (6-MP) and pyrimidines (5-fluorouracil and 5-fluorodeoxyuridine), which interfere with nucleic acid synthesis, likewise arrest antibody production and thus result in the prolonged survival of homografts on treated hosts. Studies on 8-azaguanine, a purine analogue, have shown that it also inhibits nucleic acid synthesis (Skipper *et al.*, 1951) and antibody production (Malmgren, Bennison and McKinley, 1952; Dutton, Dutton and George, 1958). Berenbaum (1960) demonstrated that 6-MP also inhibits the production of antibodies, and Schwartz and Dameshek (1960) and Meeker *et al.* (1960) have reported the protection of skin homografts in rabbits by the administration of 6-MP.

It is generally acknowledged that many antibiotics exert their growth-limiting effects by inhibiting protein synthesis, either directly or indirectly. Chloramphenicol, for example, has been noted to prevent the synthesis of DNA (Drakulic and Errera, 1959; Schneider, Cassir and Chordikian, 1960), RNA (Gros and Gros, 1956; Webster, 1957), and protein (LePage, 1953; Smith, 1953; Pardee and Prestidge, 1956; Webster, 1957; Gale, 1958) in bacteria and mammalian tissues. Because of such manifold effects of chloramphenicol, and probably other antibiotics as well, their interference with antibody production and the homograft reaction is not unreasonable.

Amino acid analogues, in so far as they have been tested, were generally less effective in protecting homografts from destruction than were the other agents already discussed. There is evidence that β-2- and β-3-thienylalanine inhibit antibody formation in the rat (Ferger and du Vigneaud, 1949; Wissler *et al.*, 1956), and thymidine uptake in DNA is inhibited by β-2-thienylalanine and ethionine (Schneider, Cassir and Chordikian, 1960). Amino acid analogues are generally agreed (Matthews, 1958; Shive and Skinner, 1958) to act either by preventing protein synthesis via interference with the utilization of natural amino acids or by becoming incorporated themselves into proteins, thus displacing their normal counterparts. Of the amino acid analogues studied in the present investigation, at least ethionine and β-2-thienylalanine have been shown to act in the latter fashion (Levine and Tarver, 1957; Munro and Clark, 1958; Munier and Cohen, 1959). Structurally defective proteins would be expected not to be biologically inactive unless the incorporated analogues occupied an indispensable position. Since considerable portions of protein molecules are known to be functionally superfluous, the relative ineffectiveness of amino acid analogues in promoting homograft survival may find an explanation along these lines of reasoning.

The ability of cortisone to protect homografts from immunological destruction is too well known to require elaboration (Morgan, 1951; Billingham, Krohn and Medawar, 1951; Krohn, 1954; Medawar and Sparrow, 1956; Scothorne, 1956; Hamer and Krohn, 1959). This hormone also depresses antibody production (Germuth and Ottinger, 1950; Kass and Finland, 1953; Berglund, 1956) and

inhibits nucleic acid synthesis (Skipper *et al.*, 1951). It has been claimed that these effects of cortisone are augmented by its interference with the release of antigens during the afferent phase of the homograft reaction (Billingham, Krohn, and Medawar, 1951; Medawar and Sparrow, 1956; Scothorne, 1956). In view of the well documented evidence in favor of the efficacy of cortisone in suppressing the homograft reaction, plus the demonstrated effectiveness of the more potent preparations (6-fluorohydrocortisone and Delta-1-hydrocortisone), the failure of cortisone to enhance the survival of scale homografts in the present experiments may reasonably be ascribed to insufficient dosages.

With reference to the mode of action of the various agents found effective in promoting extended survival of homografts, it could be argued that such results might be attributed to nonspecific toxicities rather than to effects directly related to the inhibition of antibody synthesis. Although some of the drugs tested proved to be fatal at effective doses, there is little reason to conclude that their efficacy resulted directly from their lethality *per se*. The majority of the compounds which prolonged homograft survival manifested no other toxic effects during the period of treatment. Moreover, in the case of DL-β-phenyllactic acid, a dose of 2.5 mg. was lethal without being effective in precluding the homograft reaction. Additional treatments not reported here have also failed to interfere with foreign tissue rejection at otherwise lethal doses. Thus, while inhibition of nucleic acid or protein synthesis may be fatal, other kinds of toxicity need not interfere with immunological mechanisms.

The accumulated evidence supports the contention that homograft rejection may be subject to a moratorium in the absence of the successful synthesis of nucleic acids and/or proteins. On the basis of the limited number of compounds tested, there is reason to expect that numerous other agents with comparable physiological properties might exert similar influences. Granted that there are numerous factors which inhibit antibody production and thus actually or potentially interfere with the homograft reaction, it remains to be demonstrated conclusively whether such effects are permanent or temporary. In their investigations of the beneficial effects of 6-MP on skin homograft survival in rabbits, Meeker *et al.* (1960) noted that sustained treatment was necessary to insure continued survival of the grafts. In the present experiments, a comparable conclusion seems to be indicated, for despite the survival of scale homografts in treated hosts beyond the control period, eventual though dilatory breakdown was the rule. Notwithstanding these preliminary observations, it remains as a theoretical possibility that a specific tolerance might be conferred upon an adult host exposed to a foreign antigen by selectively inactivating those antibody-synthesizing pathways specifically stimulated by the antigen. If, as Burnet (1959) contends, antibody-producing clones are descended from specific cells stimulated to proliferate by exposure to antigen, then the application of treatments designed to render such cells vulnerable to destruction or inactivation at this critical period should, perforce, result in an animal subsequently tolerant to the original antigen. Alternatively, if antibody production can continue irrespective of whether or not the involved cells are stimulated to proliferate, specific tolerance could be realized only by permanently and selectively incapacitating the biochemical pathways by which the specifically stimulated antibodies are synthesized. To achieve this without doing violence to any other mechanism of protein synthesis will be a challenging enterprise.

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Summary

1. At 28° C., the melanocytes on scale homografts in *Fundulus* are destroyed in three days by the immunological response of the host. This reaction is slower to occur at progressively lower temperatures, but is not adversely affected by splenectomy or hypophysectomy of the host, nor by daily injections of trypan blue.

2. Survival of homografts was enhanced by daily intraperitoneal injections of base analogues, potent preparations of adrenal cortical hormones, antibiotics and amino acid analogues.

3. These results are taken to indicate that the inductive and productive phases of antibody formation are particularly vulnerable to agents which interfere with protein and/or nucleic acid synthesis.

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THE LIFE-CYCLE OF PORPHYRA TENERA IN VITRO

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Cultivation of the red sea-weed *Porphyra tenera* was started in Japan several centuries ago. It is now the largest industrial cultivation of any marine product. Despite this success, more knowledge of the life-cycle and innate potencies of *Porphyra* is needed to improve methods of cultivation—to bring them under a control comparable to that achieved in land agriculture. At present this goal is unrealizable to its fullest extent. Mass-scale use of artificial fertilizers on 50 square miles of bays is uneconomical. But improvements in production and control of seeding, genetic improvement of the plant in respect to greater production and resistance to parasites, and perhaps extension of the growth period of the thallus, seem attainable goals.

Several obstacles have slowed research on the life-cycle and potencies of *Porphyra*. Until a few years ago only one part of the life-cycle was known: mysteriously, the bays abound in monospores in the autumn; these monospores, collected on bamboo or cord nets, develop into the edible, leafy thallus which is periodically harvested until March, when it fruits and disintegrates while producing carpospores. What was happening to the carpospores, and the origin of the monospores, were unknown. Those mysteries were solved after Drew (1949) discovered that the carpospores of another species, *Porphyra umbilicalis,* germinated into a filament which, in enriched sea water, produced a flimsy, sickly mat. The fact that the germ tubes produced by the carpospores are very similar to those of fungal spores, and that the older filaments of the mat are generally abnormal in appearance suggested to her that a specific host or a special substrate are needed for normal growth. Indeed several molluscan shells and even egg shells proved an excellent substrate. The filamentous thallus grows well in the shells, forming colonies identical with *Conchocelis rosea*; *C. rosea* is obviously merely a phase of the life-cycle of *Porphyra*. Kurogi (1953) and Tseng and Chang (1954) found that the carpospores of *Porphyra tenera* behaved similarly. Kurogi (1953) studied the growth of the carpospores of *Porphyra umbilicalis prox., P. suborbiculata, P. pseudolinearis* and *P. tenera*; these form "*Conchocelis*" colonies in the shells which can hardly be told from one another. In Kurogi's cultures the "*Conchocelis*" phase cultured on glass slides produced monosporangiate branches but not free monospores. However, from *Conchocelis* in oyster shells Kurogi (1953) obtained monospores which produced germlings of the leafy thallus.

The complete life-cycle of *Porphyra* was now known. This discovery renewed interest in the biology of *Porphyra,* especially the conditions for growth (Iwasaki and Matsudaira, 1958) and production of monospores (Kurogi and Hirano, 1956).

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Obstacles to speedy progress were: (*a*) inability to grow the *Conchocelis* phase outside of the shells in free conditions; and (*b*) inability to cultivate in the laboratory, out of season, the two growth phases of *Porphyra* which in nature are strictly seasonal (autumn-winter for the thallus phase and spring-summer for the *Conchocelis* phase).

As mentioned, Drew and Kurogi had grown the *Conchocelis* phase in enriched sea water on glass slides. Although the growth of *Conchocelis* was poor, Drew (1954) mentioned (p. 193) ". . . . that such free-living filamentous growths can be maintained and continue to grow indefinitely provided the culture solution is renewed regularly"; this seemed promising. Drew obtained with *P. umbilicalis* only filamentous *Conchocelis* growth on glass slides and no monosporangia were formed, while the four species of *Porphyra* (including *P. umbilicalis prox.*) studied by Kurogi produced monosporangia. The discrepancy between these results implied that good growth and fruiting of the *Conchocelis* phase in the free-living conditions might be obtained under different cultural conditions and with better media. Another reason for trying again to grow the *Conchocelis* phase of *P. tenera in vitro* was the success of Hollenberg (1958) in obtaining on glass slides in liquid media minute filamentous *Conchocelis*-like plants of *P. perforata* which produced "sporangia branchlets" and fertile "conchospores."

Preliminary Experiments

The original materials brought from Japan were a few sterilized oyster shells which had been inoculated in March, 1959, with carpospores produced by natural-grown thalli. Colonies of *Conchocelis* developed normally in the shells kept in Woods Hole sea water enriched with nitrate, phosphate, and EDTA, (medium SWI, Table I) indicating the suitability of Atlantic sea water for *Porphyra tenera.*

Table I

Enriched sea water media

	SWI	SWII
Filtered sea water	1000 ml.	1000 ml.
KNO_3	72.2 mg. (=10 mg. N)	72.2 mg. (=10 mg. N)
KH_2PO_4	8.8 mg. (=2 mg. P)	4.5 mg. (=1 mg. P)
Na_2-glycerophosphate $.5H_2O$		10.5 mg. (=1 mg. P)
Fe-EDTA (1:1 chelation)	0.5 mg. (as Fe)	0.5 mg. (as Fe)
"Tris" buffer*	500 mg.	500 mg.
pH	8.0–8.2	8.0–8.2

* Tris (hydroxymethyl) amino methane (Sigma Company).

The nutrient solution was changed fortnightly; the shells were periodically cleaned with cotton to eliminate epiphytic diatom growth and kept in subdued, continuous fluorescent light [2] (10–30 foot-candles) at 13–15° C. In September, 1959, some shells were broken in pieces and thin flakes containing one *Conchocelis* colony were thoroughly wiped clean of epiphytes with cotton and also by repeated dipping into 1.5% agarized enriched sea water containing antibiotics. The flakes were

[2] "Cool white."

then inoculated into various artificial marine media and in enriched sea water, and kept in continuous subdued light at 13–15° C. At the end of December, 1959, in two tubes of medium ASP_2NTA and in a tube of SWI + 5 μg.% indolacetic acid, a few young thalli appeared at the bottom of the test tube and on the shell flakes. Simultaneously in two tubes of SWI, tufts of free *Conchocelis* were growing out from the shell flakes. Later on (February–April, 1960), free *Conchocelis* colonies, attached to the bottom of the test tube or to the shells, appeared in the two ASP_2NTA tubes and the SWI + IAA. The young thalli and the free *Conchocelis* employed in the subsequent experiments were derived from these 5 original cultures, which are unialgal, but accompanied by bacteria and yeasts. Microbial contamination, though permanent, was minimal in all the media employed because of the lack of organic substrates and because aseptic techniques were employed throughout.

In Vitro Culture of Free-Living Conchocelis Phase

Origin of free Conchocelis

Strains of free-living *Conchocelis* were obtained in several ways: (1) from the free *Conchocelis* growing out of the shell flakes in SWI medium; (2) directly from carpospores released by mature thalli collected in Japan, shipped to New York (March, 1960), and germinated in liquid media; (3) from carpospores produced by thalli grown in artificial media *in vitro*.

At first, on the assumption that a substrate might be somehow advantageous to the *Conchocelis* phase, tufts of *Conchocelis* filaments, cut from the free growth on shell flakes in SWI, were transferred into biphasic media. To simulate the conditions in shells, the solid phase (10 ml. ASPI medium + agar 1.5%) was enriched with 0.1% $CaCO_3$, 0.01% chondroitin, or both; the liquid phase consisted of 5 ml. either of ASP7 or SWI; the *Conchocelis* tufts were implanted in the agar at the interphase. All these combinations allowed good growth at 13–15° C. and continuous subdued light and at 18–20° C. and 10 hours daily. In 2–3 months, from an initial tuft 1 mm. in length, spherical colonies of 0.5–1 cm. were obtained; later, new colonies formed at the interphase or on the glass wall. Growth was almost entirely in the liquid phase and in all the different combinations, indicating that a solid substratum rich in $CaCO_3$ or protein is unnecessary. Further experiments were done in liquid media to determine the best cultural conditions for free growth in liquid media.

Once some of these conditions were known, it became possible to germinate directly in liquid media carpospores collected from thalli grown either in nature or *in vitro*. Thalli of *P. tenera* collected in Matsukawa-ura inlet were shipped to New York in March, 1960. Following the method suggested by Professor Y. Yamada of Hokkaido University, the thalli were put between pads of absorbent cotton wet with sea water and shipped in Thermos bottles; this method avoids rotting and gives good survival. Upon their arrival in New York, the thalli were placed in enriched sea water and produced carpospores. The collected carpospores were washed several times in sterile sea water by means of capillary pipettes, and 3–5 carpospores were inoculated in test tubes containing 10 ml. of 3 types of enriched sea water (ASW8, SWI, SWII) and 9 artificial media (ASM, ASP1,

TABLE II

Artificial media composition (w./v.)

	ASP1	ASP2(NTA)	ASP6	ASP7	ASP12(NTA)*
Distilled water	100 ml.	100 ml.	100 ml.	100 ml.	100 ml.
NaCl	2.4 g.	1.8 g.	2.4 g.	2.5 g.	2.8 g.
$MgSO_4 \cdot 7H_2O$	0.6 g.	0.5 g.	0.8 g.	0.9 g.	0.7 g.
$MgCl_2 \cdot 6H_2O$	0.45 g.				0.4 g.
KCl	0.06 g.	0.06 g.	0.07 g.	0.07 g.	0.07 g.
Ca (as Cl^-)	40 mg.	10 mg.	15 mg.	30 mg.	40 mg.
$NaNO_3$	10 mg.	5 mg.	30 mg.	5 mg.	10 mg.
K_2HPO_4	2 mg.	0.5 mg.			
K_3PO_4					1.0 mg.
Na_2-glycerophosphate			10 mg.	2 mg.	1.0 mg.
$Na_2SiO_3 \cdot 9H_2O$	2.5 mg.	15 mg.	7 mg.	7 mg.	15 mg.
Na_2CO_3		3 mg.			
Fe (as Cl)		0.05 mg.			
B_{12}	0.02 μg.	0.02 μg.	0.05 μg.	0.1 μg.	0.02 μg.
Biotin					0.1 μg.
Thiamine					10 μg.
Vitamin mix 8**	0.05 ml.		0.1 ml.		
Vitamin mix S3***		1 ml.		1 ml.	
PII Metals****	1.0 ml.	3 ml.		3 ml.	1 ml.
SII Metals†					1 ml.
P8 Metals††			1 ml.		
Tris buffer	0.1 g.	0.1 g.	0.1 g.	0.1 g.	0.1 g.
Nitrilotriacetic acid		(10 mg.)		7 mg.	(10 mg.)
pH	7.6	7.8	7.4–7.6	7.8–8.0	7.8–8.0

* Developed by L. Provasoli for tropical species of dinoflagellates.

** One ml. of Vitamin mix 8 contains: thiamine HCl, 0.2 mg.; nicotinic acid, 0.1 mg.; putrescine 2HCl, 0.04 mg.; Ca pantothenate, 0.1 mg.; riboflavin, 5 μg.; pyridoxine 2HCl, 0.04 mg.; pyridoxamine 2HCl, 0.02 mg.; *p*-aminobenzoic acid, 0.01 mg.; biotin, 0.5 μg.; choline H citrate, 0.5 mg.; inositol, 1.0 mg.; thymine, 0.8 mg.; orotic acid, 0.26 mg.; B_{12}, 0.05 μg.; folic acid, 2.5 μg.; folinic acid, 0.2 μg.

*** One ml. of Vitamin mix S3 contains: thiamine HCl, 0.05 mg.; nicotinic acid, 0.01 mg.; Ca pantothenate, 0.01 mg.; *p*-aminobenzoic acid, 1 μg.; biotin, 0.1 μg.; inositol, 0.5 mg.; folic acid, 0.2 μg.; thymine 0.3 mg.

**** One ml. of PII metal contains: ethylenediamine tetracetic acid, 1 mg.; Fe (as Cl), 0.01 mg.; B (as H_3BO_3), 0.2 mg.; Mn (as Cl) 0.04 mg.; Zn (as Cl), 0.005 mg.; Co (as Cl), 0.001 mg.

† One ml. of SII metals contains: Br (as Na), 1.0 mg.; Sr (as Cl), 0.2 mg.; Rb (as Cl), 0.02 mg.; Li (as Cl), 0.02 mg.; I (as K), 0.001 mg.; Mo (as Na), 0.05 mg.

†† One ml. of P8 metal contains: Na_2 versenol, 3 mg.; Fe (as Cl), 0.2 mg.; Mn (as Cl), 0.1 mg.; Zn (as Cl), 0.05 mg.; Co (as Cl) 0.001 mg.; Cu (as Cl), 0.002 mg.; Mo (as Na), 0.05mg.; B (as H_3BO_4), 0.2 mg. Versenol = hydroxyethyl-ethylenediamine triacetic acid.

ASP2, ASP2NTA, ASP6, ASP7, ASP12, ASP12NTA and D; Table II). *Conchocelis* growth was obtained in most of these media except ASW9 and ASP2. ASP1, ASP6, ASP12NTA, ASP12 and ASW8 gave very good growth; ASP7, D, and SWII were less good; SWI very poor.

Young germlings of *P. tenera* (5 mm. long) cultured at 14–16° C., and illuminated 13 hours a day with 400–500 foot-candles of incandescent light, did not grow normally (see later) and produced carpospores from which *Conchocelis* colonies developed.

Suitable media and cultural conditions for free-living growth of Conchocelis

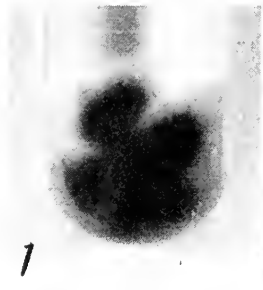
Several artificial media and enriched sea waters permit continued growth of the *Conchocelis* phase. In decreasing order, ASP12NTA, ASP2NTA, ASP12, ASP6, and ASP7 are the most suitable artificial media, and SWII and SWI the enriched sea waters. *Conchocelis* cultures easily last 6 months; the color of the colonies varies in different media: pinkish-red in ASP12NTA, ASP12, and ASP6; pale brown in ASP2NTA, dark brown in SWII, and pinkish-grey in ASM. The color is more intense in the center of the colony, probably because of the presence there of intensely pigmented monosporangial branches. The type of medium influences growth rate and monosporangia formation. In decreasing order, growth was fastest in ASP12NTA, SWII and ASP7 and slower in ASP2, ASP12, and MEC3. Monosporangia were formed and monospores liberated earlier in ASP12NTA, and in decreasing order in ASP12, ASP7, ASP2, SWII, SWI. The temperature range is between 10 and 26° C.; the optimum between 13 and 20°. Single pieces of the filament of the *Conchocelis* phase (~1 mm.) transferred in new media grew into new *Conchocelis* colonies vegetatively. It was possible in this way to subculture the *Conchocelis* phase: 5 serial transfers (one every 2–3 months from February to December, 1960) resulted in good growth. Quite likely the *Conchocelis* phase can be grown indefinitely as free-floating colonies in liquid media.

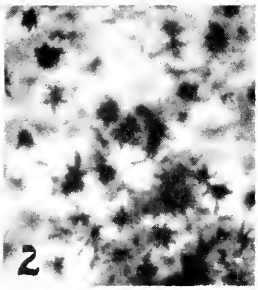
The *Conchocelis* colonies in test tubes of liquid media generally grew at the bottom of the tube attached to the glass wall and appeared as fuzzy balls 4–10 mm. in diameter (Fig. 1). In larger containers, where they grow free-floating in the medium, they were stellate, often reaching a diameter of 10–15 mm. (Fig. 2). The *Conchocelis* phase can be grown, but poorly, also on agar slants in screw-cap tubes.

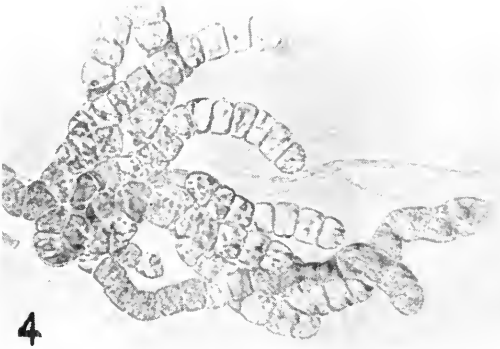
At the beginning of this work the free-living *Conchocelis* colonies were grown in subdued light (20–40 ft.c.) to simulate natural conditions; under these conditions growth was quite slow. Later, in surveying the effect of light intensity, it was found that growth was greatly increased by higher light intensities—the higher, the better (maximum tried, 350 ft. c.). Incandescent and fluorescent light were equally effective; however, the color of the *Conchocelis* was different: reddish in fluorescent light and cool brown-black in incandescent light. Continuous illumination also favored growth. Under these conditions (350 ft. c. continuous fluorescent light) mass cultures were obtained in 2-liter Erlenmeyer flasks and in tall, 4-liter bottles (Fig. 3) by gradual transfer in increasingly larger containers (10 ml. inoculated into 100 ml.; 100 ml. in 1 liter, etc.).

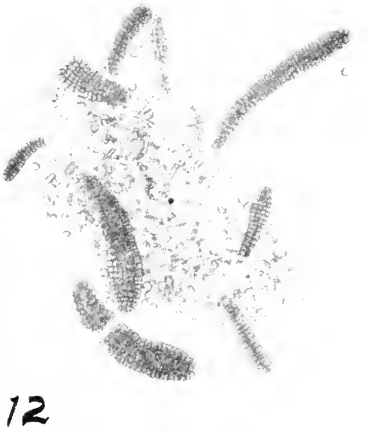
Effect of photoperiodism on monosporangia and monospore production

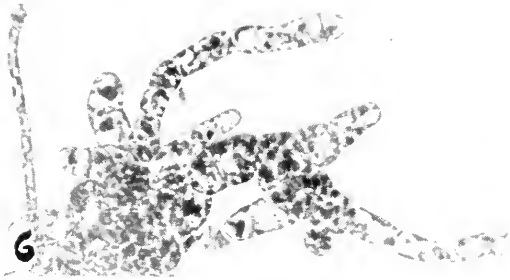
Kurogi's experiments (1959) indicated that photoperiodism may govern monosporangia production and monospore liberation in *Conchocelis* grown in shells. The following experiment was set to test the effect of photoperiodism on free-living *Conchocelis*. With fluorescent light of 150–250 ft. c., a daily photoperiod of 8–11 hours induced formation of monosporangia in 2–3 weeks and young thalli in 3–8 weeks from the time of inoculation into new media of pieces of *Conchocelis* filaments (Figs. 5, 6, 7). No substantial difference was found in cultures grown

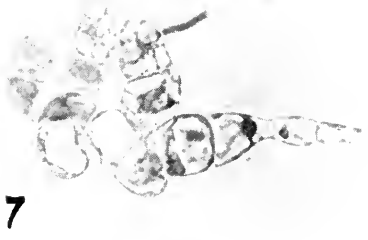












FIGURES 1–7, 12.

at 13–15° C. or 18–20° C. in high light (150–250 ft. c.) for a daily photoperiod of 8–11 hours. When the light intensity was reduced to 30–50 ft. c., the appearance of young thallus germlings was greatly retarded in the 8-hour photoperiod (> 96 and < 184 days) and apparently prevented in the 11-hour photoperiod (no leafy thalli in 180–240 days).

Under continuous fluorescent light at intensities of 150–250, 60–100, and 10–20 ft. c., both at 13–15° and 20–26°, neither spores nor thallus germlings were found during the two experiments which lasted, respectively, 180 and 240 days. Growth of *Conchocelis* filaments and colonies proceeded normally, and may, indeed, be favored by continuous light; very good mass cultures were obtained in continuous light. Intensely purple, inflated portions similar to monosporangia, appeared after a month or more in cultures of 60–250 ft. c. At that time these structures were thought to be small monosporangia. Unfortunately the observations of these experiments were done at great intervals (one month or more), and through the walls of the test tubes using a dissecting microscope. Only later, when it became evident that these structures were not producing spores, was a simple experiment tried: a *Conchocelis* colony grown for two months in continuous light at 13–15° C. was transferred to new medium and illuminated 8 hours a day; after 5 weeks many thallus germlings (5–8 mm. long) were growing alongside the *Conchocelis* colony.

Evidently, maturation of monosporangia, release of monospores, or both, are induced by a short photoperiod and prevented by continuous light.

The sporangia produced in continuous light (250–350 ft. c.) in the mass cultures seemed to be morphologically different from the monosporangia produced under short-day conditions. Sporangia cells in continuous light have thicker walls and length of the cells is usually about half their diameter; some cells are quadrate (Fig. 4). They are very similar to the "plantlets" described for *P. umbilicalis* var. *laciniata* by Drew (1954, p. 203, Fig. 4c).

On the contrary, the cells of the short-day monosporangia often have elongated cells and the appearance of monosporangia is much more twisted (Figs. 5, 6, 7) because of the lateral branches. Are the continuous-light sporangia undeveloped or abnormal monosporangia, or are they a new type of sporangium? The evidence at hand does not exclude either possibility.

The aforementioned experiment of transferring a *Conchocelis* colony from continuous light to short-day is indicative but not conclusive; only one observation was made 38 days after the transfer: young thalli of 5–8 mm. were found. The formation *de novo* of true monosporangia is not excluded because under the same light and temperature conditions (exp. *II*, Table III) young thalli appeared between 22 and 31 days in a culture started with pieces of *Conchocelis* filaments (no length was noted in the protocols; they were probably 2–3 mm. long).

FIGURE 1. Colonies of free-living *Conchocelis* in artificial medium.
FIGURE 2. Free-floating *Conchocelis* (detail of Figure 3).
FIGURE 3. Mass culture of *Conchocelis* in aerated 3-liter bottle, continuous light.
FIGURE 4. Sporangia formed in continuous illumination.
FIGURE 5. Typical monosporangia formed in short day conditions (8–11 hours daily).
FIGURES 6, 7. Same detail.
FIGURE 12. Young thallus germlings, and monospores.

The hypothesis that the *Conchocelis* phase can produce other sporangia besides the monosporangia has already been advanced by Drew for *Porphyra umbilicalis* var. *laciniata* (1954) and *Bangia fuscopurpurea* (1958) to explain *Conchocelis* infections in sterile shell derived from other *Conchocelis*-infected shells. This possibility is greatly reinforced by our experiments with *P. tenera*. More than 5 serial transfers were done directly from *Conchocelis* to *Conchocelis* in test tubes or in mass culture without passing through the thallus phase or carpospores: in every case, inoculating pieces (in test tubes) of *Conchocelis* filaments or entire colonies (in mass cultures) led to numerous new colonies. These experiments also do not prove conclusively that the increase in the number of *Conchocelis* colonies is due to the production of special spores developing into new *Conchocelis* colonies,

TABLE III

Effects of short-day and continuous light conditions on Conchocelis *phase**

Temperature	Light** period	Light intensity**	Appearance of sporangia†	Appearance of foliaceous thalli†	Remarks
13–15° C.	8 hr.	150–250 ft. c.	*I* 19 days *M.* *II* <22 days *M.*	<46 days >22–<31 days	good growth of thalli (1–1.5 cm.) 3 cm. thalli in 96 days
		30–50 ft. c.	*I* 27 days *M.* *II* <48 days *M.*	none up to 84 days >96–<184 days	discontinued at 84 days small thalli
18–20° C.	11 hr.	150–250 ft. c.	*I* 19 days *M.* *II* 23 days *M.*	>56–<84 days 31 days	good growth of *Conchocelis*-thalli soon bleached
		30–50 ft. c.	*I* 19 days *S2* *II* 23–31 days *S2*	none up to 240 days none up to 184 days	large *Conchocelis* colonies
13–15° C.	continuous	150–250 ft. c.	*I* 35 days *S1–S2* *II* 31 days *S1–S2*	none up to 240 days none up to 184 days	††
		30–50 ft. c.	*I* 84 days *S2* *II* 72–96 days *S2*	none up to 240 days none up to 184 days	
20–26° C.	continuous	60–100 ft. c.	*I* 35 days *S2* *II* 31 days *S2*	none up to 240 days none up to 184 days	
		10–20 ft. c.	*I* 56 days *S2* *II* *S2*	none up to 240 days none up to 184 days	(Figs. 8–11)

* Media: ASP7 and SWII. Results of two separate experiments (*I* and *II*). *M* = monosporangia; *S1*, see Figure 4; *S2*, see Figures 8–11.
** Fluorescent: "cool white."
† Days from date of inoculation. Inoculum = a small piece of *Conchocelis* filament.
†† At 50 days a *Conchocelis* colony was transferred to new medium and to 8 hours of light. In a month monospores and thalli appeared.

because even small pieces of *Conchocelis* filament, which can grow into a full new colony, could have been present. However, the short-celled sporangia produced by *P. tenera* in continuous light (Fig. 4) or the sporangia-like swollen cells described by Drew for *Bangia* (1958, Fig. 3, p. 366) may be a new type of sporangium whose spores produce a new *Conchocelis* colony.

Other very strange structures are produced in continuous (10–100 ft. c.) or 11 hours subdued fluorescent light (30–50 ft. c.) at 13–15° C. and 20–26° C. (Table III). The similarity of the latter sporangia with fungal structures is striking (Figs. 8, 9, 10, 11; sporangia (?) *S2* of Table III).

The variety of structures created in different lights and temperatures shows that *P. tenera* has unusual powers of adaptation. The *Conchocelis* phase can now

be grown free, making the morphological observations easy. This permits a wider analysis of the unusual morphological versatility of *P. tenera* as well as of the possible deviations from the normal life-cycle induced by various lights and temperatures.

The Leafy Thallus Phase

Some cultural conditions for the growth of the thallus had been determined previously (Iwasaki and Matsudaira, 1958, and unpublished).

(1) Leafy thalli grow normally in enriched sea water (Miquel's sea water), while they are short and unhealthy when grown in filtered, unenriched inshore sea water.

(2) High-intensity, incandescent light is required for normal continued growth; growth is, however, slower than in natural sunlight. Young plants grown in fluorescent light die in a few days.

(3) Young plants grow normally when illuminated 8–10 hours daily but die quickly when grown in continuous light.

These results were on the whole confirmed by the present investigation.

Effect of media on leafy thallus growth

Thalli (1–2 mm.) derived from monospores produced by free-living *Conchocelis* (Fig. 12) were grown in enriched sea water and artificial media at 14–16° C.,

Table IV

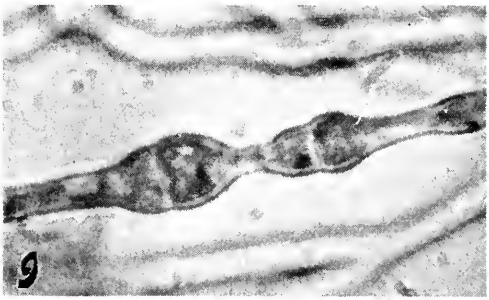
Thallus growth (two-month)

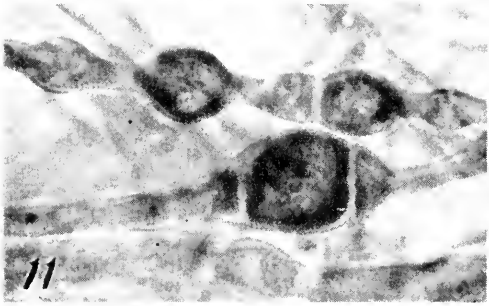
Media	Growth	Color
ASP1	10 × 40 mm.	brown
ASP2	8 × 50 mm.	
ASP12	20 × 40 mm.	red-brown
ASP12NTA	8 × 20 mm.	
SWI	8 × 80 mm.	reddish
SWII	20 × 35 mm.	pale brown

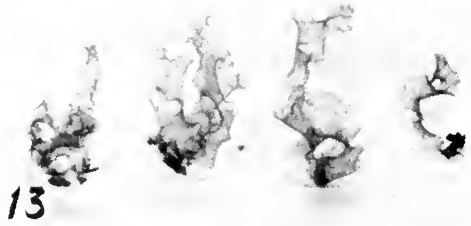
and illuminated 9 hours daily with 400 ft. c. of incandescent light. The experiment was done in test tubes (20 × 120 mm.) containing 10 ml. of medium; once a month the medium was replaced aseptically with 10 ml. of fresh medium. Good normal growth was obtained in two months in some artificial media and in enriched sea water (Table IV and Fig. 13). Narrow long thalli were obtained in SWI and most artificial media, broader thalli in SWII and ASP12. The 1957 experiments were done during the season in which the thalli grow in nature (fall-winter). On the contrary, the new experiment was done between May and August, 1960, indicating that normal thalli can be grown out of season if the light period is suitable (8–11 hours daily).

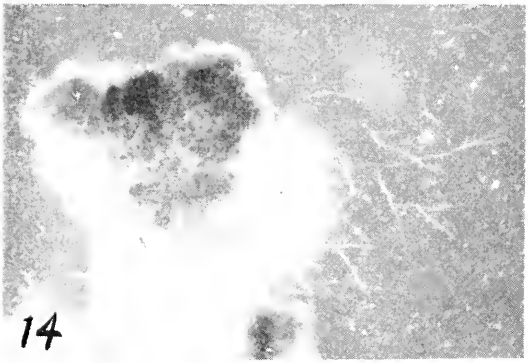
Effect of long-day conditions on leafy thalli

In retrospect, the importance of the photoperiod for *Porphyra tenera* might have been suspected because the two phases of the life-cycle of *P. tenera* correspond so sharply to the seasons. The leafy thallus grows in the short-day seasons









FIGURES 8, 9, 10, 11. Inflated cells (sporangia?) produced in subdued light.

FIGURE 13. Two-month-old thalli grown in test tube. From left, medium SWI, SWII, ASP12, ASP1.

FIGURE 14. Young thallus degenerated under long-day conditions (13 hours daily). Lower part bleached; large pigmented cells at top; *Conchocelis* filaments germinating from "spores."

FIGURE 15. Root-like projections growing out of a young thallus grown in SWII under long-day conditions.

(autumn-winter), the *Conchocelis* phase in the long-day seasons (spring-summer). Furthermore, the transition between the two phases of the life-cycle coincides with the equinox (Fig. 16). On the contrary a great part of the temperature range (7–21° C.) is common to the two phases: normal thalli grow in nature between 3–21° C. and the *Conchocelis* between 7 and 25° C. Therefore, only the lower zone (3–7° C.) may be suspected to affect *Conchocelis* growth and the upper zone (21–25° C.) thallus growth. These considerations, and the already known effects of continuous light on thallus growth (Iwasaki and Matsudaira, 1958) and short day on monosporangia formation (Kurogi, 1959) suggested trial of growth under long-day conditions.

Five young germlings (0.5 mm.), derived from monospores of free-living *Conchocelis*, were inoculated in each tube of the following media: SWI, SWII, ASP1, ASP2, and ASP12. They were incubated at 14–16° C. and illuminated 13 hours daily with 400–500 ft. c. of incandescent light. The controls were grown

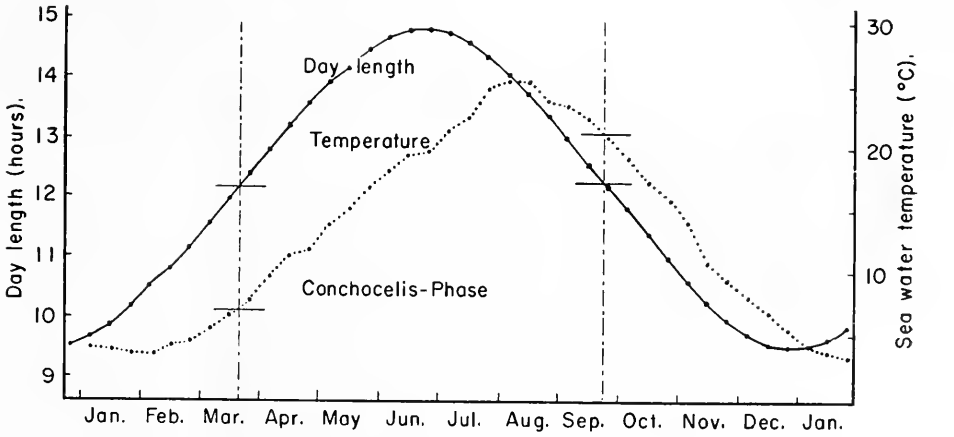


FIGURE 16. Day length at Sendai and average sea water temperature in Matsushima Bay.

under similar conditions but illuminated 8 hours daily. The control gave, as in the previous experiment, normal thalli of the narrow shape.

The thalli in long-day conditions grew very slowly and very soon became thick and irregular in shape. After 20 days or more, the thalli became pale, leaving numerous scattered big reddish-colored cells. The thallus around the edges assumed the appearance of a callus tissue (Fig. 14). After 40 days (in ASP2) or more, spores were released. Since these spores germinated into filaments which later formed well-developed *Conchocelis* colonies, we assume that, at least functionally, they are equivalent to carpospores. These events differ slightly in time and amount of growth in the various media except SWII. In SWII the thallus after 27 days started to produce root-like projections (Fig. 15) which branched out into thinner filaments; after two months the thallus became covered with *Conchocelis* colonies. Apparently 13 hours of daylight, which corresponds at the latitude of Sendai (39° N) to late April, already inhibits normal thallus growth and in-

duces the formation of structures functionally equivalent to carpospores. This experiment was repeated later with similar results.

In another experiment in tall covered containers (10 cm. diameter; 7 cm. high) containing 200 ml. of ASP1, young thallus buds (1–2 mm.) were grown for one month (April 22–May 25) at 14–16° and illuminated 8 hours daily with 400 ft. c. of incandescent light: the thalli, which had reached by then an average size of 3 cm.^{2}, were illuminated for 10 days (May 25–June 4) with 100–200 ft. c. of incandescent light: on alternate days, 8 hours daily followed by one day in continuous light. After this period of alternating photoperiods, the culture was grown in 100–200 ft. c. and 8 hours daily of fluorescent light. In a month (July 5) big, dark cells appeared, scattered at the edges of the leafy thalli which were stunted and curled. Ten days later these cells produced germ tubes which developed into *Conchocelis* colonies. Two months (August 5) after the alternating light treatment, many colonies of *Conchocelis* were growing free on the bottom of the dish and covering the stunted disintegrating thalli. After 82 days (August 25) mature monosporangia were formed and a few days later the monospores were released and produced thallus germlings. These germlings on September 10 had already reached an average size of 1 cm.^{2}

The complete life-cycle was obtained in 5–6 months.

It is remarkable that the leafy thalli of the second generation grew normally, though slowly, in fluorescent light and at low intensities (100–200 ft. c.). This is by no means an isolated case of thallus growth in fluorescent light: all the cultures of *Conchocelis* grown at 13–20° C. for 8–11 hours daily of fluorescent light eventually released monospores (1–4 months, depending upon light intensity). These spores gave rise to leafy thalli reaching 2–10 mm. before they became pale and died. The arrest in growth of these leafy thalli was probably due to lack of nutrients: the medium in these experiments was not changed monthly, as was done for the experiment on thallus growth in different media. Ability of the thalli to grow in fluorescent light may be an adaptation to utilization of fluorescent light acquired during the *Conchocelis* phase: the *Conchocelis* phase does not require incandescent light. This adaptability, whatever the cause, reflects again the great plasticity and versatility of *P. tenera*.

Discussion

These results may help solve some of the problems of life-cycle and growth potencies of *P. tenera*. Solutions here, in turn, may improve the farming of this sea weed. The first report of the entire life-cycle of a *Porphyra* obtained *in vitro* is the one of Hollenberg (1958). He obtained from carpospores *Conchocelis*-like filaments which formed sporangia and liberated spores (16 days from carpospore germination). These spores in turn developed into blade-like plantlets (young thalli). Since the cultures were grown during the summer in north light, it is possible that the very rapid formation of sporangia and the poor growth of the *Conchocelis* phase were due to light conditions. While this paper was being written, the paper on the *Conchocelis* stage of *P. umbilicalis* by Kornmann (1960) appeared. Like us, Kornmann obtained the complete life-cycle *in vitro*. He started in November, 1959, with a "plantlet" (probably an immature, or abnormal monosporangium) cultured in Erdschreiber. The "plantlet" became fertile and made

monospores which did not develop. Only a few cells of this structure remained vegetative and reproduced in a month another "plantlet" (without "rhizoids") which produced many monospores. Of these, only 6 germinated into leafy thalli which in a month and a half reached 1.5–2 mm. in length. The thalli formed "Bällchen" from which thin filaments grew out; the filaments, by division, produced "zweige" (his Figure 5 = "plantlet" = monosporangia?). From the "zweige" arose as side-branches ("seitliche Verzweigung") thin filaments which in free culture produced a confused ball of yarn (verworrene Knäuel = free *Conchocelis*) or enveloped the original plantlet. These filaments grow also as a typical *Conchocelis* in calcareous shells.

Unfortunately, no data are given of the light period under which the cultures were grown. From his Figures 2 and 5, the "plantlets" are very similar to monosporangia. If so, the thin filaments (which are *Conchocelis* filaments) should produce, and not be produced by the monosporangia (as Kornmann states in Figure 5 and the text). But the structure in Figure 5 could be equivalent to the sporangia which were produced in our *Conchocelis* colonies grown in continuous light (our Fig. 4). As mentioned, these sporangia are suspected of producing spores germinating into a new *Conchocelis*. Kornmann's light conditions seem also to be inadequate for thallus growth because, as in our thallus cultures under long-day conditions, *Conchocelis* filaments arise from the thallus (Kornmann, Fig. 3B) or big colored cells are formed (Kornmann's "Bällchen" which can be seen at the base of the thallus of Figure 3, C) from which *Conchocelis* filaments arise. Kornmann's Figure 1C represents, most likely, true monosporangia and *Conchocelis* filaments.

Whatever the interpretation, it is seen that, both in Kornmann's and in our experiments, the life-cycle can be obtained *in vitro*. Detailed morphological studies are planned to solve some of the many questions; *e.g.,* what is the typical morphology of the true monosporangia of *Conchocelis* grown free—how do they differ from those produced in shells? What are the mysterious "plantlets" of Drew, and of Figures 1A and 2A of Kornmann—are they sporangia whose spores develop another *Conchocelis* phase, or abnormal monosporangia? What are the deviations from the natural life-cycle in shells that develop when the *Conchocelis* phase is grown free and in different day-lengths and light-intensities? What are the big, dark cells formed in the degenerating thalli under long-day conditions?

The present research confirms and extends previous results on the effect of the photoperiod on *P. tenera*. As mentioned, the *Conchocelis* phase grows in nature during the long-day seasons and the leafy thallus phase in short-day seasons. The leafy thallus phase is apparently a short-day plant: growth is arrested and the thallus degenerates when exposed to 13 hours of light daily. The *Conchocelis* phase is not strictly a long-day plant: *in vitro* it grows, but slowly, under short-day (8-hour) conditions and in subdued light. However, high light, longer day (11-hour), and especially continuous light enhance growth vigorously. The incomplete data available indicate that the photoperiod governs the formation of monosporangia and the liberation of monospores. Our *in vitro* experiments confirm fully the results of Kurogi (1959) obtained with *Conchocelis* grown in shells. He found that photoperiods of 10 and 12 hours of light (corresponding to conditions of winter, spring and autumn, respectively) induce an abundant formation of monospores, while 15 hours of light daily did not enhance the formation of monosporangia.

Furthermore, the *Conchocelis* which were liberating monospores in 10-hour photoperiods continued for only a few days, and then stopped liberating monospores, when transferred to 15 hours of light; conversely the long-day (15-hour) *Conchocelis* began to liberate monospores after they were transferred to short-day (10-hour) conditions. Similarly the *in vitro* experiments on free-living *Conchocelis* show that short-day (8-, 11-hour) induces early formation of monosporangia and liberation of monospores. Continuous light, or subdued 11-hour photoperiods, induce the formation of interesting and different sporangia, or peculiar inflated cells in the *Conchocelis* filaments, whose fate and origin need further investigation.

The preliminary experiments on the thallus indicate that the photoperiod also governs the formation of carpospores; 13 hours of light daily induce cessation of growth and degeneration of the leafy thallus, followed by formation of carpospores or their physiological equivalents. Exposure of full-grown thalli to different photoperiods is now needed to define precisely the effect of the photoperiod on carpospore production.

These findings emphasize the need of determining the effect of photoperiods on the life-cycle and alternation of generations in sea weeds. Föyn (1955) had observed that the northern species of *Ulva* (*lactuca*) can grow normally in continuous light, while the southern Mediterranean species (*Thuretí*) dies in such conditions.

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Summary

1. The complete life-cycle of *Porphyra tenera* was obtained *in vitro*.

2. Chemically defined media or enriched sea water permit good growth of these unialgal (not bacteria-free) cultures.

3. Under suitable light and temperature, the complete life-cycle is completed in 5–6 months. Both the *Conchocelis* and the thallus phases may be grown out of season.

4. The *Conchocelis* phase grows well free in liquid media; a calcareous substrate is unnecessary. *Conchocelis* colonies grown in liquid media when free-floating, are stellate and round, but mold-like when attached to glass walls. They are brown-black or purple-red, depending on the composition of the medium. Rapid and abundant growth of the free *Conchocelis* is elicited by high-light intensities. Fluorescent light is a good light source.

5. Monosporangia formation and release of fertile monospores are induced by short-day conditions (8–11 hours daily); monosporangia and germinating monospores develop after 1–2 months from the inoculation of the *Conchocelis* filaments. In continuous light, *Conchocelis* growth is rapid but the sporangia produced are somehow different from the ones produced in short-day conditions.

6. In continuous light, the number of colonies increases rapidly after transfer to new media. This could be due to formation of new colonies from small pieces of filaments. However, even though free spores were not found, it is not excluded that new *Conchocelis* colonies may have been derived from special spores.

7. The *Conchocelis* phase was cultured for one year by transferring free *Conchocelis* colonies or pieces of filaments every two months in new media. Mass cultures with good yields were obtained in continuous fluorescent light.

8. The leafy thallus, derived from monospores grown in shells, grows well and normally in artificial media, at 13–18° C. and in high intensity incandescent light of 8–11 hours daily, but not in fluorescent light.

9. A photoperiod of 13 hours daily inhibits growth of young thalli (1–2 mm.). The thalli became thick, curly, degenerate, assume a callus appearance, bleach almost completely except for scattered groups of dark-pigmented, big cells which produce spores germinating into *Conchocelis* filaments. In one type of enriched sea water (SWII), the thalli, after thickening, and while degenerating, produce rhizoid-like structures which give rise to *Conchocelis* filaments.

10. In nature, the *Conchocelis* phase grows in the long-day seasons, the leafy thallus phase grows in the short-day seasons; and the transition between the two phases is almost exactly at the equinox. On the contrary, no correlations exist between temperature and the phases of the life-cycle: a large temperature zone (7–21° C.) is common to the two phases. Similarly, our preliminary experiments show that the length of the photoperiod has remarkable effects on the *Conchocelis* and leafy-thallus phases of *P. tenera*. The photoperiod governs, besides growth, the formation of the spores producing the next phase of the life-cycle. It is reasonable, therefore, to suppose that like land plants, some sea weeds, or phases of their life-cycle, may be long- or short-day plants.

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RESPIRATION RATES IN PLANARIANS. III. THE EFFECT OF THYROID COMPOUNDS ON OXYGEN CONSUMPTION[1]

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Iodinated proteins have been found throughout the invertebrate world (Roche, 1952; Gorbman *et al.*, 1954), primarily in the form of mono- and diiodotyrosine, although in a number of insects (Limpel and Casida, 1957) and in *Musculium*, a fresh-water fingernail clam (Gorbman *et al.*, 1954), a high percentage of the protein-bound iodine has been shown to be in the form of thyroxine. None of these compounds has been demonstrated unequivocally to take part in physiological processes in the invertebrate animal (Goldsmith, 1949; Gorbman *et al.*, 1954), but recent reports indicate the question is not settled. Wingo and Cameron (1952) found that thyroxine hampered the multiplication of a ciliate protozoan, *Tetrahymena geleii*, but increased the rate of oxygen uptake above that of parallel control cultures. Thyroxine added to the diet of rice moth (*Corcyra cephalonica*) larvae is reported to have increased the oxygen consumption requirement, although thyroglobulin was without effect (Srinivasan *et al.*, 1955).

The presence of iodinated proteins in planarians has not been investigated, but several workers have reported a positive action of thyroid compounds on physiological activities in this group. Castle (1928) observed that *Phagocata* (*Planaria*) *velata* was attracted to and fed readily upon macerated sheep thyroid, and subsequently decreased in size even more rapidly than worms subjected to starvation. Goldsmith (1937), studying the effect of endocrine feeding on regeneration and growth in *Dugesia tigrina* (*Planaria maculata*), noted no significant differences in the head regeneration time in the gland-fed animals, but found that thyroid-fed individuals increased in size to a lesser extent than the liver- and pituitary-fed forms. The influence of thyroxine on eye formation in *Phagocata gracilis* was investigated (Weimer *et al.*, 1938) in pieces of planarians cut at different levels and allowed to regenerate in a saturated thyroxine solution. Once the reconstitution process had begun, the rate of eye formation was reported to be much higher for the pieces in thyroxine.

No reports are available of the effect of thyroid hormones on oxygen consumption in planarians. Phenylthiourea, an anti-thyroid agent, has been shown, however, to exert a depressing effect on planarian respiration (Jenkins, 1961). In view of these findings an investigation was undertaken to ascertain the effect of certain thyroid compounds on respiration rates in *Dugesia dorotocephala*, a common fresh-water planarian.

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Design of Experiment

The planarians used in this study were large, sexually mature animals, collected from Buckhorn Springs[2] in Murray County, Oklahoma. They were maintained in pans of lake water, provided with an aerator, at a constant temperature of 20° C. Experimental animals were taken on the seventh day after feeding and were not fed during the course of the experiment.

Compounds used for this investigation were thyroxine (T_4), 3,5,3′-triiodothyronine (T_3),[3] and 3,5-diiodotyrosine (DIT). In order to determine the concentration to be used, groups of cut posterior ends of planarians were allowed to regenerate in a graded series of molar solutions of each of the compounds in lake water, and the regenerated animals examined for signs of any abnormalities. In worms in both the thyronines, eye spots were visible under a dissecting microscope by the third day, compared to the fifth day for the animals in water and in diiodotyrosine. It was noted, however, that the worms in the 3×10^{-5} *M* dilution of triiodothyronine appeared to have a slight thickening across the head behind the eyes. This was not apparent in the 2×10^{-5} *M* solution; the latter was therefore chosen as the higher concentration for the experiment.

All three chemicals were made up at this concentration so their effects could be compared. In addition, a solution of half the molarity given above was used for each in order to test whether or not a more dilute solution would have an appreciable physiological effect. The controls were cultured in lake water.

The procedure was similar to that employed for observing the effect of goitrogens on oxygen consumption in planarians (Jenkins, 1961) with the following exceptions: The Latin square method was used in order to provide maximum randomization. Seven replicate experiments were performed. Each replicate experiment employed seven groups of five planarians each; one group for each of the two concentrations of the three chemicals used, and one water control. On the day that a replicate experiment was begun, 35 of the largest specimens in one stock pan were selected and divided randomly into the seven unit groups.

For each replicate experiment, oxygen consumption determinations were made as follows:

Day 0: Oxygen consumption was measured over a period of three hours with all seven groups of animals in water. At the close of the day's readings, each group was placed in an individual fingerbowl of water until the following day.

Day 1: The Warburg flasks were prepared with the solutions of thyroid compounds (or of culture water) to be used. The planarians were placed in the flasks and manometer readings were made during the first 3½ hours of exposure to the chemicals. At the termination of the day's readings, each group was placed in a fingerbowl containing the same concentration of thyroid compound as that in which oxygen consumption was to be determined during the remainder of the experiment.

Further readings for periods of three hours were made on the second, fourth, and sixth days. The worms were placed in fresh thyroid compound solutions every second day.

[2] Acknowledgment and thanks are due to Oscar Lowrance, owner of Buckhorn Springs property, for permission to collect the planarians.

[3] Supplied through the courtesy of the Sigma Chemical Company, St. Louis, Missouri.

Results

The oxygen consumption of each group of planarians was calculated according to standard methods (Umbreit *et al.*, 1949) and the results are given in Table I. Little variation was shown among the groups in response to treatment on any one day, the normally-occurring slight downward trend shown by the controls being apparent in each of the experimental groups. The data show significantly that,

Table I

Effect of thyroid compounds on oxygen consumption of planarians

Treatments	μl. O_2/gm./hr.				
	Days				
	0	1	2	4	6
Water	130	128	131	120	121
Thyroxine, 2×10^{-5} *M*	133	130	130	121	120
Thyroxine, 1×10^{-5} *M*	132	129	131	120	124
Triiodothyronine, 2×10^{-5} *M*	129	133	128	124	124
Triiodothyronine, 1×10^{-5} *M*	131	130	131	121	120
Diiodotyrosine, 2×10^{-5} *M*	133	132	128	123	119
Diiodotyrosine, 1×10^{-5} *M*	126	130	130	124	125

under the conditions of this experiment, there is no demonstrable effect on the oxygen consumption of the planarians.

Discussion

Although neither iodotyrosines nor iodothyronines, which are widely distributed among invertebrates, have been found to influence physiological processes in these animals, it is obvious that there is some homeostatic regulation in organisms without a thyroid, and that metabolic processes do occur and are regulated within the animal. Whether thyroxine or its analogs play any part in this regulation remains to be established. The prevailing opinion at the present time is that invertebrate tissues are insensitive to the action of thyroid hormones. Findings which are not in agreement, such as moth larvae showing an increased metabolic rate when fed with thyroxine (Srinivasan *et al.*, 1955), have not been confirmed in other invertebrates.

Although evidence of the physiological activity of thyroid hormones in metazoan invertebrates is inconclusive, there is increasing evidence that one-celled organisms respond markedly to these chemicals. The findings of Wingo and Cameron in regard to *Tetrahymena geleii* have been mentioned above. Gutenstein and Marx (1957) have demonstrated that respiration of yeast cells (*Saccharomyces cerevisiae*) is significantly accelerated by thyroxine and inhibited by a specific thyroxine antagonist. Augmentation of oxygen consumption has also been observed in *Escherichia coli* subjected to the influence of T_3 and T_4 (Roche *et al.*, 1959), although the respiratory action of the two iodothyronines does not seem bound to a metabolic transformation of the hormones.

In some instances it appears that invertebrates are able to metabolize thyroid compounds in much the same manner as the tissues of vertebrates do. The hepatopancreas of the mollusks, *Mytilus galloprovincialis* and *Octopus vulgaris*, has been shown to degrade T_3 by deiodination and oxidative deamination, followed by oxidative decarboxylation (Covelli *et al.*, 1960). This is considered to be a metabolic degradation of the hormone rather than a physiological activation. The formation of thyroxine metabolites by *Escherichia coli* has also been reported (Gräsbeck *et al.*, 1960) and explained on the basis that both *E. coli* and other micro-organisms are able to oxidatively deaminate many amino acids.

The question of extrathyroidal iodine metabolism is closely related to the problem of invertebrate tissue responses to thyroid hormones. The discovery that thyroxine could be recovered from iodinated casein (Ludwig and von Mutzenbecher, 1939) was followed by early reports (Chapman, 1941; Morton *et al.*, 1943) that newly-formed thyroxine-like compounds could be demonstrated in the tissues of thyroidectomized rats. The observation that the same limited series of iodine compounds is formed when any of a large number of proteins is iodinated, whether in the thyroid, in artificially iodinated proteins, or in the iodoproteins of invertebrates (Reineke, 1949) appeared to confirm this idea, but it has recently been discredited (Taurog *et al.*, 1960) on the basis that the concentrations of iodine used in the early experiments were far above the physiological range.

The evidence to date strongly indicates that tissues of invertebrates are insensitive to the action of thyroxine and its analogs. The negative results obtained in the present experiment support this view. However, the continual recurrence of reports which substantiate the opposite view to some extent, such as the augmentation of oxygen consumption in protistans, noted above, prevents the complete acceptance of the view that invertebrate tissues are wholly insensitive to thyroid compounds. Since most reports of a positive response of non-chordate tissues to thyroxine and its analogs are limited to those experiments in which groups of like cells are used, either as tissues from metazoans or as concentrated groups of one-celled organisms, it is possible the elucidation of the cellular metabolism of thyroxine may bring to light principles which will aid in solving the question of regulation of metabolic processes in thyroidless organisms. It seems reasonable to suppose that the same fundamental pattern of metabolic regulation may be found throughout the animal kingdom.

Summary and Conclusions

1. Using the Latin square method, a study was made of the effect of the thyroid compounds diiodotyrosine, triiodothyronine, and thyroxine on respiration in planarians. No statistically significant effect was found with any one of the three chemicals under the conditions of the experiment.

2. The regulation of metabolic processes in invertebrates is discussed briefly. The suggestion is made that the same fundamental pattern of metabolic regulation may be found throughout the animal kingdom, despite the fact that no evidence of this is demonstrated in the present paper.

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HISTOPHYSIOLOGICAL STUDIES ON THE CORPUS ALLATUM OF LEUCOPHAEA MADERAE. III. THE EFFECT OF CASTRATION [1, 2]

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In the insect, *Leucophaea maderae,* and in related species reproductive processes are controlled by an intricate neuroendocrine mechanism (Scharrer, 1958a, 1959; Engelmann, 1960). Each reproductive cycle is initiated by a hormone from the corpora allata. Concurrently with alternating phases of ovarian activity and quiescence, the corpora allata become active and inactive at regular intervals. They remain inactive as long as they are restrained by nervous stimuli from the brain. The reactivation of the corpora allata at the appropriate time is controlled by a variety of factors, among them feed-back from the reproductive organs. The exact nature of this feed-back is still unknown, but it seems that a humoral factor is involved that acts by way of the central nervous system. As a rule, corpus allatum activation occurs when the ovaries contain undeveloped eggs, except during "pregnancy," *i.e.*, as long as an ootheca is present in the uterus. The active corpus allatum, found in animals with growing eggs, shows a considerable increase in volume and other distinct changes in its histology as compared with the inactive gland characteristic of newly emerged or pregnant animals (Engelmann, 1957; Scharrer and von Harnack, 1958).

The individual components of this neuroendocrine control system can be analyzed by experimental interference at various levels, for example, by severance of the nerves restraining the corpora allata (Scharrer, 1952), or by gonadectomy (von Harnack and Scharrer, 1956). Such procedures disrupt the normal functional and structural periodicity of the corpora allata.

In the absence of the ovaries, the corpora allata of *Leucophaea* continue in a state of activity beyond the normal period. The question arises whether this condition surpasses the normal degree of activity of the corpus allatum cells, and whether the lack of intermittent inactivation of the gland may perhaps result in permanent structural change or even damage.

The present paper deals for the most part with a morphological analysis of the corpus allatum of gonadectomized females of *Leucophaea* ranging in age from emergence to senescence. The situation in males seems less interesting, since their corpus allatum fails to respond to gonadectomy as in females.

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Material and Methods

The present study includes 154 adult females and 26 males of the roach, *Leucophaea maderae*, gonadectomized under varying environmental conditions in our laboratories in Cleveland, Denver, and New York between 1944 and 1959. The large majority of the females (147) were operated upon at various nymphal stages, the remaining seven shortly after they had become adults. In the ovariectomized nymphs, the interval between operation and emergence ranged from 1 to 152 days. In the group operated in the adult stage, the longest interval between emergence and castration was 18 days. The completeness of the gonadectomy was verified by examination of the excised tissues under the dissecting microscope, by testing the experimental animals individually for possible reproduction by placing them in separate jars containing normal males, and by autopsy at the time of fixation. The condition of the accessory sex glands was also recorded; excessive amounts of secretory material are characteristic of gonadectomized females.

Fixation was scheduled at intervals ranging from an adult age of nine to 457 days. The material thus encompasses the entire adult life span. The total period after gonadectomy, therefore, ranged from a minimum of 32 to a maximum of 491 days. The majority of the animals (143) had been castrated for over 100 days.

A smaller number of males was included in this study; their corpora allata are more uniform in structure, and presumably less complex in function, than those of females. All of the 26 male castrates were operated upon as nymphs, 12 to 104 days before emergence. The adult age at the time of fixation varied between 0 and 366 days. The total castration period thus ranged from 39 to 432 days, with all except three specimens falling in the group above 100 days.

The histological procedures and the methods of quantitative evaluation of the corpora allata were essentially the same as those described in the first paper of this series (Scharrer and von Harnack, 1958). Most cases were fixed in Helly's solution. The Halmi-Dawson "aldehyde fuchsin" method was used with the addition of Weigert's hematoxylin as a nuclear stain. The present study includes tissues treated with the standard periodic acid-Schiff (PAS) technique. The sections were kept in the periodic acid solution and in the Schiff reagent for 10 minutes each. The duration of pretreatment with buffered saliva ranged from 30 minutes to two hours. Certain additional specimens were fixed in 10% formalin, alcohol-formol-acetic acid (AFA), or Carnoy's solution.

Among the female castrates, 57 representative cases were selected for complete quantitative analysis, *i.e.*, volumes of the corpora allata, nuclear numbers, and nuclear-cytoplasmic ratios were determined. In the remaining 98 animals only volumetric determinations were made. Since organ volume, accompanied by histological inspection of the relative amount of cytoplasm, is a good indicator of the degree of activity of the corpora allata, these specimens serve to substantiate the conclusions drawn from the study of the more completely analyzed group.

In the males, volumetric determinations were used as an indication of corpus allatum activity, since the normally low variability of their corpora allata was not significantly altered after gonadectomy. In the largest and smallest glands of this group, nuclear numbers and nuclear-cytoplasmic ratios were calculated in order to delimit the range of variability within the male castrates.

Additional methodological matters, such as hemicastration, will be mentioned in the text.

Results

a. Females

When the volumes of the corpora allata of all gonadectomized females studied were plotted according to adult age (Fig. 1), several facts became apparent.

All age groups with the exception of the youngest and the oldest showed a considerable spread in corpus allatum size. Starting from glands whose volume

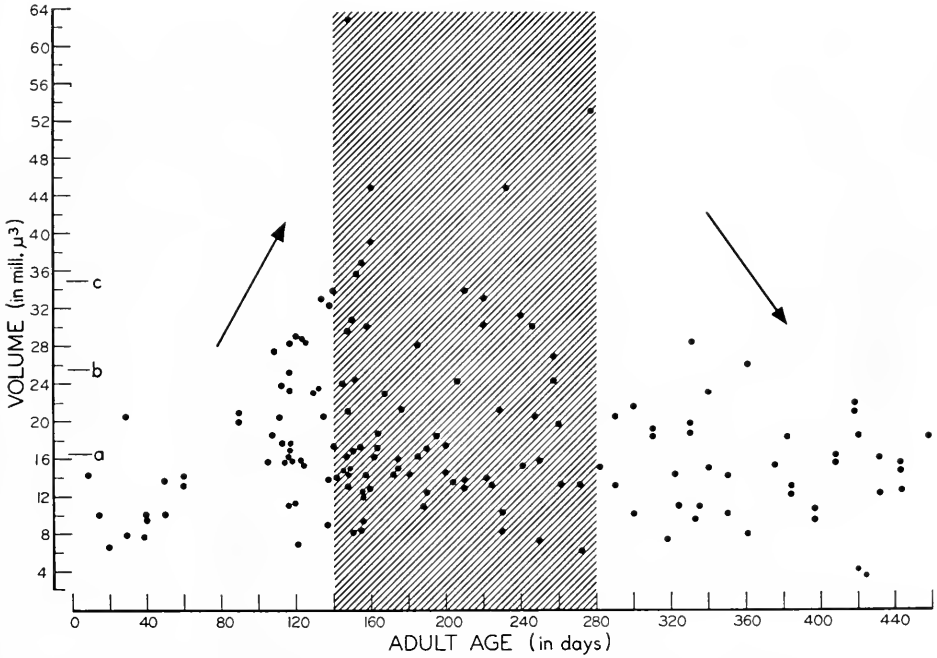


Figure 1. Diagram showing the size variation of corpora allata of 154 ovariectomized *Leucophaea* arranged according to adult age. Each dot represents the measurement of the paired glands from one individual. The arrows indicate that, with increasing age, the volumetric maxima rise stepwise to reach peak values in a mid-period (140–280 days; shaded area), and gradually decline in old age. For comparison, three maximum levels of corpus allatum size in groups of non-castrate animals are indicated on the scale: a, First activity cycle preceding first ovulation of normal controls; b, Second activity cycle following first parturition in normal controls; c, Response to feeding following drastic starvation.

matches that of the smallest inactive glands in normal controls, through specimens appearing moderately or fully active, the range extends to organs much larger than any observed in the previous studies of this series.

These supernormal values seem to be unique for gonadectomized females. They are, therefore, of primary interest, even though their number in the present series is comparatively small. A total of 26 animals surpasses the maximum size of the corpora allata characteristic of the second reproductive cycle of normal controls.

Of these, seven exceed also the higher level reached by females which had been returned to a normal diet after prolonged starvation (von Harnack, 1958). The possible reasons for the scarcity of excessively high corpus allatum values as well as the great variability of corpus allatum size within age groups will be discussed below.

If one follows, in consecutive age groups, the distribution of the maxima in corpus allatum size a pattern becomes apparent which is indicated by the arrows in Figure 1. Comparable to the situation in normal post-emergence females, there is, with increasing adult age, a gradual volumetric increase. However, in contrast to the intact animals with their predictable periodic return to corpus allatum inactivity, the growth trend continues for a longer time in the ovariectomized animals. At about 100 days of adult age, their highest value for the corpus allatum reaches the limit typical of the second reproductive cycle of normal females. One hundred and forty days after emergence the maxima in the castrates match the still higher top level of those females which had received a normal diet after drastic starvation (von Harnack, 1958).

The "giant" corpora allata, found so far exclusively in castrate females, all occur in the age group between 140 and 280 days. After this period the maxima gradually decline throughout the remaining adult life span whose termination is represented by a female killed at the high adult age of 455 days.

The interpretation of these volumetric data should be based on the following considerations. Our previous studies of the activity cycles of the corpora allata of *Leucophaea* have shown a remarkable capacity of these glands to change their structure according to physiological conditions, a responsiveness which expresses itself by alternating volumetric increase and decrease. It is difficult to assess the range of individual variation, both as to the degree of response and the time required by each specimen to react in a given situation. Since this variability from individual to individual exists in normal animals kept under uniform laboratory conditions (Scharrer and von Harnack, 1958), one may expect it to be even more pronounced in experimental animals such as the starvation series (von Harnack, 1958) or in the present, in which the normal cyclic pattern of the corpora allata has been disrupted by the removal of an important target organ, the ovary.

Furthermore, the value obtained for a given specimen fixed at any given age does not permit conclusions as to its past history or (theoretical) future potentiality. For example, small corpora allata in an animal of—let us say—200 days of adult age may have become hyperactive early, as did those of the female fixed 148 days after emergence, and may subsequently have regressed. It is equally possible that a sample represents a slowly responding type which would have eventually reached a peak of the kind indicated by the excessively large gland fixed at the adult age of 280 days. At the time of fixation, a corpus allatum devoid of feedback stimuli from the ovary may even have passed through more than one phase of activation. This possibility is suggested by the observation that corpus allatum implants free from central nervous control may change from inactivity to activity and vice versa (Engelmann, 1960). Certain as yet undetermined extrinsic or intrinsic conditions may modify the effect of castration on the corpus allatum (see also Doane, 1960). Finally a specimen, for reasons unknown, may have been altogether unresponsive to the abnormal situation created by gonadectomy.

In view of all these considerations, it is not surprising that a relatively large number of cases in the present experimental series show corpora allata that lie within the normal range of volumetric variation. Theoretically, the chances of "hitting" a gland at its peak value are small, especially if the state of maximal activation were of short duration.

Thus it is the occurrence rather than the frequency of abnormally high volumetric values which is of primary significance. Their existence demonstrates the capacity of the corpus allatum of *Leucophaea* to respond to abnormal physiological states which, in degree, far surpasses even that observed under drastically changed nutritional conditions. The most plausible explanation for this pronounced histophysiological response in gonadectomized specimens seems to be the persistent lack of ovarian feed-back stimuli. The prolonged effort of the corpora allata to stimulate a target organ which is no longer present presumably results in morphological changes indicative of hyperfunction.

The assumption made here, that corpus allatum volumes are not maintained at peak levels indefinitely, is supported, but not proved, by the absence of abnormally high volumetric values among all gonadectomized females of the series whose adult life span surpasses 280 days. Since no periodic changes in afferent stimuli, like those from gonad and ootheca in normal females, can account for the inactivation of the corpora allata of castrates, one may conclude that states of excessive glandular stimulation eventually subside on their own account.

The next question of interest concerns the changes in nuclear-cytoplasmic ratio in relation to corpus allatum volume (Fig. 2). It may be recalled that, as a general rule established in normal adult females, the number of nuclei per unit of corpus allatum tissue decreases with increasing organ volume. One may inquire whether the same holds for the corpora allata of gonadectomized females and, if so, whether the shift in nuclear-cytoplasmic ratio continues in those female castrates whose corpus allatum volume surpasses the maxima of the normal physiological range.

In all those gonadectomized females whose corpus allatum size, at the time of fixation, was within that of the normal physiological range, the relationship between organ volume and nuclear-cytoplasmic ratio compared well with that in unoperated controls. In the smallest corpus allatum from a castrate animal (3.6 million μ^3) the number of nuclei per mm.^{2} was calculated as 17,251. In a control gland of similar size (3.4 million μ^3) the corresponding figure was 16,765. In both the experimental and the normal series, these figures gradually decreased with increasing organ size to about one fourth of the initial level. In the castrates, glands with volumes paralleling the upper limit of the normal physiological range had, for example, 4389 nuclei per mm.^{2}, the corresponding count for normal specimens being 3770 nuclei per mm.^{2}. Young and old gonadectomized females with corpora allata of similar size did not show differences in nuclear-cytoplasmic ratios. Thus, generally speaking, the level of "activity," as determined by the histological criteria discussed so far, is approximately the same in pairs of specimens with comparable corpus allatum size, irrespective of age, or the presence or absence of ovaries.

If the trend in shifting nuclear-cytoplasmic ratios were to continue at the same rate beyond the confines of the physiological size range, the relative cytoplasmic content and thus the degree of "activity" in the largest corpora allata of the gonadectomized series would indeed be unusual.

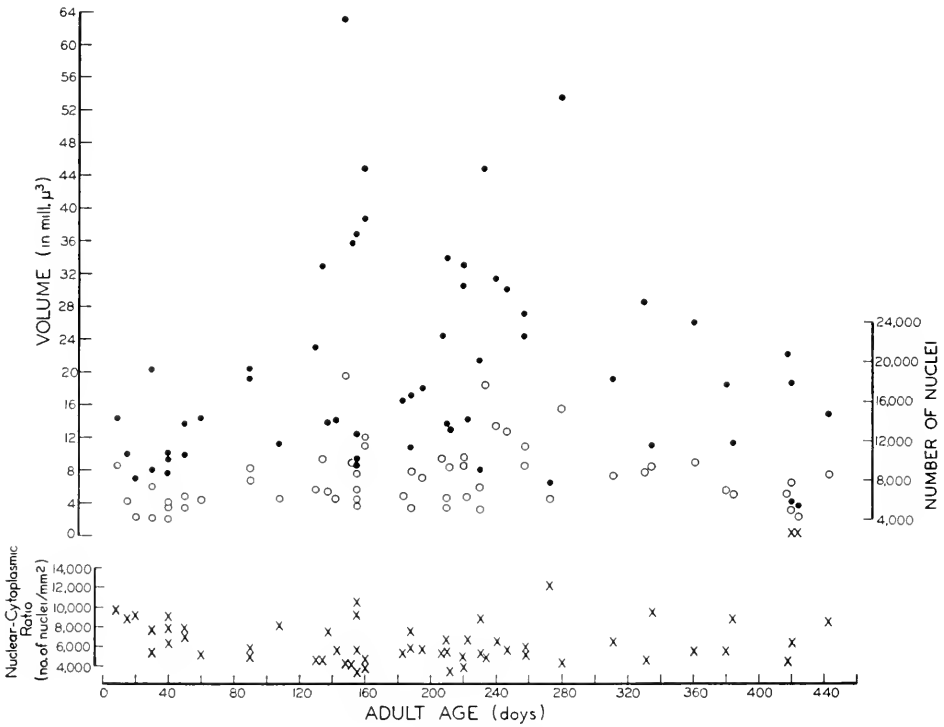


FIGURE 2. Diagram summarizing quantitative analysis of corpora allata of 57 samples of ovariectomized *Leucophaea* selected so as to illustrate the range of variation throughout the adult life span. ● = volume of both corpora allata; o = number of nuclei calculated for both glands; x = nuclear-cytoplasmic ratio.

The following data show that this is not the case. The smallest figure, 3486 nuclei per mm.^{2}, found among the oversized corpora allata of the castrates, is not significantly lower than the minimum of the control series (3698 nuclei per mm.^{2}). Therefore, one must conclude that the increase in relative cytoplasmic content with rising corpus allatum size reaches a plateau at a volumetric level equal to the maximum of the control group. Beyond this point, there seems to be no more drastic change in nuclear-cytoplasmic ratio, a situation which is also demonstrated by the fact that the nuclear counts in gonadectomized specimens continue to rise

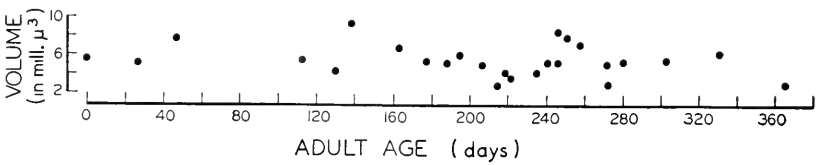


FIGURE 3. Diagram showing the volumetric values of corpora allata of 27 gonadectomized males. Compared with the situation in females, no clear-cut castration effect is noted. The range of variation is small and bears no relationship to adult age.

with increasing corpus allatum volume. They reach a maximum (18,736), which considerably surpasses that in normal controls.

Thus it appears that the increase in corpus allatum volume following ovariectomy, once it reaches abnormal proportions, differs from the growth process signalling activation of the gland in the course of its normal functional cycles. In the normal gland, the increase in nuclear number is surpassed by that in cytoplasmic content. By contrast, both nuclear number and cytoplasmic content increase proportionally during the period of overgrowth of the corpora allata of castrates.

In accordance with their considerable size variation, the corpora allata of castrate females differ widely in their morphology. As may be expected, they lack clear-cut signs of cyclic activity such as those that can be identified in normal females (Scharrer and von Harnack, 1958). Thus, for example, mitotic figures and pycnotic nuclei, although not frequent, occur throughout the life span with the exception of very young castrate adults.

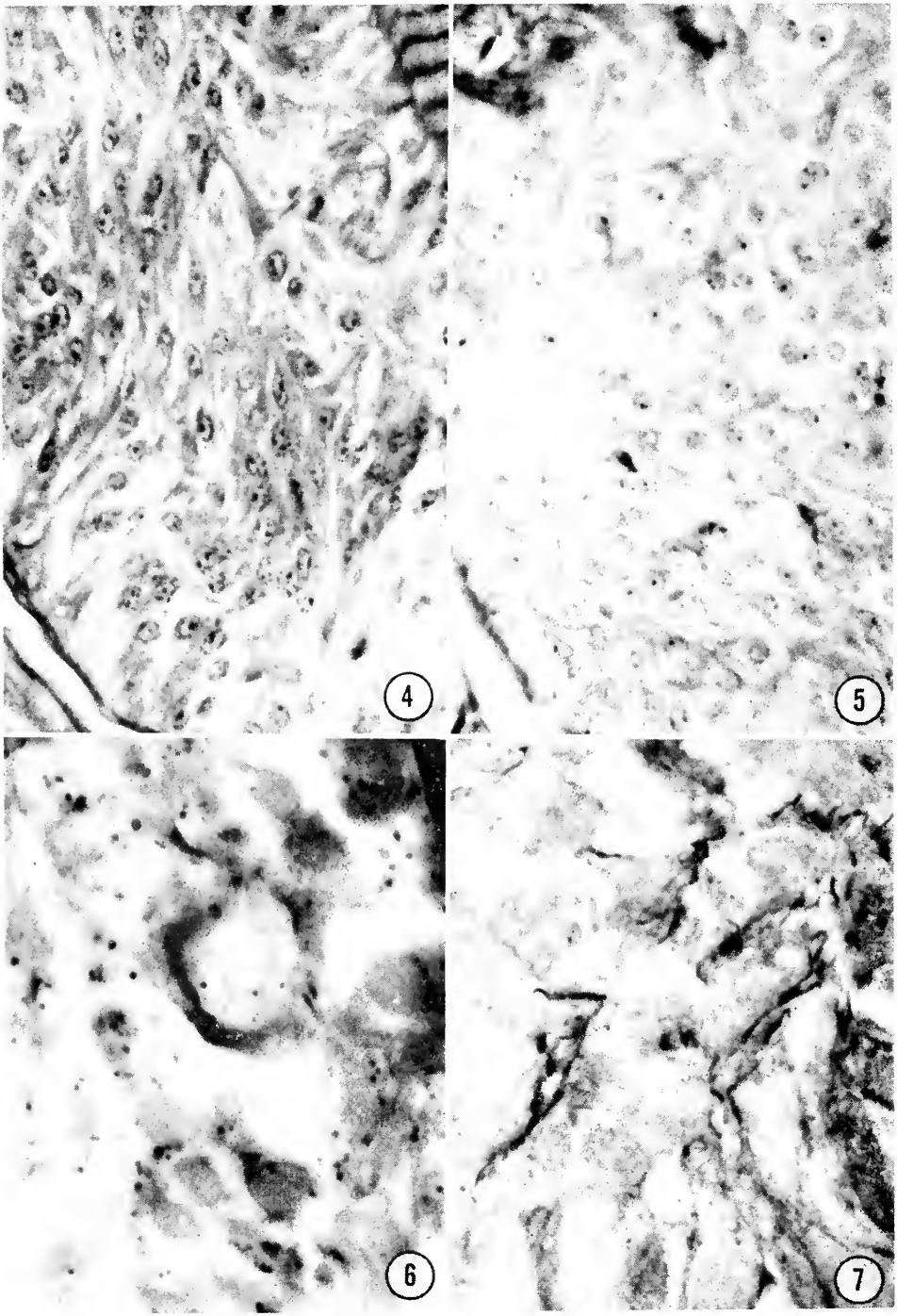
Aside from this apparent difference in periodicity, the quantitative data as well as histological features presented so far indicate that, within a comparable volumetric range, the glands of castrates resemble those of normal controls in several ways. A small corpus allatum, be it from a newly emerged normal or an old gonadectomized animal, has densely packed nuclei and shows very little cytoplasmic detail. With increasing size of the corpora allata conspicuous histological changes which are related to the gradually shifting nuclear-cytoplasmic ratio occur in both the normal and the experimental groups.

Cells filled with small, aldehyde fuchsin-positive granules, similar to those described in normal animals (Scharrer and von Harnack, 1958), are found in many of the castrates, especially those with medium-sized and large corpora allata. As a rule such cells are not numerous, and a careful search of serial sections is necessary for their detection. The youngest castrate female in which such cells were recorded had been killed 30 days after emergence. From this point on no preferential age distribution of positive cases was apparent. The present study did not add anything to the understanding of the functional role of these cells. The possibility that they are hemocytes is being explored.

More important in the corpus allatum cells of castrate as well as normal females are inclusions which stain lavender or pale green with the Halmi-Dawson method used. They are present after fixation in Helly's as well as formalin, AFA, and Carnoy's solution. In saliva-treated PAS preparations these granules stain intensely positive. This and the results with the other techniques used so far suggest that they belong to the category of glyco- or mucoproteins.

Matched pairs of experimental and normal animals show the following relationships. In younger castrates the PAS-positive granules are not conspicuous; they compare in number and size with those of normal controls. However, at an adult age of two months and over, the amount of this material in castrates with good-sized corpora allata significantly surpasses that in unoperated females (Figs. 6 and 7).

The examination of several other tissues revealed that this phenomenon is not restricted to the corpus allatum (Scharrer and von Harnack, 1960). In the fat body, the difference between ovariectomized and normal specimens is considerable. The wide range of possible variation in the content of PAS-positive, saliva-resistant material is illustrated by Figures 10 and 11. It should be stated, however,



FIGURES 4–7.

that not all castrates reach the degree of accumulation shown in Figure 10, while on the other hand normal controls may contain more inclusions than those depicted in Figure 11. In spite of these variations, there can be no doubt that the average amount of this material is considerably increased several months after gonadectomy.

Comparable PAS-positive inclusions are also noticeable in the muscles of gonadectomized females with an adult age of over 40 days. They are particularly conspicuous in areas near the insertion of a muscle fiber in the chitinous skeleton (Fig. 12). In normal animals very few or no such inclusions are observed in the musculature.

Another example is the central nervous system where castrates also show a greater amount than normal females of PAS-positive material other than glycogen. This material is seen in varying amounts within numerous ganglion cells. However, the most pronounced difference occurs in a special type of neurosecretory cell (B cell) of the subesophageal ganglion which normally contains only few granules in the periphery of the perikaryon (Fig. 9). With few exceptions, in castrates this pair of cells accumulates large amounts of cytoplasmic granules which stain intensely with the PAS technique (Fig. 8). In Halmi-Dawson preparations these granules are distinguished by their green coloration. These cells have been called "castration cells" in an earlier study (Scharrer, 1955). Within the limited scope of the histological tests employed so far, the inclusions in the fat body, musculature, and nervous tissue resemble those of the corpora allata. Morphologically they differ in that they are larger.

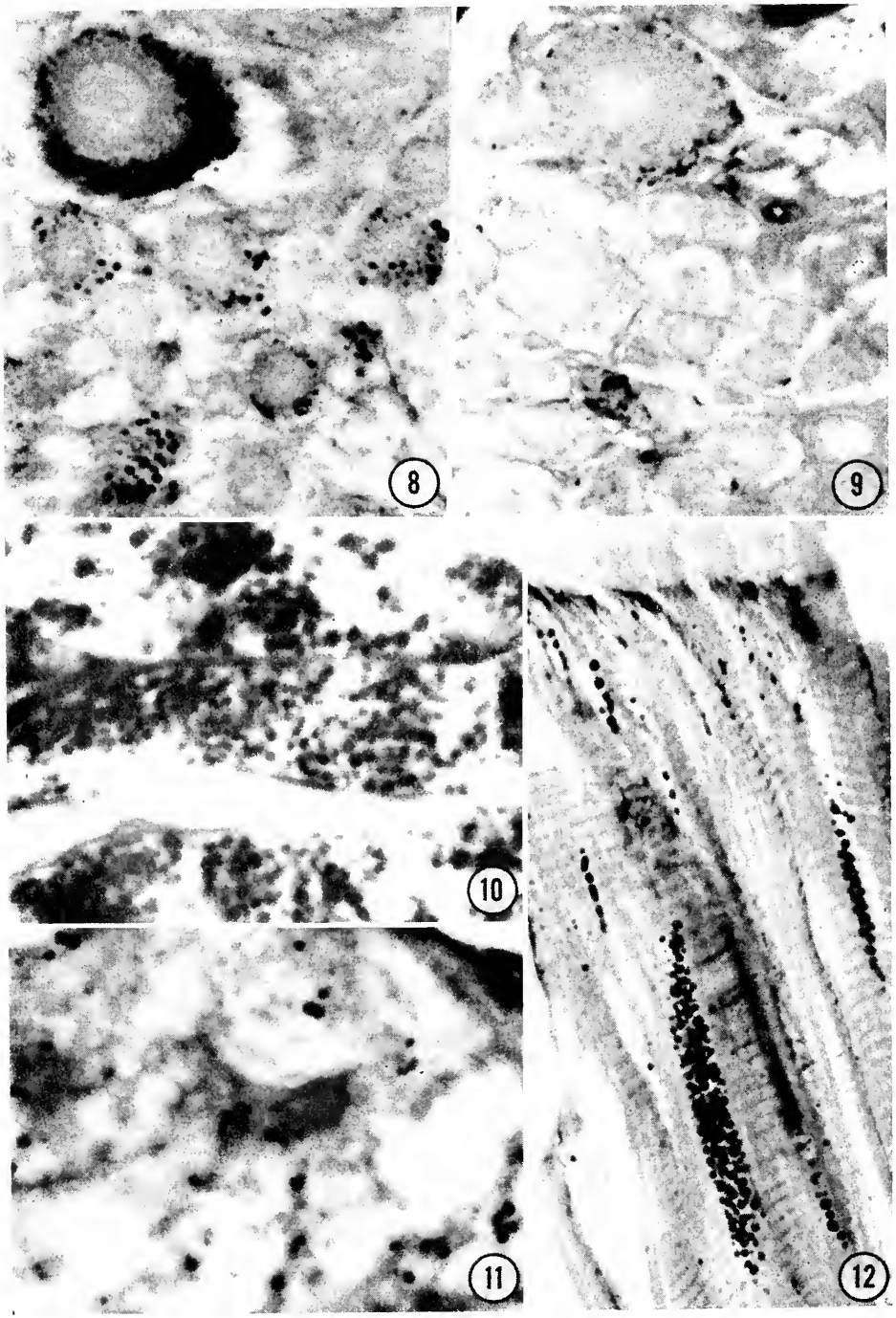
Sections of corpora allata with high cytoplasmic content may show varying degrees of vacuolization. Also, individual cells may appear partially separated from each other and thus more easily identified than in smaller specimens where cell boundaries can be detected only in electron micrographs. When the glands reach abnormally high volumes in castrates, the form and arrangement of individual cells undergo further characteristic changes. The orientation of cell processes toward the periphery of the gland and the probable relationship of this arrangement to the release of secretory material into the surrounding hemolymph in highly active normal corpora allata have already been reported (Scharrer and von Harnack, 1958). In the largest castrate specimens, many cells appear "stretched" to the point of becoming spindle-shaped. The resulting pattern of corpus allatum structure is quite characteristic (compare Figs. 4 and 5). To what extent this might

FIGURE 4. Section through corpus allatum of gonadectomized female of *Leucophaea*. Adult age 148 days; volume of corpora allata 62.9 million μ^3 (maximal value of series). Note characteristic pattern of spindle-shaped cells. Helly, 7 μ, modified aldehyde fuchsin. $\times 450$.

FIGURE 5. Control section for Figure 4; same technique. Active corpus allatum of normal female fixed at adult age of 125 days; 15 days after parturition; ovarian eggs nearly mature; volume of corpora allata 18.7 million μ^3. Note irregular arrangement of cells (the existence of cell boundaries, difficult to see in the light microscope, will be demonstrated in a future publication on the ultrastructure of the corpus allatum). $\times 450$.

FIGURE 6. Section through corpus allatum of castrate female showing numerous PAS-positive granules. In addition to these intracellular inclusions, other PAS-positive elements seen are a small part of the corpus allatum sheath (upper right corner) and intercellular material within the gland. Adult age 228 days. Formalin, 7 μ, saliva pretreatment, PAS. $\times 1200$.

FIGURE 7. Control section, treated with same technique as in Figure 6, through active corpus allatum of normal female of same adult age; 10 days after parturition; development of ovarian ova well under way. Note relative paucity of PAS-positive granules. Intercellular elements are comparable to those in Figure 6. $\times 1200$.



FIGURES 8–12.

be a sign of "pathological" change remains undetermined. Aside from this feature, glands of extraordinary size show few cytological peculiarities not also observed in smaller glands.

In our material several unoperated cases with a history of ovarian dysfunction, related to senility or of unknown origin, were of particular interest for comparison with castrates. As might be expected, these "normal" animals displayed several of the cytological features, though not always all, characteristic of the experimental group. For example, one specimen with the exceptional adult age of 599 days had abnormally large corpora allata (vol. 37 million μ^3, as compared with 25.5 for the maximum control value) with a normal "active" appearance except for the presence of a giant nucleus. In this case, PAS-positive granules were more abundant than normally, in the corpora allata as well as the central nervous system and the fat body, but not in the musculature. In another case, which had reached the adult age of 300 days without signs of reproductive activity, the number of granules was increased in the musculature as well as the corpus allatum and the fat body.

In view of the castration effects just described it was of interest to study the situation after unilateral gonadectomy. Fifty-two females whose right ovary had been removed before emergence produced offspring at normal intervals but, on the average, only one-half of the number of young borne by unoperated control animals (Scharrer, 1958b). In their histology, the corpora allata of this experimental group resembled those of normal adult females. According to the stage in the ovarian cycle at which the animals were fixed, the structural criteria used in this study indicated varying degrees of glandular activity. The fat body, musculature, and nervous tissue also showed no abnormalities. Thus, hemicastration apparently fails to lead to those morphological changes characteristic of females without both ovaries or with severe disturbances in gonadal function.

b. Males

The corpora allata of the 26 castrated males studied showed no drastic effects of the operation. The volumetric values in this group (range: 2.8 to 9.4 million μ^3; mean: 5.5 million μ^3) are only somewhat higher than those of 46 normal control specimens (range: 2.1 to 9.0 million μ^3; mean: 4.9 million μ^3). The total number of nuclei calculated for the smallest (3486) and the largest glands (3867) of the

FIGURE 8. Section through subesophageal ganglion of castrate female showing abundance of PAS-positive granules. These occur in various ganglion cells, but are particularly concentrated in the large B-type neurosecretory cells designated as "castration cells"; one of these in upper left corner of Figure. Same animal and same technique as in Figures 6 and 10. × 600.

FIGURE 9. Control section through same area as in Figure 8 of subesophageal ganglion of normal adult female. Note that B-type neurosecretory cell and adjacent ganglion cells contain only a few PAS-positive granules. Formalin, 7 μ, saliva pretreatment, PAS. × 600.

FIGURE 10. Section through cephalic fat body of castrate female. Same animal and same technique as in Figures 6, 8, 12. Note numerous PAS-positive granules. × 1400.

FIGURE 11. Control section for Figure 10 through cephalic fat body of normal female. Same animal and same technique as in Figure 7. In contrast to that shown in Figure 10, this specimen contains few granules. × 1400.

FIGURE 12. Longitudinal section through cephalic musculature of castrate female. Same animal and same technique as in Figures 6, 8, 10. Note conspicuous accumulations of PAS-positive granules. Normal controls show only rare granules. × 560.

gonadectomized series differs only little, and consequently the range of nuclear-cytoplasmic ratios is considerable (15,571 nuclei per mm.^{2} in the smallest, and 5117 in the largest gland). Thus the corpora allata of castrate males resemble those of normal males and females in that the relative amount of cytoplasm is increased when the glandular volume rises.

In normal adult males, fluctuations in corpus allatum volume occur during the entire adult life span (Scharrer and von Harnack, 1958). Similarly, castrates show no significant variations of "activity" as determined by organ volume and nuclear-cytoplasmic ratio during adult life (Fig. 3). Neither is there, within the range of the time intervals of the experiment, a relationship between these values and the total (nymphal plus adult) length of the castration period. The animal with the largest glands happens to have an adult age of 138 days, that with the smallest one of 273 days. It may also be of interest to illustrate the relationships between male and female castrates by selecting a comparable pair of specimens. In the male with the largest corpora allata (9.4 million μ^3) the total nuclear count was 3896 (5117 nuclei/mm.^{2}), in a female with corpora allata of the same size it was 3994 (6110 nuclei/mm.^{2}).

Inasmuch as the corpora allata of male *Leucophaea* show hardly any volumetric response to changing physiological conditions, either during the normal life cycle or following castration, no dramatic changes in glandular histology were expected. The glands from castrate males compare in their cellular structure with those of normal males and of females whose corpora allata are inactive or only mildly active. The amount of saliva-resistant PAS-positive material present in castrate males does not surpass that in normal males or females. This applies to the cell inclusions in the corpora allata as well as those in the fat body, musculature, and nervous tissue.

Discussion

The present study has demonstrated significant changes in the histology of the corpora allata in response to gonadectomy in females of *Leucophaea*. By contrast males showed at best only a very mild reaction. The latter result is in line with previous negative results in males of several other insect species (Day, 1943; Bodenstein, 1947; Johansson, 1958). The slight rise in the average volume of the corpora allata of male castrates of *Leucophaea* might be dismissed as insignificant, except that it seems to parallel a similar responsiveness in normal adults. In these, a mild stimulation of the glands occurs during the post-emergence period, *i.e.*, at a time when in normal females a much more pronounced (pre-ovulatory) activation of the corpora allata was observed (Scharrer and von Harnack, 1958). Possibly these data indicate a limited capacity in male insects to respond to changes in the internal milieu comparable to that displayed, for example, by the male mammary gland. The lack of clear-cut histological correlations between the corpora allata and gonadal activity in males of *Leucophaea* is not surprising because all other available data speak for the absence, in the male sex, of a physiological role of these glands in the control of reproduction (Scharrer, 1946). But even in a case, such as in the gregarious phase of *Schistocerca,* where "sexual maturation" in the male is controlled by the corpora allata, these glands seem unaffected by gonadectomy, at least as far as physiological criteria are concerned (Loher, 1960).

The data in the literature are less consistent regarding the situation in female insects. Several investigators noted a volumetric increase in the corpora allata

of castrates (Pfeiffer, 1940; Thomsen, 1942; Day, 1943; Bodenstein, 1947; Johansson, 1958; for indirect evidence see also Vogt, 1942; Lukoschus, 1956; Doane, 1960; Gill, 1960). Studies in other species failed to reveal increased volumes following ovariectomy (Day, 1943; Kaiser, 1949). In view of the small proportion of cases with excessively large corpora allata in the series described in the present paper and their restriction to a certain age group, it seems reasonable to suggest that in the investigations quoted above castration effects may have escaped detection. This was certainly the case in two previous papers dealing with *Leucophaea* (Scharrer, 1946; Lüscher and Engelmann, 1955).

On the other hand, species differences have to be taken into consideration. This refers not only to volumetric responses, but to the manner in which these come into effect. For example, in two Diptera, *Calliphora* (Thomsen, 1942) and *Lucilia* (Day, 1943), the higher volume in the corpora allata of castrates is due to an increase in cell size, but not in cell number, whereas in *Leucophaea* an increase in cell number, as well as cell size, in conjunction with glandular activation has been definitely established.

The possible reasons for the low frequency of corpora allata showing pronounced castration effects in our present material have been discussed earlier in this paper. The same interpretation may apply to other species for which a considerable overlap between the volumetric values of gonadectomized and normal specimens has been reported (Thomsen, 1942; Bodenstein, 1947). Similarly, according to Doane (1960) [4], the hypertrophy of the corpus allatum in the sterile mutant adp/adp of *Drosophila* is an unstable condition. These observations, in addition to the present more detailed study, make it seem unlikely that the corpora allata of ovariectomized females stay active indefinitely as was postulated for *Leucophaea* by Lüscher and Engelmann (1955).

The role of the PAS-positive, saliva-resistant granules observed in various tissues of *Leucophaea* is still uncertain. The discussion of their possible significance must take into account the following points:

(1) Cytoplasmic inclusions whose histochemical characteristics suggest that they belong to the category of glycoproteins are not restricted to the corpora allata, but occur in a variety of other tissues. Among these, the fat body, the musculature, and the nervous system have been tentatively explored in this study. The tests applied so far are too limited to permit any conclusions regarding the extent of chemical relationship among these cell inclusions. The granules occur in both sexes. Their numbers show some individual variation, presumably in relation to different physiological states. It seems possible that some of them, *e.g.*, in the corpus allatum or in the neurosecretory cells, are glycoprotein hormones or their precursors comparable to those of the delta cells of the vertebrate adenohypophysis.

(2) Attention should be focused on the fact that these "glycoprotein" granules increase in number and size in the corpora allata of gonadectomized females, as compared with normal controls. Concomitantly, the fat body, musculature, and

[4] We wish to thank Dr. W. W. Doane, Yale University, for permitting us to read her thesis before its publication. Part of it has appeared in print since the present paper went to press. See: Doane, W. W. 1960. Developmental physiology of the mutant *female sterile (2) adipose* of *Drosophila melanogaster*. I. Adult morphology, longevity, egg production, and egg lethality. J. Exp. Zool., **145**, 1–21; and Doane, W. W. 1960. Developmental physiology of the mutant *female sterile (2) adipose* of *Drosophila melanogaster*. II. Effects of altered environment and residual genome on its expression. J. Exp. Zool., **145**, 23–41.

central nervous tissue of female castrates show conspicuous deposits of this material, whereas it may be almost absent in normal animals. Furthermore, the tissues examined show a certain uniformity as to the degree of their response. A given animal with a particularly high content of inclusions in the fat body also has proportionately more of them in the other tissues involved than does another animal in which these changes are altogether less conspicuous. Another site showing castration effects of this kind is the hemolymph, but their analysis must await future study.

By contrast, male castrates do not differ from intact males with respect to the amounts of this material present in the various tissues mentioned. This difference between the sexes represents an additional illustration of an already known situation, *i.e.*, the apparent absence of a corpus allatum-gonad axis in male *Leucophaea*. Thus, the data in castrate and normal males can hardly contribute to the understanding of the functional relationships among the "glycoprotein" granules in different tissues. Our interest, therefore, centers on the more dynamic situation in the females.

Here, the marked increase of these inclusions after gonadectomy, as well as the parallelism in the response of several different types of tissue, suggests a common denominator. Obviously, the appearance of these castration effects is related to the absence of the ovaries.

One possible explanation of the phenomenon is that the increase in the number and size of these cytoplasmic inclusions in animals without ovaries is an expression of a metabolic change (see, for example, Sägesser, 1960). Perhaps "precursor materials" (Pfeiffer, 1945), normally intended for yolk production, become deposited in abnormal quantities in tissues of castrate females. If this were true in the case of *Leucophaea*, allatectomized females, in which the ovaries and accessory sex glands are arrested (Scharrer, 1946), should show an increase in cell inclusions in the fat body, musculature, etc. comparable to that in castrates. Preliminary exploration of such animals, as well as female castrates subjected to prolonged starvation, suggests that the cell deposits in question are not, or at least not exclusively, surplus metabolites and that a role of the ovary more specific than that of "depository" for yolk material may be involved. Perhaps the castration effects reported are related to the absence of an ovarian hormone, an interpretation which is also strengthened by recent observations in *Drosophila* (Doane, 1960).

The effects of gonadectomy on the corpora allata in female insects discussed here show certain parallelisms among crustaceans and vertebrates; in these both sexes are involved. Parasitic or surgical castration in isopods, in which no organ analogous to the corpus allatum is known, leads to hypertrophy of the sinus glands (Yamamoto, 1955, Oguro, 1960). In mammals, such as the rat, the absence of the gonads elicits changes in the anterior pituitary indicative of glandular hyperfunction. Among such castration effects have been reported not only hypertrophy and increased gonadotropin content, but also characteristic changes in the morphology and cytochemistry of the gonadotrophs resulting in "castration cells" (see Hellbaum and Greep, 1940; Ladman and Barrnett, 1956; Takewaki, 1956). It is of particular interest that these cells, like the corpus allatum cells of ovariectomized *Leucophaea*, contain more glycoprotein than those of normal controls (Catchpole, 1949/50; Purves and Griesbach, 1955).

Summary

1. The corpora allata of *Leucophaea maderae* display an impressive capacity to respond to alteration of the internal milieu resulting from ovariectomy. Compared with the periodic structural changes of these glands in the course of normal reproductive cycles, the morphological characteristics of the corpora allata of castrate females are quite unpredictable. There is considerable spread from low organ volumes indicating glandular inactivity to excessively high values so far unmatched in non-castrate animals.

2. Only a small proportion of ovariectomized animals have abnormally large corpora allata, and these are confined to an intermediate period of the adult life span. This suggests that their growth response lacks permanency. Its apparent cause is the persistent absence of feed-back stimuli from the ovaries. The reasons why this castration effect on the corpus allatum eventually subsides are unknown.

3. The rise in relative and absolute cytoplasmic content with increasing organ size, observed earlier in normal animals, applies equally to gonadectomized females whose corpora allata at the time of fixation did not exceed the maximum of the control values. Above this level, the growth pattern differs in that no further drastic change in nuclear-cytoplasmic ratio occurs, *i.e.*, nuclear numbers and cytoplasmic content increase at the same rate.

4. Compared to those of normal controls, the corpora allata of castrate females contain progressively increasing amounts of saliva-resistant PAS-positive granules, probably glycoprotein in nature. A conspicuous accumulation of comparable cytoplasmic inclusions also occurs in various other tissues, such as the fat body, musculature, and nervous system. The functional relationships of these variously located inclusions are still uncertain, but the parallelism in their frequency suggests a common denominator. Whether they accumulate because of the absence of an ovarian hormone, or merely in response to metabolic changes following castration, is as yet undetermined.

5. In contrast to females, male castrates of *Leucophaea* do not differ significantly from normal controls as to the volume and cytoplasmic content of their corpora allata. The same applies to the presence of PAS-positive material in various tissues. This difference between the sexes in response to castration is undoubtedly related to the absence of a corpus allatum-gonad axis in male *Leucophaea*.

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INTRA- AND INTERSPECIFIC DIFFERENCES IN RATE OF OXYGEN CONSUMPTION IN GOBIID FISHES OF THE GENUS GILLICHTHYS [1]

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Many investigators have searched for adaptive physiological differences between intraspecific units. The growing literature has been reviewed a number of times (Thorpe, 1940; Timofeef-Ressovsky, 1940; Dobzhansky, 1951, Ch. 6; Prosser, 1955, 1957; Dehnel, 1955; Moore, 1957).

Recently attention has turned to physiological variation in relation to latitudinal distribution (Roscoff, Station Biologique, 1957; Vernberg 1959a, 1959b; Vernberg and Tashian, 1959). The results from most of the past research of this type cannot be applied to studies of intraspecific divergence (Prosser, 1957). Unfortunately, the early workers either did not consider thermal acclimation (Bullock, 1955, for review), or purposely acclimated the animals to the temperatures at which they were found (*e.g.,* Rao, 1953). Thus latitudinal and acclimation effects have not been clearly separable. Developmental rates of embryos from latitudinally separated populations (Dehnel, 1955) may be independent of latitudinal difference in environment, but only if temperature at the time of yolk formation is without effect on the metabolism of the yolk-consuming embryo. To appreciate genetically adaptive divergence in physiology as a function of latitude, the animals must be acclimated to, and compared at, the same temperatures.

The present study was undertaken to establish the degree of physiological divergence, if any, between widely separated populations of *Gillichthys mirabilis* Cooper. The results of an extensive morphological examination of several populations of this species, and of *Gillichthys seta* (Ginsburg), (Barlow, 1961, and unpublished data) constitute the point of departure for the program at hand. Oxygen consumption, a highly integrated metabolic phenomenon, was the physiological system chosen for the investigation.

[1] A revised portion of the dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of California, Los Angeles. Procedural details, and further figures and tables, are available in the dissertation.

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Material and Methods

Gillichthys seta doubtless is the species most closely related to *G. mirabilis* (Barlow, 1961). In occurrence, *G. seta* is restricted to the northern part of the Gulf of California. It is found only along the exposed coast in the highest intertidal zone among the streams and pools of rocky reefs.

The habitat of *G. mirabilis* is the intertidal region of coastal sloughs. Typically, this is a muddy region exposed to air at low tide. Some of the fish remain at low tide in crab burrows, or the likes, and breathe air; *G. seta* also is capable of aerial respiration.

The distribution of *G. mirabilis* embraces several faunal regions. The northmost populations in central California co-exist with a low-boreal fauna; the southmost populations in the Gulf of California occur within a tropical Panamic region; and the northern part of the Gulf of California, where *G. mirabilis* is also found, has a depauperate, mixed fauna. Thus *G. mirabilis* is arranged in disjunct populations over three roughly parallel, north-south, coast lines.

Distribution along a north-south coast ordinarily corresponds to a temperature gradient, warmer to the south. This applies to the Pacific Coast, and in the winter to the Gulf of California. In the late summer, however, the gradient in the Gulf is either reversed, cooler to the south, or the temperatures are everywhere about the same. As a result, the annual range of mean temperatures in the Gulf of California increases from south to north. The climate of the Salton Sea continues this trend of increased temperature extremes to the north.

In 1930 *G. mirabilis* was introduced into the Salton Sea. The parental stock came from San Diego Bay, just a few miles south of the slough at Los Peñasquitos. The fish have become well established in the Salton Sea in spite of the many peculiar features (Carpelan, 1958) of this environment. Judging from the number of aberrant specimens, the Salton Sea is a marginal habitat for *G. mirabilis*.

Treatment of the animals

The fish were held in sea water in 20- and 40-gallon tanks. The water was aerated, and filtered continuously, and was replaced with fresh sea water two to three days prior to each experiment.

The fish were fed exclusively on fresh squid, which they ate voraciously. The number of days between feedings, as well as the feeding time, were purposely kept irregular to minimize cyclic behavior. They were not fed just prior to an experiment; this interval was adjusted according to the size of the fish and the acclimation temperature (at 10°, intervals of seven to twelve days, and at 24°, of two to four days; longer periods for longer fish), so that the fish when tested were flat-bellied, and production of fecal material was low.

The minimum acclimation time was two weeks at 24° ± 0.5°, and four weeks at 10° ± 0.05°. Experience indicated acclimation was complete sooner than that, especially at 24°. In changing fish from one temperature to the other, they were held for one week at the intermediate temperature of 17°, as was done in the experiments by Brett (1952). Illumination was continuous. For both species, only male or immature fish were used.

Apparatus

Special chambers for the Warburg apparatus were constructed, from ground-glass fittings, to accommodate the elongate fish, and in various sizes (45-cc., 85-cc., and 250-cc.) to maintain the proper ratio between fish and chamber volumes.

Reduction of the oxygen tension in the chamber was circumvented by installing an oxygen well, a collapsible bag made of No. 6, 100 gauge, Saran film, on the air inlet of the manometer. Each time the stopcock was opened to balance the manometer fluid, pure oxygen, at atmospheric pressure, replaced that utilized.

Experimental procedure

Each fish was blotted gently, then weighed in the chamber. Filtered sea water was added in an exact amount, equal to about 5% of the flask volume (enough water to wet the belly and the floor of the mouth of the fish). The flask was then flushed with oxygen, potassium hydroxide solution was quickly introduced into the cup, and the flask was affixed to the manometer. The bath was darkened and the manometers were stationary. Temperatures were controlled to within $\pm 0.025°$. The initial temperature was always the same as the acclimation temperature of the fish.

The animals were placed in the bath in the evening, and the first reading was taken twelve hours later between 0830 and 0930 hours. To determine the adjustment period, however, readings were commenced 30 minutes after placing the fish in the bath.

Oxygen uptake was recorded at the acclimation temperature during the thirteenth and fourteenth hours in the chamber. The bath temperature was then steadily raised 7° during one hour. Two hours elapsed at the new temperature before the oxygen uptake was recorded during another two-hour period, the eighteenth and nineteenth hours in the flasks.

Excepting the fish from Puerto San Carlos, no animal was used a second time in the same series of acclimation experiments.

The respiratory rates were compared at 10° and 17° for four populations of *G. mirabilis* acclimated to 10°. From north to south, the fish were from San Francisco Bay (Alviso Ponds), latitude 37° N., in central California; Los Peñasquitos, latitude 33° N., in southern California; the Salton Sea, latitude 33° N.; and San Felipe ("El Marino" slough), latitude 31° N., in the northern part of the Gulf of California. Though the Salton Sea and Los Peñasquitos are on about the same latitude, the warmer climate of the Salton Sea is considered here to be more "southern." San Felipe is not much farther south than southern California but is in a region with a warmer over-all climate.

Fish from the same four places were also acclimated to 24° and tested at 24° and 31°. A group of fish from Puerto San Carlos, latitude 28° N., near Guaymas in the central part of the Gulf of California, was included in the latter comparison. The *G. mirabilis* from San Carlos were small when obtained. In spite of growing well in aquaria, the distribution of sizes of the San Carlos fish included much smaller animals than in the other samples. Consequently they were not included in the analysis of co-variance within the species *G. mirabilis*. Comparisons between *G.*

mirabilis and *G. seta* were based on experiments with *G. mirabilis* from San Carlos, and *G. seta* from Puertecitos, latitude 30° N.

Treatment of the data

The decision to accept or reject the stated statistical hypothesis was based on the 5% critical level in order to minimize type II errors (Hoel, 1954, p. 33). Acceptance of a hypothesis such as equal means does not imply the populations in question were proven to be the same.

The mean rate of oxygen consumption was employed throughout. Minimum rates were only 3% to 5% below the mean.

Regression lines were fitted by least squares to the logarithms of the experimental data. It was convenient to express this rectilinear relationship as oxygen consumption per unit weight, instead of per total weight. The slope is then negative, as indicated in the expression: Rate $= a\ \mathrm{Wt}^{-b}$, where a is the rate when weight (Wt) equals one, and b is the regression coefficient.

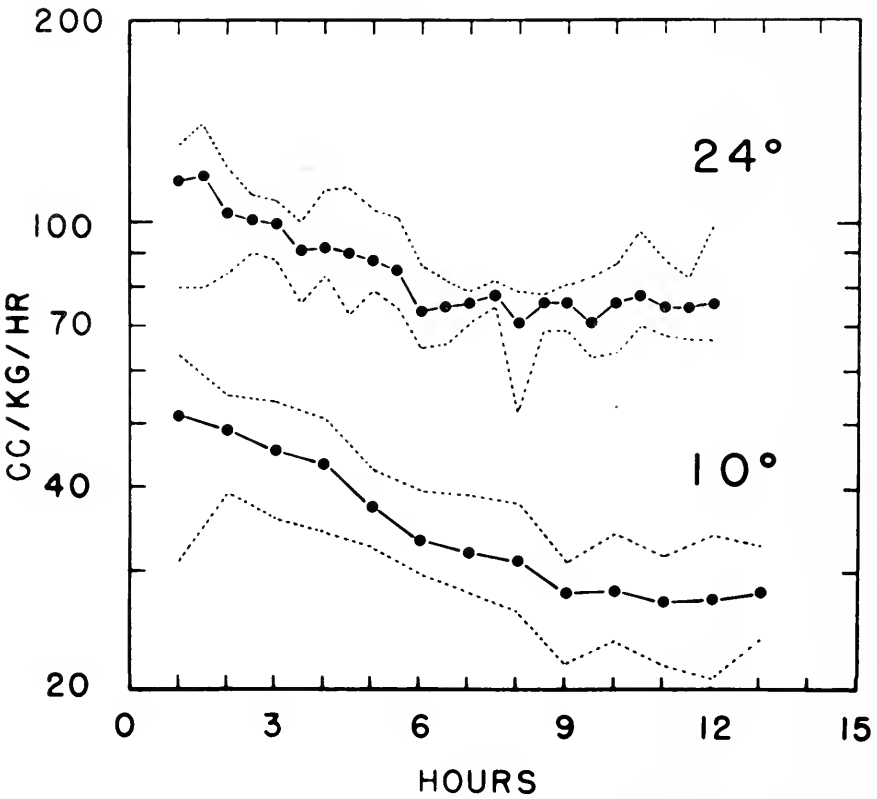

FIGURE 1. Time to steady rate of oxgen consumption for *Gillichthys mirabilis* from Los Peñasquitos. Solid lines connect means and dashed lines connect ranges. Individual rates were adjusted to the eight-gram intercept using regression coefficients of −0.28 for the 10° group and −0.56 for the 24° group (n = 5); the procedure is discussed in Roberts (1957).

Results

Uniformity of results

The rate of oxygen consumption remained the same from month to month in a given population. Fish from the Salton Sea, tested in January, April, and May, showed no perceptible differences. And fish from San Carlos re-tested after periods of growth, continued to manifest rates appropriate to their earlier determined regression lines (Fig. 4).

The oxygen added to the chambers at the beginning of each experiment apparently did not disturb the fish. Controls with air, instead of oxygen, metered into the chambers showed no differences.

At 10° the initial rate of oxygen uptake was high, but reached a constant level within nine to ten hours (Fig. 1). The adjustment time at this temperature was independent of the size of the fish relative to the volume of the chamber.

The initial rate was also high at 24°. For all fish in small or medium size chambers, adjustment was achieved within six to ten hours (Fig. 1). The adjustment time in the largest chambers, however, varied depending on the ratio of the fish size to chamber volume. In the large chambers oxygen consumption was reasonably regular within eight to ten hours if the fish were larger than 15 grams. Because of this, only fish equal to or larger than 20 grams were used in large chambers at 24°.

Wells (1935a) investigated the adjustment time of *G. mirabilis,* but in water instead of air. The results of his four experiments show excellent agreement with

Table I

Relation of oxygen consumption to body weight in Gillichthys mirabilis *and* G. seta

Temperature	10° C.			17° C.		
Parameter	n	a	−b	n	a	−b
G. mirabilis						
San Francisco	46	36.9	0.186	34	68.6	0.138
Los Peñasquitos	53	43.7	0.277	43	86.2	0.286
Salton Sea	46	31.0	0.160	36	62.6	0.193
San Felipe	35	57.1	0.346	35	79.5	0.247
Temperature	24° C.			31° C.		
Parameter	n	a	−b	n	a	−b
G. mirabilis						
San Francisco	36	254	0.577	36	300	0.526
Los Peñasquitos	49	256	0.560	40	331	0.530
Salton Sea	36	310	0.626	36	419	0.622
San Felipe	34	246	0.568	34	351	0.613
San Carlos	54	248	0.451	42	318	0.487
G. seta						
Puertecitos	24	177	0.289	24	289	0.257

TABLE II

Results of co-variance tests on differences in rate of oxygen uptake in Gillichthys mirabilis *and* G. seta *Entries refer to* G. mirabilis *unless stated otherwise.* F = *F ratio, d.f.* = *degrees of freedom,* $_{0.95}$ = *5% critical value*

	Temperature	F	d.f.	$F_{0.95}$	Decision
Hypothesis: means same after adjustment for body weight					
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	10° C.	8.50	3,175	2.66	Reject
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	17° C.	6.58	3,143	2.67	Reject
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	24° C.	1.93	3,150	2.67	Accept
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	31° C.	5.46	3,141	2.67	Reject
G. mirabilis (San Carlos)/*G. seta* (Puertecitos)	24° C.	28.6	1,76	3.97	Reject
G. mirabilis (San Carlos)/*G. seta* (Puertecitos)	31° C.	1.07	1,63	3.99	Accept
Hypothesis: Same slope within the groups					
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	10° C.	2.65	3,172	2.66	Accept
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	17° C.	3.58	3,140	2.67	Reject
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	24° C.	0.68	3,147	2.67	Accept
San Francisco/Los Peñasquitos/Salton Sea/San Felipe	31° C.	2.25	3,138	2.67	Accept
G. mirabilis (San Carlos)/*G. seta* (Puertecitos)	24° C.	7.65	1,74	3.97	Reject
G. mirabilis (San Carlos)/*G. seta* (Puertecitos)	31° C.	12.6	1,62	4.00	Reject

the curves in Figure 1, when recalculated in cc. O_2/kg./hr. and plotted on semi-logarithmic graph paper.

For all groups, the mean rate of oxygen consumption remained consistent over a 12-hour period after adjustment, and no cyclic phenomena were evident. During the readings the rate of uptake of each fish fluctuated, resembling fluctuations noted for other fishes by Spoor (1946) and by Pritchard (1955).

Influence of size on oxygen consumption

As is so often the case (examples in Dehnel, 1960), the smaller the animal, the greater its oxygen consumption per unit weight. In *G. mirabilis* and in *G. seta* this relationship between size and metabolism is more pronounced than in most fishes, the values of b being large (see Discussion). Moreover, both inter- and intraspecific differences in the estimate of b were found in the genus *Gillichthys*.

The slopes of the lines at 10° were nearly the same for the various populations of *G. mirabilis*, though the differences approached the 5% level of significance (Tables I, II). The slopes for the populations steepened from the northmost (−0.19) to the southmost (−0.35), excluding the Salton Sea group (Table I, Fig. 2), but these differences were of doubtful significance.

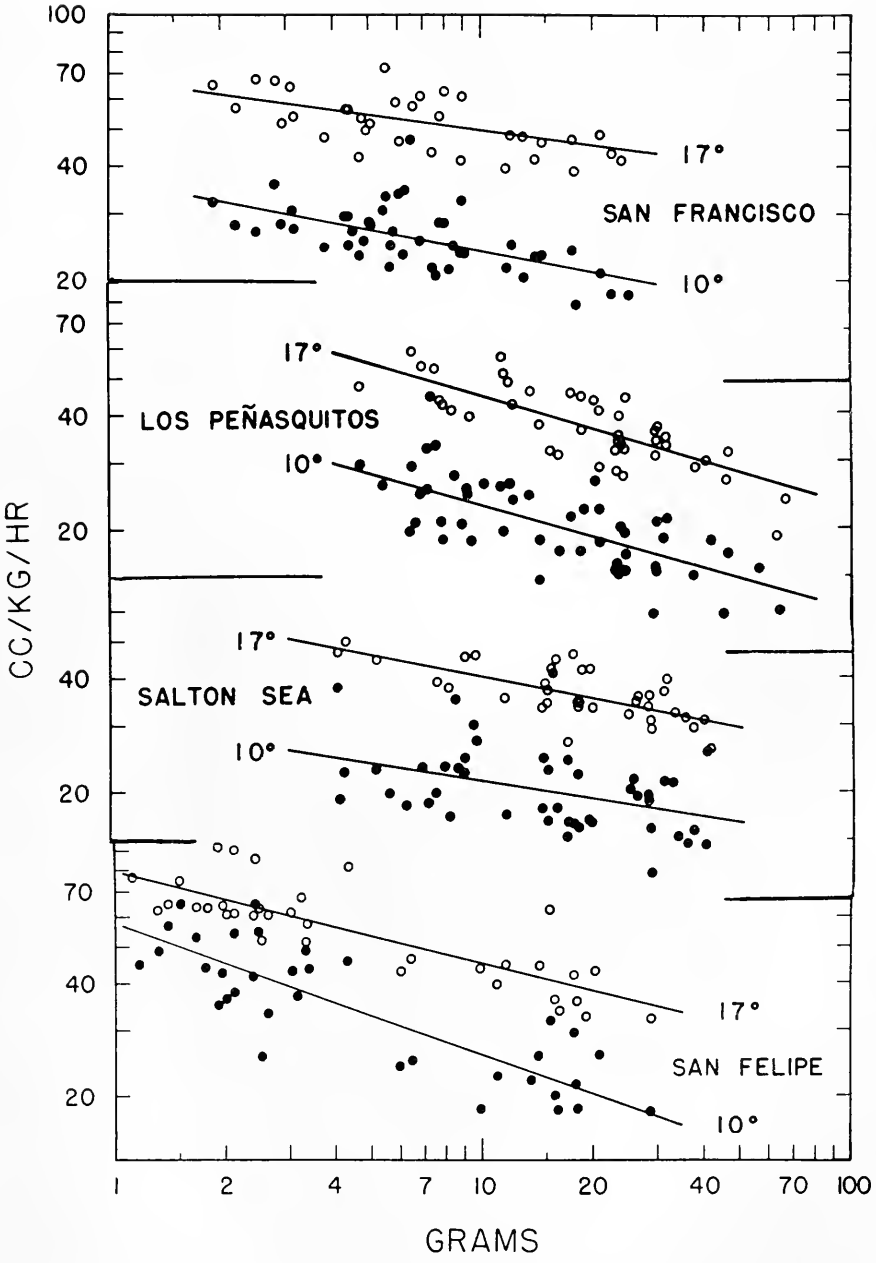

FIGURE 2. Relation between oxygen consumption and body weight of *Gillichthys mirabilis* from San Francisco, Los Peñasquitos, Salton Sea, and San Felipe at 10° (closed circles) and 17° (open circles).

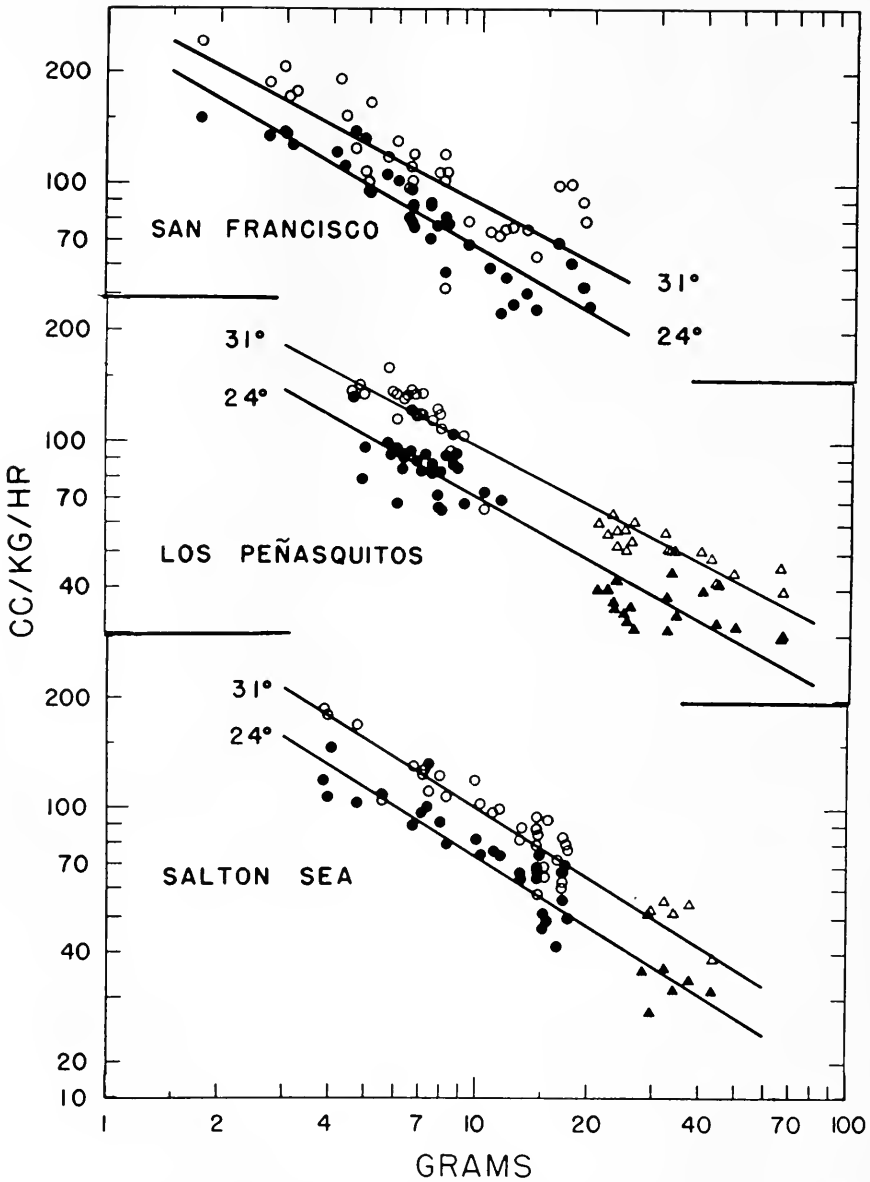

FIGURE 3. Relation between oxygen consumption and body weight of *Gillichthys mirabilis* from San Francisco, Los Peñasquitos, and Salton Sea at 24° (closed symbols) and 31° (open symbols); in small and medium chambers (circles), and in large chambers (triangles). Chamber size not indicated for San Francisco.

At 17° the slopes for the various populations were significantly different (Table II), though the relationship between them was similar to that observed at 10° (Table I, Fig. 2). The value of b was low for fish from San Francisco and Salton

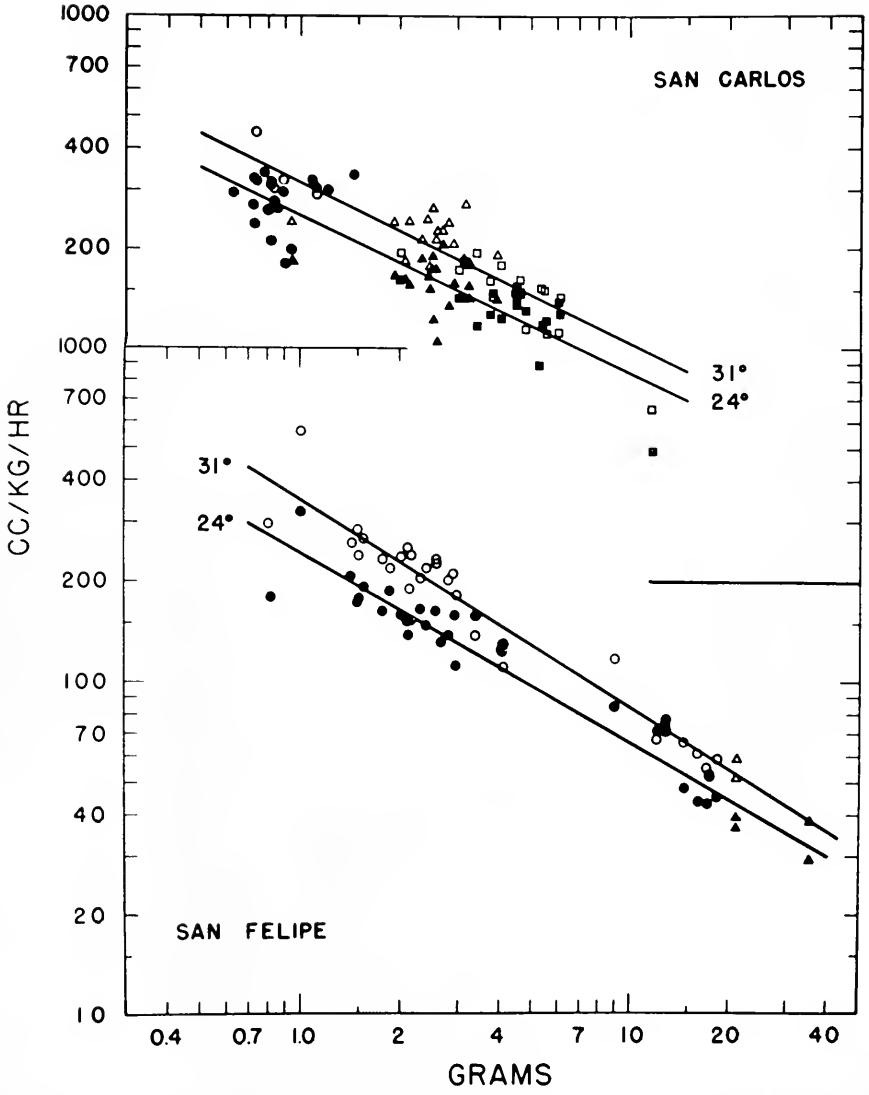

FIGURE 4. Relation between oxygen consumption and body weight of *Gillichthys mirabilis* from San Felipe at 24° (closed symbols) and 31° (open symbols); in small and medium chambers (circles), and in large chambers (triangles); and of *G. mirabilis* from Puerto San Carlos, at 24° (closed symbols) and 31° (open symbols), in small and medium chambers (not indicated); tested on 21 Feb., 1958 (circles), 26 March, 1958 (triangles) and 15 May, 1958 (squares).

Sea, and higher for those from San Felipe and Los Peñasquitos. Thus the increase in temperature sharpened the differences but did not alter their direction. No trend with latitude was noted for the slopes at 17°.

The values of the constant b were statistically uniform at 24° (Tables I, II;

Figs. 3 and 4). The fish from San Carlos were not included in the statistical analysis, but the slope of their data (−0.45) was lower than that of the four principal populations.

Even the stress of a higher temperature, 31°, failed to reveal significant differences between the slopes (Tables I, II; Figs. 3 and 4). The value of *b* for the San Carlos fish (−0.49) diverged from the other groups in the same direction as at 24° in that it was lower again.

Comparisons of the slopes obtained from *G. seta* and from *G. mirabilis* (San Carlos), showed that the two species differed at 24° and at 31° (Tables I, II; Figs. 4 and 5). The separation at 31° was particularly noteworthy because at this temperature the mean rates of oxygen consumption (see below) were not significantly different for the two species.

Both at 24° and at 31°, the constant *b* for young *G. mirabilis* (−0.45 and −0.49) was closer to that of *G. seta* (−0.24 and −0.26) than were the slopes for larger *G. mirabilis* (−0.56 to −0.63, and −0.53 to −0.62). Compared to adult *G. mirabilis,* the slope was lower for smaller *G. mirabilis,* and lower still for *G. seta.*

Mean rates of oxygen consumption

At 10° the mean rates of oxygen consumption of the four populations of *G. mirabilis* were significantly different (Table II, Fig. 2); one regression for all the data also was not statistically acceptable (statistical parameters for this test are not indicated in Table II). This divergence apparently was caused by the smaller fish.

Within the experiments at 17°, the mean rates of oxygen consumption of the four populations were not the same, nor was one regression adequate (Table II, Fig. 2). The divergence, however, seemed to be independent of fish size.

The means of the rates of oxygen consumption at 24° (not including the San Carlos sample) showed no differences, and the hypothesis of one regression line was just acceptable (Table II, Figs. 3 and 4). The larger fish from San Carlos fell on about the same regression as did the fish from the four other populations, whereas the smaller San Carlos fish seemed to lie slightly below this regression.

Under stress of a higher temperature, 31°, differences arose between the mean rates of consumption of the four populations, and one regression also was statistically unsatisfactory (Table II, Figs. 3 and 4). No trend between the means of populations was discernible. The rates of the smaller fish from San Carlos once more appeared to lie below the regression for the other groups.

The oxygen uptake of *G. mirabilis* from San Carlos was compared with that of *G. seta* from Puertecitos. At 24° the means were significantly different, but at 31° the average rates were the same (Table II).

Influence of temperature on oxygen consumption

Four sets of Q_{10} comparisons were made for respiratory rates. Two categories involved the acute changes 10° to 17°, and 24° to 31°. Another category was the comparison of rates at 10° and at 24° after acclimation, the chronic changes. Finally, the rates for fish acclimated to 10° but tested at 17° were contrasted with the rates for fish acclimated to 24° but tested at 31°. These relationships are summarized in Table III. Q_{10} was used only as a descriptive device.

TABLE III

Temperature coefficients (Q_{10}) *for* Gillichthys mirabilis *and* G. seta

	10° C. to 17° C.		24° C. to 31° C.	
	1 g.	10 g.	1 g.	10 g.
G. mirabilis				
San Francisco	2.42	2.84	1.27	1.50
Los Peñasquitos	2.64	2.56	1.45	1.59
Salton Sea	2.73	2.45	1.45	1.57
San Felipe	1.60	2.22	1.66	1.43
San Carlos	—	—	1.43	1.47
G. seta				
Puertecitos	—	—	2.01	2.24

	10° C. to 24° C.			17° C. to 31° C.		
	1 g.	10 g.	50 g.	1 g.	10 g.	50 g.
G. mirabilis						
San Francisco	3.97	2.09	1.33	2.87	1.52	0.97
Los Peñasquitos	3.53	2.22	1.61	2.62	1.75	1.32
Salton Sea	5.17	2.40	1.41	3.89	1.92	1.17
San Felipe	2.84	1.97	1.52	2.89	1.58	1.04

Rates used in the calculations of the Q_{10} values were obtained from intercepts of the regression lines. Most comparisons were made at the 10-gram intercept because this value was near the median of the size ranges for most samples. The temperature coefficients at the one-gram intercept, too, were listed to facilitate comparisons with other published results. Vagaries of the slope due to chance can be magnified by comparisons made outside the size distribution of the fitted lines, and may lead to erroneous conclusions.

When the temperature was raised from 10° to 17°, the Q_{10} of fish acclimated to 10° had a value greater than 2.0 in all populations at the 10-gram level. This was appreciably higher than the Q_{10}'s recorded for animals acclimated to 24°.

The Q_{10}'s for *G. mirabilis* (10-gram fish) from San Felipe were almost always the lowest for that species. The other Gulf population of *G. mirabilis,* from San Carlos, also had a commensurately low coefficient for the one temperature interval at which it was tested. Thus, the *G. mirabilis* from the Gulf of California had lower temperature coefficients than those from the Pacific Coast, or Salton Sea.

Within the north to south sequence of populations in California (San Francisco, Los Peñasquitos, and Salton Sea), the predominant trend was higher coefficients to the south. But for the acute change in temperature among the 10° acclimation groups, the trend was just the opposite. In general, the values for Los Peñasquitos and Salton Sea were closer to one another than to that for San Francisco, particularly for acute changes.

Gillichthys seta clearly had a higher temperature coefficient than any population of *G. mirabilis* at 24° (Table III). No *G. seta* were tested at 10°.

An additional set of experiments was performed to establish whether or not the

high chronic Q_{10}'s observed in the cold-acclimated fish could be ascribed to the removal of cold depression. The three populations of *G. mirabilis* from California acclimated to 10° were tested at 3.5° intervals from 10° to 27.5°. The program was the same as described in Material and Methods.

Cold depression was not a factor in the temperature response of the cold-acclimated fish. As seen in Figure 6, the Q_{10}'s remained approximately the same between 10° and 24°.

Prosser (1958) has presented a classification of types of metabolic responses to temperature changes in relation to cold- and warm-acclimation. To provide such a comparison, data from a warm-acclimated (24°) group of *G. mirabilis* from San Francisco were included in Figure 6. This curve, however, has been improvised from two different experiments, and requires an explanation.

The points representing rate of oxygen consumption at 24° and 31° were derived from the lines fitted to the results of the standard experiment (Fig. 3), and are reliable.

The values for 17° and 34.5° were obtained as follows. The fish in the respirometers were slowly (eight hours) cooled from 24° to 17°, held at 17° four hours, recorded two hours, then tested at 3.5° intervals up to 34.5°, following the usual program. The values at 17° regularly fell near the projected 24°–31° line. Warming the water to 20.5° and 24°, on the other hand, resulted in pronounced individual differences. These seemed to be of two types: (1) some of the fish manifested high Q_{10}'s, followed by a decrease in rate at a higher temperature, then an increase again at a still higher temperature, whereas (2) the others showed what could be called the anticipated regular increment. I have exercised my judgment and selected out those data that I believe to be reliable, *i.e.*, all the recordings from the San Francisco fish taken at 17° and 34.5°, but none in between.

The change in Q_{10} revealed by a comparison of the two kinds of curves in Figure

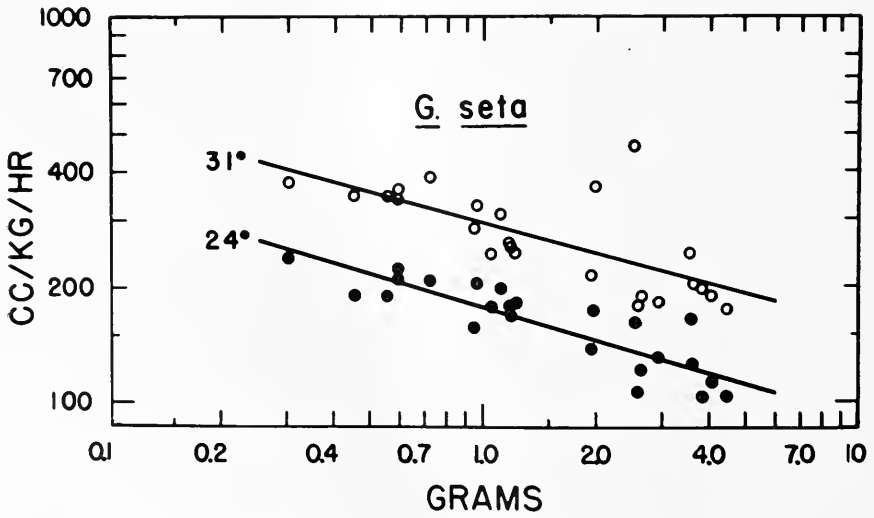

FIGURE 5. Relation between oxygen consumption and body weight of *Gillichthys seta* from Puertecitos at 24° (closed circles) and 31° (open circles).

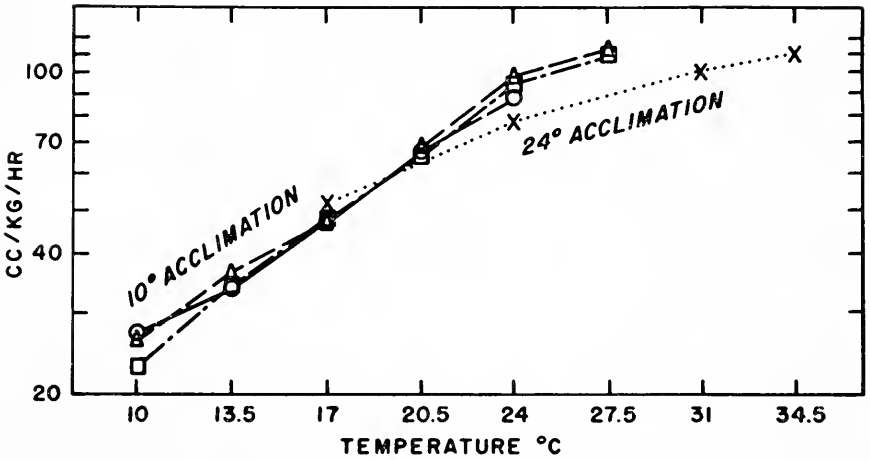

FIGURE 6. Influence of cold- and warm-acclimation on metabolic responses of *Gillichthys mirabilis* to temperature changes. Each point represents the mean rate of oxygen consumption for ten fish, weighing from 4 to 10 grams. Individual rates were adjusted to the 8-gram intercept using the appropriate values of *b* listed under 10° and 24° in Table I; the procedure is discussed in Roberts (1957). Acclimation to 10°: circle = San Francisco population, triangle = Los Peñasquitos population, square = Salton Sea population. Acclimation to 24°: X = San Francisco population.

6 would be classified in the system proposed by Prosser (1958) as Type III B, rotation counter-clockwise with cold acclimation. Clockwise rotation evidently is more common than counter-clockwise (Prosser, 1958). The curves for warm- and cold-acclimated fish intersect between 17° and 20.5°. The curve (not shown) for the warm-acclimated fish from Los Peñasquitos intersects the curves for cold-acclimated animals at 20.5°.

Evidently little or no translation is involved. This would be more apparent if the warm-acclimated fish had been tested over a lower temperature range. Incidentally, above 34° the oxygen uptake in many of the 24°-acclimated fish decreases, and between 35° and 37° many die (further experiments not reported here).

Influence of size on temperature coefficients

There was no apparent difference in Q_{10} between large and small *G. mirabilis* for acute temperature changes. But the temperature coefficients after acclimation were influenced greatly by the size of the fish involved. The chronic Q_{10}'s were strikingly higher in the smaller fish (Table III; Figs. 2 to 4). Apparently acclimation was more effective in larger fish. The 50-gram fish, acclimated to 24° and tested at 31°, commonly had lower respiratory rates than fish of the same size acclimated to 10° and tested at 17°. The size of the fish, therefore, is important in chronic, but not acute, metabolic adjustments.

DISCUSSION

Evidence in fishes for intraspecific differentiation of adaptive physiological systems has received infrequent but notable attention. Heuts (1947, 1956) described

the physiological adaptations to salinity and temperature in the embryos of three races of the stickleback, *Gasterosteus aculeatus*. Minamori (1957, and earlier papers cited there) studied in detail various aspects of developmental physiology in races of the spinous loach, *Cobitus taenia*. The races were arranged in a series according to temperature adaptation. The forms ecologically adapted to higher temperatures, tolerated higher temperatures and developed more slowly at intermediate temperatures than did the cold-adapted forms. Hart (1952) investigated geographic variation in heat tolerance in twelve species of fresh-water fishes. In three species, only, significant differences were apparent between subspecies. In field experiments, Wohlschlag (1957) discovered the sympatric, but ecologically separate forms of an arctic whitefish, *Coregonus sardinella,* had different rates of oxygen consumption. But these "forms" of *Coregonus* probably would be considered distinct species by Svärdson (1953). Guppies, *Lebistes reticulatus,* from inbred lines had different susceptibilities to high temperatures (Gibson, 1954). Under extreme environmental conditions some genotypes of carp, *Cyprinus* (sp.?), were more viable than others (Kirpichnikov, 1945).

Differences between populations, after acclimation, usually are attributed to genetic dissimilarities. The evidence for this thesis is indirect, but consistent (references in Introduction, and Fry, 1957a). The role of the environment in modifying the degree of reversibility, however, is not known.

The temperatures associated with early development allegedly can alter the thermal resistance of guppies, *Lebistes reticulatus* (Gibson, 1954). The thermal resistance of fish reared at 25° and at 30° was similar, but those cultured at 20° were less resistant. At the lowest temperature only two female guppies could be induced to give birth, and at the highest temperature mortality was appreciable. The fish reared at the high, and especially at the low, temperatures could have been selected for by the effects of the thermal environment. Other experiments in the same paper demonstrated different heat resistances in different genetic lines. Males and females also had dissimilar tolerances, and the sex ratios in the experiments on thermal history were not given. The influence of the early thermal experiences on the metabolism of adult poikilotherms remains, then, an open question.

The results reported here, nonetheless, are interpreted as though metabolic differences between fully acclimated animals from naturally occurring populations, indicate heritable dissimilarities.

Influence of size on oxygen consumption

Although the regression coefficients relating oxygen consumption to weight were not especially informative at the intraspecific level, they were most revealing for species relationships. The slope of the regression for the small species, *G. seta,* obviously was lower than that for the adults of *G. mirabilis* (24° acclimation). The disparity was less apparent when the data from adult *G. seta* were compared with those for sub-adult *G. mirabilis*. As noted below, the value of b (*i.e.*, $-b$) tended to decrease at small sizes in *G. mirabilis* (acclimated to 24° only). Morphologically, adult *G. seta* retain many characteristics which in *G. mirabilis* are juvenile (Barlow, 1961). The low regression coefficients for oxygen uptake in *G. seta* might also represent a retention of a juvenile feature.

Von Bertalanffy (1951) discussed the relation between regression coefficients

and phylogeny. He opined that some higher taxonomic categories could be characterized by the value of *b*. Apparently this does not hold for gobiid fishes since the slopes differ between species, and change with acclimation as well.

Certain difficulties were encountered with the data obtained from fish acclimated to 24°. The relationship between the logarithms of oxygen consumption and body weight was not strictly rectilinear over the size range of the experimental animals. There was a tendency for the negative value of *b* to decrease slightly in the smaller *G. mirabilis*, and also in the large adults, though less so. As Zeuthen (1955) suggested, it may be best to regard *b* as a tangent to some more complicated curve.

To complicate matters, the value of *b* in *G. mirabilis* acclimated to 10° was relatively constant over the entire size range of the experimental animals. The low temperature of 10° is a normal winter temperature for *G. mirabilis*, but surely it is suboptimal for this species in terms of activity and growth; the fish seem well suited to 24°. Possibly, the low temperature of 10° effects a physiological compensation for the unnatural continuous illumination. Or, at 10° metabolism is less affected by growth and activity. The values of *b* obtained from the cold-acclimated fish are more in accord with most of those reported for other fishes.

Zeuthen (1953) suggested that −0.22 probably is a representative value for *b* among fishes. Fry (1957b) cited −0.10 to −0.20 as usual values for *b*, but also noted five instances where values of from −0.33 to −0.50 had been reported. The value of *b*, −0.50 to −0.60 for *G. mirabilis* acclimated to 24°, then, is higher than that usually considered typical for fishes. The value of *b* (−0.15) reported by Scholander *et al.* (1953), was derived from a composite of several species. The data for some of the species, however, appear to show a slope greater than −0.15 (*Abudefduf saxatilus, Scarus croicensis*). Wohlschlag (1957) recorded a slope of −0.60 for a coregonid fish, though he attributed the high value to the small size of the sample. Recent investigations into other animal groups have revealed values of *b* well above −0.33 predicted from the surface volume relationship (references in Zeuthen, 1953; Mann, 1956; Dawson and Bartholomew, 1956; Vernberg, 1959a; Dehnel, 1960; Velma Vance, personal communication).

Mean rates of oxygen consumption

Physiological responses to stress may reveal differences when measurements within the temperature range to which the animals are adjusted might fail (Prosser, 1957). The means of the rates of oxygen consumption in *G. mirabilis* were the same only at 24°, which probably lies within its optimal temperature range. Differences were apparent when the temperature was raised to 31°. The lowest temperature, 10°, must have been stressful: activity was greatly reduced, although social behavior, frontal display by males, was observed. Fish acclimated to 17° were only slightly less active than those at 24°. Nonetheless, the sudden 7° increase in temperature from 10° to 17° probably stressed the metabolic system of the cold-acclimated fish.

While differences in mean rates of oxygen consumption at a given temperature appeared to indicate divergences between populations of *G. mirabilis*, the direction of the departures seemed to be irregular. At 10°, only, there may have been evidence for higher metabolic rates to the south in smaller fish. This was particularly noticeable in the animals from San Felipe, that had been tested in late spring. The

other populations had been examined during the winter. But if there had been a residual seasonal adjustment, the rates for the San Felipe group should have been low in comparison to the winter groups. As seen, just the opposite occurred.

The oxygen consumption of *G. mirabilis* is similar whether the fish are in or out of water. Though not measured in water, the rates reported here for aerial respiration of *G. mirabilis* agree with those found by Wells (1935a) for respiration in water of the same species, when allowances are made for differences in thermal acclimation. The rate of oxygen uptake of the Hungarian mud-minnow, *Umbra lacustris (krameri?)*, also is the same whether entirely aquatic, or a combination of aquatic and aerial (Geyer and Mann, 1939).

The rates of oxygen uptake in *Gillichthys mirabilis* and *G. seta* accord with the values reported for other species of air-breathing Gobiidae. The rates at 24.5° for *Periophthalmus vulgaris* (seven grams) and *P. dipus* (16 and 17 grams) given by Schöttle (1931) coincide nicely with the regression relating oxygen consumption to weight for *G. mirabilis*. The rate for *P. schlosseri* (110 grams) was about 100% higher than would have been expected for *G. mirabilis* of that size, but agreed well with the extension of the regression for *G. seta*. The data of Schöttle were reduced by 50% before making the comparisons because she recorded the rates during the first hour; the values for *G. mirabilis* decreased by about this amount during the 12-hour adjustment period.

Influence of temperature on oxygen consumption

The responses to temperature changes seemed to be of more adaptive significance than the absolute level of oxygen consumption. While the mean rates showed no interpretable differences, the disposition of the Q_{10}'s suggested a meaningful pattern.

The temperature coefficient data infer the following arrangement of populations: *G. mirabilis* from the north and central regions of the Gulf of California are alike, but differ from the other groups considered by having consistently lower Q_{10}'s; *G. mirabilis* from San Francisco Bay are similar to, but somewhat apart from, the other two populations in California in that their Q_{10} is highest at low temperatures, but relatively low at high temperatures; *G. mirabilis* from the Salton Sea and Los Peñasquitos show a correspondence when responses to acute changes are compared, but are dissimilar when temperature coefficients of acclimated rates are considered.

Temperature coefficients generally are higher with adaptation to higher temperatures (but see Dehnel, 1960): northern species or forms usually have lower coefficients than their southern counterparts (Rao and Bullock, 1954; Minamori, 1957; Heuts, 1956; Tashian, 1956; Dehnel, 1955). In some respects *G. mirabilis* was just the opposite: warm-acclimated fish had low Q_{10}'s in all the groups, and cold-adapted ones had high Q_{10}'s. A further inconsistency was that the Q_{10}'s were higher in northern populations when the fish were cold-acclimated. Certain data, however, were in accord with the hypothesis of higher Q_{10}'s in more southern populations. Warm-acclimated *G. mirabilis* from the Pacific Coast and Salton Sea had higher Q_{10}'s to the south. But the two Gulf of California populations had even lower coefficients than the northmost Pacific Coast population, San Francisco. Certainly the Gulf habitats experience much higher maximum temperatures and a greater range, too, than do those in San Francisco Bay. The temperature patterns in the Gulf and on the Pacific Coast are not equatable.

The most obvious assumption is that the low sensitivity to temperature changes observed in *G. mirabilis* from the Gulf of California is an adaptation to the more variable climate there. Yet *G. seta* from the upper Gulf lives in a habitat subject to even greater thermal oscillations; and the Q_{10}'s for *G. seta* are higher than for any population of *G. mirabilis* under the same conditions. This may not be a fair comparison because it involves another species with different behavior and ecology.

The adaptive significance of the nature of the Q_{10}'s in *G. mirabilis*, and in *G. seta*, cannot be appreciated until more information is at hand, both with regard to the general and the specific aspects of the problem. It might be presumed, though, that within the species *G. mirabilis* low sensitivity to temperature change is an adaptation to higher temperatures, and high sensitivity an adjustment to lower temperatures. This is at variance with the conclusions of Scholander *et al.* (1953) which were based on interspecific comparisons, and also with the hypothesis put forth by Rao and Bullock (1954).

Influence of size on temperature coefficients

The few available data for fishes indicate that size affects Q_{10} in different ways depending on the particular species, and probably on the experimental procedures. The temperature coefficient was said to be independent of size in goldfish, *Carassius auratus* (Fry and Hart, 1948). Within a given race of the loach, *Cobitis taenia*, temperature coefficients of embryos were independent of egg volume (Minamori, 1957). At low temperatures size had no role in the determination of Q_{10}'s in the killifish, *Fundulus parvipinnis*, but the smaller fish were more sensitive at higher temperature intervals (Wells, 1935b; re-evaluated on the basis of relative changes). In another cyprinodontid fish, *Cremichthys baileyi*, from a warm spring, small individuals were more sensitive to a temperature drop than were large ones (Sumner and Lanham, 1942). In the brook trout, *Salvelinus fontinalis*, size had little influence on the response to chronic changes in temperature when standard metabolic rates were compared, except at the highest temperature interval where small fish were more responsive. When active, however, the smaller trout had higher temperature coefficients than the larger ones (Job, 1955). Measured by opercular rates, large carp, *Cyprinus carpio*, were more responsive to long term (chronic) temperature changes than were small individuals (Meuwis and Heuts, 1957).

In *Gillichthys mirabilis*, and in *G. seta*, temperature coefficients were not size-dependent in short term (acute) temperature changes. The relatively greater increase in respiratory rate of small *G. mirabilis* acclimated to 24° as contrasted to 10° may indicate that large *G. mirabilis* are metabolically more homeostatic than small, though only when allowed time to adjust. Meuwis and Heuts (1957) noticed a similar relationship in the carp, except the smaller fish, not the larger, were more homeostatic.

Comparison of physiological and morphological findings

The two species involved, *G. mirabilis* and *G. seta*, are distinct but difficult to distinguish from one another (Barlow, 1961). Ichthyologists, unless experienced with this group, frequently have difficulties separating them. In many respects *G. seta* represents an extension of the morphological cline manifested by the widely

distributed populations of *G. mirabilis* (Barlow, unpublished data). Physiologically, however, the two species diverge where their distribution overlaps, the Q_{10}'s and the regression coefficients, *b*, showing marked character displacement.

Within the species *G. mirabilis*, the greatest structural differences between populations were for the most part discernible only by means of statistical analysis. The aberrant Salton Sea population, of course, was an exception, but need not disturb the thesis being put forward here. Furthermore, the morphological investigation involved the analysis of many characters, such as measurements of several body parts, counts of the various fins and the vertebrae, and extent and nature of squamation. Among the multitude of possible physiological parameters, only oxygen consumption was studied. Considering this, it is fair to say the magnitude of intraspecific variation in oxygen uptake was commensurate with that observed in the morphological study.

The pattern of divergence, the "species structure" of *G. mirabilis*, became apparent only when many intervening populations, in this case 17, were compared morphologically. It is questionable if much sense could have been made of the morphological data from only those five populations analyzed experimentally. Indeed, before the series of populations had been investigated, three nominal species had been proposed for the two under discussion, and the situation was not at all clear (Barlow, 1961).

Beyond this, it might be pertinent to ask if the experiments were designed properly to reveal meaningful differences in the respiratory rates. Perhaps more informative data could be obtained by acclimating the animals to a controlled regime of oscillating temperatures, simulating the natural situation. After all, the most obvious thermal dissimilarity in the locales involved is the range of temperatures experienced by each, not the mean. Constant temperature, not to mention illumination, is an unreal circumstance for the animals; the absence of these modulating influences might even be inimical to certain metabolic processes.

In closing, the lack of differences in the nature of the oxygen uptake at 24° should be emphasized. From numerous field observations, I believe this temperature is near the thermal optimum for the species *G. mirabilis* throughout its geographic range. The similarities at 24°, therefore, might have been expected. The dissimilarities in the populations were manifested as the temperature departed from this value. The environment of each population evidently is unique in its combination of various temperatures (especially maxima and minima), photoperiod, salinity, and so on. Probably the singular features of each habitat have stamped their character on the gene pool of the population, effecting a slight, though significantly adaptive, divergence in the metabolic response to temperature.

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Summary

1. Rate of oxygen uptake was determined for moist fish in a Warburg apparatus. Fish acclimated to 10° were tested at 10° and 17°; those acclimated to 24° were tested at 24° and 31°. Five populations of *G. mirabilis* were investigated (San Francisco Bay in central California, Los Peñasquitos in southern California, San Felipe in the northern Gulf of California, Puerto San Carlos in the central Gulf, and the Salton Sea). One population of *G. seta* (from the upper Gulf) was studied at 24°.

2. Rate of oxygen consumption per unit weight was plotted against body weight. The regression coefficient, *b*, differed between populations for *G. mirabilis* though not in an interpretable fashion. The value of *b* was low at 10° and 17° (−0.14 and −0.35), and high at 24° and 31° (−0.45 and −0.63). The slopes were much lower for *G. seta* at 24° and 31° (−0.29 and −0.26) than for *G. mirabilis*.

3. The mean rates of oxygen uptake were different for the populations of *G. mirabilis* at all temperatures except 24°. The average rate for *G. seta* at 24° was lower than that for *G. mirabilis*, but the rates were about the same for both species at 31°.

4. The temperature coefficients, Q_{10}, varied from population to population of *G. mirabilis* in what seemed to be a pattern. The coefficients were lower in groups from the Gulf of California at almost all temperature intervals. Within the Pacific Coast and Salton Sea populations, the Q_{10}'s for the northern population were highest at the low acclimation temperature, and lowest at the high acclimation temperature. For all groups, the Q_{10}'s were highest when cold-acclimated (2.2 to 2.8), and lowest when warm-acclimated (1.4 to 1.6). The coefficient for warm-acclimated *G. seta* was high (2.0).

5. There was no apparent difference in temperature coefficients between large and small *G. mirabilis* for acute temperature changes. The smaller fish manifested appreciably higher Q_{10}'s than did the larger fish, however, when rates were compared after acclimation.

6. The degree of intra- and interspecific differences in the nature of the oxygen consumption was roughly of the same order of magnitude as the morphological differences reported in another article.

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THE ABSENCE OF DAILY PHOTOSYNTHETIC RHYTHM IN SOME LITTORAL MARINE ALGAE

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A photosynthetic periodicity in algae has been reported by several workers in recent years. Investigations with natural populations of planktonic forms have shown a marked variation in the ability of these organisms to fix carbon dioxide at different times of day. The rate of photosynthesis is reportedly highest in the middle and late morning, and lowest in the late afternoon and evening, a difference of several fold occurring (Doty and Oguri, 1957; Yentsch and Ryther, 1957; Shimada, 1958). In the coenocytic alga *Hydrodictyon* it has been found that a photosynthetic rhythm established with light-dark cycles persists for two or three periods in continuous light, the period of the induced rhythm depending upon that of the previous light-dark regime; thus, it is in this case not an inherent but an imposed or conditioned rhythm (Pirson, 1957). In *Chlorella* it has been found possible to establish synchronous cultures by means of light-dark cycles (Tamiya, 1957; Sorokin, 1960). In this case the rate of photosynthesis varies with the age of the cells, younger cells in general showing a rate higher than that of older cells. In view of these observations it was thought desirable to determine whether some of the multicellular algae show a photosynthetic rhythm; therefore, the following experiments were carried out at the Hopkins Marine Station during June and July of 1960.

Materials and Methods

The algae utilized in this study were common littoral marine genera collected in the vicinity of Pacific Grove, California. The organisms were maintained in running sea water in a laboratory for a period of not greater than 24 hours prior to the start of the tests. The tissues were placed in one-liter jars completely filled with natural sea water and exposed for a period of one-half hour to incandescent light (2 bulbs) of an intensity approximating 800 foot candles. The temperature within the sampling jars was held constant at about 15° C. by submerging them in a battery jar through which sea water was circulated rapidly during the course of the light exposure. The sampling jars were turned around once during the exposure period to stir the contents. At the conclusion of the exposure, the oxygen content of the water within the sampling jars was determined by the Winkler technique, and the rate of oxygen production per gram fresh weight calculated. Values presented are for net oxygen production, uncorrected for respiration; however, since respiration is low in relation to photosynthesis at the light intensity used (about 10% as shown by occasional dark-bottle checks), variations in respiration would be small

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compared to total oxygen production. In these experiments the initial exposure was made in the middle of the morning between 9:00 and 10:30 A.M., the second exposure in the early afternoon between 12:00 and 1:30 P.M., and the third in the late afternoon between 4:30 and 6:30 P.M. The final exposure was made the next morning between 6:30 and 9:00 A.M., the same piece of tissue being used in all four exposure periods.

Two of the genera, *Ulva* and *Porphyra*, were subjected to cycles of 12 hours of white light followed by 12 hours dark for a period of four to ten days to see if a photosynthetic rhythm could be induced in the same manner as in *Hydrodictyon* and *Chlorella*. After the light-dark treatment, the rate of photosynthesis with continuous light and with intermittent light (5 minutes light, 5 minutes dark) was measured by affixing the tissue to a platinum electrode as described previously (Haxo and Blinks, 1950). An electrical potential (0.5 volt) was applied to the electrode, the rate of oxygen production being reflected in the current flow. The latter was recorded on a Varian Graphic Recorder. A flow of sea water around the tissue was maintained throughout this experiment. The light intensity was approximately 80 foot candles.

Results

The results of experiments with four of the genera tested for a naturally occurring diurnal rhythm are summarized in the accompanying table. It is clear that no rhythm such as that observed in the plankton was detected in the organisms tested here. In addition to the species listed in the table, the following organisms were tested and did not show any photosynthetic rhythm: *Prionitis lanceolata, Endocladia muricata, Gigartina papillata, G. canaliculata, Porphyra perforata, Rhodoglossum affine, Fucus furcatus, Coilodesme californica,* and *Egregia menziesii.* In some cases small variations of 5 to 10% were observed, but these are minor compared to the differences of several fold reported for the plankton and for *Hydrodictyon.* In a few cases the late afternoon value was significantly lower than that of the previous morning, but in these cases an even lower value was observed the next morning, indicating poor survival of the tissue rather than a rhythm.

The samples of *Porphyra* and *Ulva* subjected to the regular light-dark cycles also showed no subsequent photosynthetic rhythm. In the case of *Porphyra* the ability to photosynthesize in either intermittent or continuous light did not change during the course of the day. In *Ulva* the rate of photosynthesis was at first high on exposure to light after a dark period of several hours, gradually dropping to a lower steady level after about two hours of constant or intermittent illumination. The lower level was maintained as long as the illumination was continued, so that

Table I

Rate of oxygen production in cubic centimeters per hour per gram fresh weight

Organism	Morning	Early afternoon	Late afternoon	Next morning
Hesperophycus harveyanus	.15	.19	.19	.19
Chaetomorpha aerea	.35	.30	.38	.33
Ulva sp.	1.08	1.16	1.10	1.16
Iridaea flaccida	.34	.38	.35	.35

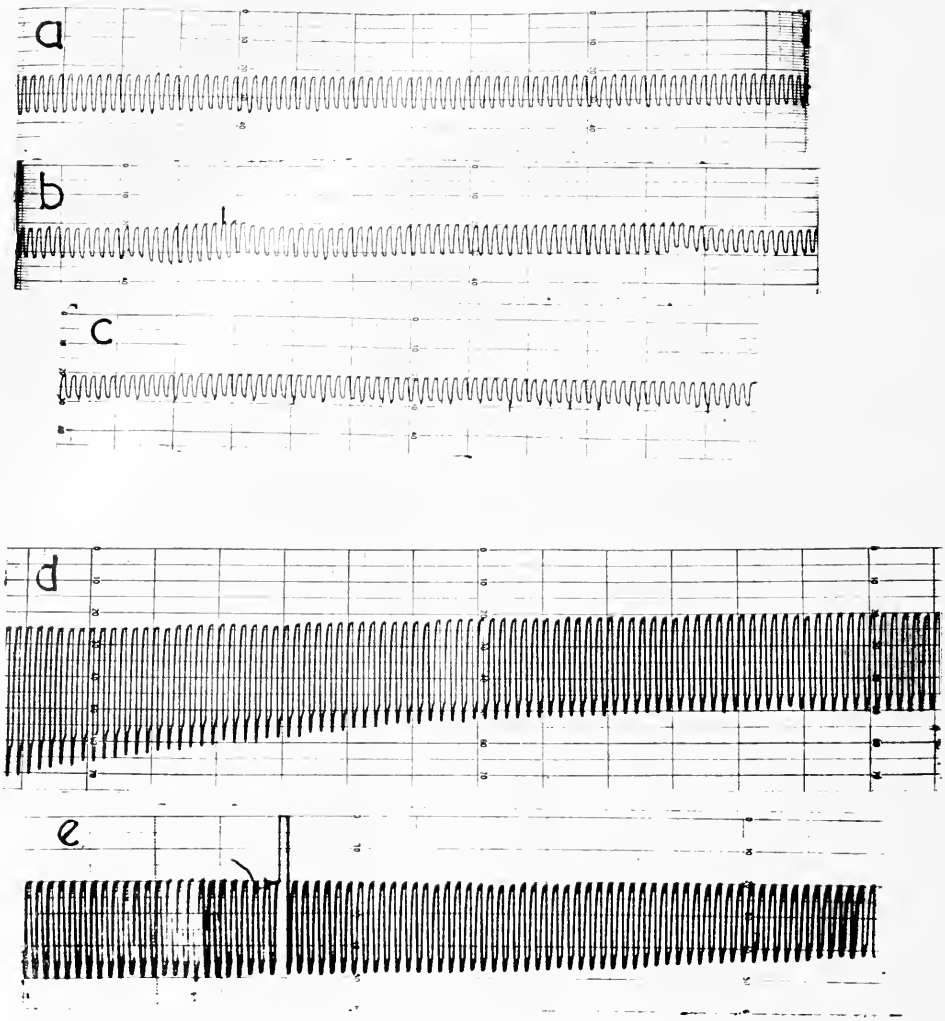

FIGURE 1. Recording of photosynthetic oxygen production by *Ulva* sp. in intermittent light (80 F.C.); 5 minutes light alternated with 5 minutes dark. a, b, and c constitute a continuous 40-hour sequence; d and e another 28-hour sequence. No diurnal rhythm is apparent, only a slow falling away of the original photosynthetic rate. Tissues were previously exposed to 12 hours light, 12 hours dark for 10 days. Time marks one hour apart; downward excursion of the recorder is proportional to oxygen production.

no rhythm was occurring. The accompanying figure shows typical patterns displayed by *Ulva*. Similar records were taken for *Porphyra*.

DISCUSSION

It is apparent from the results presented here that no diurnal rhythm occurs in the organisms tested, and that in *Ulva* and *Porphyra* no photosynthetic periodicity is

induced by a 12 hour light-12 hour dark regime. In the latter connection, no information is available as to whether or not cell division is synchronized by the light-dark cycles, although the absence of any detectable photosynthetic rhythm tends to argue against such synchronization. No explanation appears to be at hand for the diurnal periodicity reported by Doty and Oguri and others. The rhythm in *Hydrodictyon* may be the result of physiological differences in the cells at different stages of development, as Tamiya (1957) has suggested.

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CONTROL OF FLASHING IN FIREFLIES. I. THE LANTERN AS A NEUROEFFECTOR ORGAN

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The ability of some fireflies to produce remarkably uniform and brief flashes of light is of much interest in relation to cellular control mechanisms. Neural involvement has been implicit or explicit in most theories of flash regulation, but there are two main ideas as to its ultimate mechanism. According to one view the luminescence of the photocyte is controlled by limiting the access of oxygen by supposed mechanical valves (tracheal end cells). According to the other view the terminal nerves stimulate the photocyte directly. The oxygen theory has been dealt with in previous papers (Buck, 1948; Hastings and Buck, 1956). The present series of papers explores responses to electrical stimulation.

Work by Macartney (1810), Macaire (1821), Todd (1826), Joseph (1854), Kölliker (1858), Owsjannikow (1868), Bellesme (1880), Arnold (1881), Verworn (1892), Perkins (1931), Brown and King (1931), Snell (1932), Alexander (1943) and others showed that voluntary luminescence is abolished by decapitation and can be elicited by electrical stimulation. Dubois (1886), Heinemann (1886), Fuchs (1891), Lund (1911) and Gerretsen (1922) attempted to localize the stimulation to specific efferent nerves, though at a very crude level. Steinach (1908) reported evidence of summation of subliminal stimuli, and Chang (1956) recorded facilitated series of responses from both the larval and adult firefly and compared them with responses in muscle. Hanson (1961) showed that regional excitation corresponds to the gross innervation of the lantern, and Carlson (1961) found that even the "pseudoflash," caused by passive entry of oxygen into an hypoxic lantern, is affected by prior neural stimulation. Nonetheless, the type and degree of neural involvement in flash control remain uncertain because of ignorance of where the nerves in the lantern terminate. Thus, nerves might control either end cells (oxygen control) or photocytes directly, and the hypoxic effect might be a direct limitation or, as Brücke (1881) suggested long ago, an inactivation of control nerves.

In view of this impasse it seemed possible that understanding of the role of nerve in triggering the firefly flash might be gained by analyzing the flash as an effector event and exploring its variation with changing stimulus parameters. Subsequent papers in this series will deal with central nervous aspects of excitation, peripheral nervous phenomena, and effector unit response. Preliminary summaries of some of the work have been given (Case and Buck, 1957, 1958, 1959a, 1959b).

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Materials and Methods

The fireflies investigated were adults of *Photinus pyralis* (Linné) from Maryland and Iowa, *Photinus marginellus* Le Conte and *Photinus consanguineus* Le Conte from Woods Hole, *Photinus punctulatus* Le Conte from Iowa, and both larvae and adults of the most common photurid (tentatively identified as *Photuris versicolor* Barber) from each of the three localities. (Identifications were made via the papers of Barber and McDermott (1951) and Green (1956).) Adult specimens were netted in the field and used within a week, meanwhile being kept unfed in humidified jars at room temperature. Larvae were collected on lawns near streams and kept in petri dishes on moist paper, either at room temperature for immediate use or at 5–10° C. for use weeks or months later.

Preparations included intact individuals, decapitated specimens, and isolated lanterns. To prepare the adult lantern, which consists of ventral plaques in abdominal segments 6 and 7, these segments were excised and all viscera overlying the photogenic organ, usually including the terminal ganglia of the ventral nerve cord, were removed. The larval light organs, a pair of small oval spots on the ventrolateral surface of abdominal sternite 8, were excised with part of the surrounding sternite.

Stimuli were delivered by Grass S-4 stimulators via radio-frequency isolation units and electrodes of unshielded 30-gauge silver or platinum-iridium wire. Intact or decapitated specimens were de-legged and anchored on dental wax with wire staples spanning the body transversely. Isolated light organs were laid across a parallel electrode pair in a small moist chamber.

Two methods were used in temperature experiments. In one, the specimen, with electrodes in position, was bound to the bulb of a thermometer reading to 0.01° suspended in air in a test tube immersed in a water bath; in the other the specimen was attached to a thermistor thermometer readable to 0.5°, in a small copper air chamber through the walls of which water was circulated from a large temperature-stabilized reservoir.

Light emission was followed with an RCA 931-A photomultiplier leading directly to one channel of a dual beam oscilloscope and photographed simultaneously with a second trace carrying a stimulus marker.

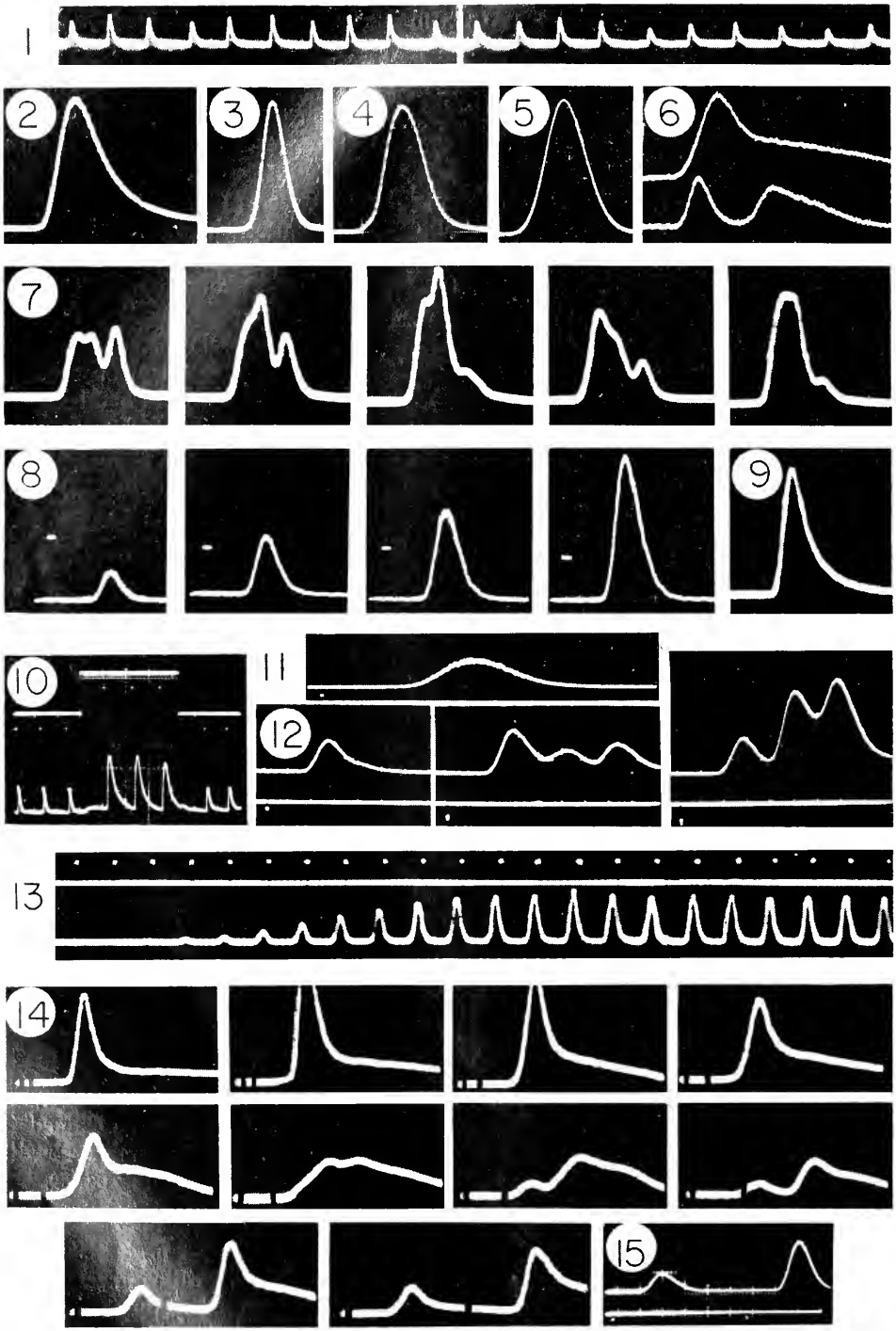
In all except the temperature experiments the preparation was mounted on the stage of a stereoscopic microscope magnifying up to 120×. Unless otherwise specified, all experiments were done at 25 ± 3° C.

Results

Both spontaneous and induced flashing exhibit a high degree of consistency, and most of the illustrations and data presented are typical of responses obtained in dozens or hundreds of measurements. Instances of refractoriness associated with apparent central nervous phenomena will be considered in the following paper.

1. *The spontaneous flash*

Spontaneous flashing is seen only in individuals with brain still connected with cord. As a general rule it does not occur in either field or laboratory below 18–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20° C. Under the conditions of most of our experiments (animal immobilized; electrodes inserted), spontaneous flashes were generally infrequent, although single responses could sometimes be induced by various types of mechanical stimulation. *Photuris* was exceptional in its spontaneous activity, some specimens flashing with almost clock-like regularity. Figure 1 shows the first 10 and last 10 of 80 consecutive flashes given by a male of *Photuris* in 32 seconds. In this series the mean interflash interval was 405 ± 3.2 milliseconds (S.E. = 0.8% of mean) and mean flash intensity was 21.3 ± 0.5 arbitrary light units (S.E. = 2.4% of mean).

Insofar as flash kinetics are concerned, oscilloscopic observation confirms field impressions in showing characteristic differences between species. Thus, the durations of flashes of comparable magnitude may vary from 200 msecs. or less in *Photuris* (Fig. 9) to a second or more in *Photinus pyralis* (Fig. 2). Single flashes may have nearly symmetrical accretion and decay phases (Figs. 3, 4, 5) or show marked skewing (Figs. 2, 9). Flashes may be either single or multiple (Figs. 6, 7). We have already emphasized the usual constancy of flash type within a species (Figs. 1, 13, 32 etc.) but it is also relevant to the control problem to point out differences that may occur between the flashes of the two sexes of one species (Figs. 6 *vs.* 3 *vs.* 9), between different individuals of the same sex and species (Figs. 3 *vs.* 9; 6), and even between successive flashes of one individual (Fig. 7). Sometimes these variants have ready explanations, such as differences in degree of disturbance of the specimens, or asynchrony or variable involvements of different lantern regions; but some are not presently understood.

In most species the spontaneous flash under laboratory conditions appears comparable to that emitted in natural flight. However, in *Photuris*, which is a variable and confusing genus even in field behavior (Barber and McDermott, 1951), the free-flying male of the Woods Hole variety typically gives a rapid four-peaked twinkle every second or two, whereas the female gives a long lingering glow at irregular intervals; in the laboratory both sexes usually give single flashes, although

FIGURES 1–15. Note that in photos of oscilloscope traces the time scale (S) is given as the width of the print. Some stimulus artifacts have been retouched. (1) Woods Hole *Photuris*, male. First and last 10 of a series of 80 spontaneous flashes. S = 8 sec. (2) Maryland *Photinus pyralis*, male. Spontaneous flash. Light did not regain base level for 1 second. S = 735 msecs. (3) Woods Hole *Photuris*, male. Spontaneous flash. S = 220 msecs. (4) *Photinus marginellus*, male. Spontaneous flash. S = 450 msecs. (5) Maryland *Photuris*, male. Spontaneous flash. S = 130 msecs. (6) Woods Hole *Photuris*, female. Two frames of spontaneous flash of two individuals. S = 300 msecs. (7) *Photinus consanguineus*, male. Five successive spontaneous flashes. S = 640 msecs. (8) Woods Hole *Photuris*, male. Responses of isolated lantern to 5, 7, 8 and 9 volts, all at 10 msecs. S = 200 msecs. (9) Woods Hole *Photuris*, male. Spontaneous flash. S = 240 msecs. (10) Woods Hole *Photuris*, female, decapitated, electrodes in light organ. Eight successive responses to 10 msecs./15 v. with the fourth, fifth and sixth occurring in a 4 v. D.C. field. S = 10.5 secs. (11) *Photuris* larva, decapitated, electrodes immediately anterior and posterior to light organ. Induced glow, 10 msecs./15 v. S = 3.6 secs. (12) *Photinus punctulatus*, female, isolated lantern. Three frames showing responses to 4, 8 and 10 v./4 msecs. S = 350, 500, 500 msecs. (13) Woods Hole *Photuris*, sex unknown, decapitated, electrodes in lantern. Response to stimulus train of 4 msecs./5 v. at 10 per second. S = 2.2 secs. (14) Woods Hole *Photuris*, female, decapitated, electrodes in lantern. Responses to paired stimuli of 6 msecs./15 v. with intervals of 15, 20, 30, 40, 50, 60, 70, 90, 150 and 200 msecs. S = 360 msecs. except 430 msecs. for last two frames. Stimulus indicated by break in photomultiplier trace. (15) Maryland *Photinus pyralis*, male, decapitated, electrodes immediately anterior and posterior to lantern. Responses to two equal stimuli 1200 msecs. apart. S = 2 secs.

at least the female occasionally produces compound flashes (Fig. 6) as does the female of the Maryland *Photuris* (Fig. 8 of Hastings and Buck).

In larvae of most lampyrid fireflies the photogenic organ is much smaller and simpler than the adult lantern, and light is emitted as a long glow rather than a short flash. No spontaneous glows were recorded, because of their very irregular timing and low frequency, but the kinetic contrast between larval and adult luminescence is well illustrated by the induced glow (Fig. 11; note time scale). Actually, the duration of the larval glow is usually even longer in nature—often several seconds—but in view of our observations at different temperatures (see below) this is quite possibly accounted for by the temperature of the soil surface being considerably below 25°, particularly on the damp evenings in early autumn when larvae are most easily observed.

2. Induced flashing : general

Although, as will be shown, it is possible to alter flash duration, intensity and form by changing stimulus parameters, a single, moderate electric shock usually elicits a flash indistinguishable from the normal spontaneous flash characteristic of the species. Seemingly normal flashes can result with the electrode pair at any level of the body from head to lantern, but are usually most reliably produced with ab-

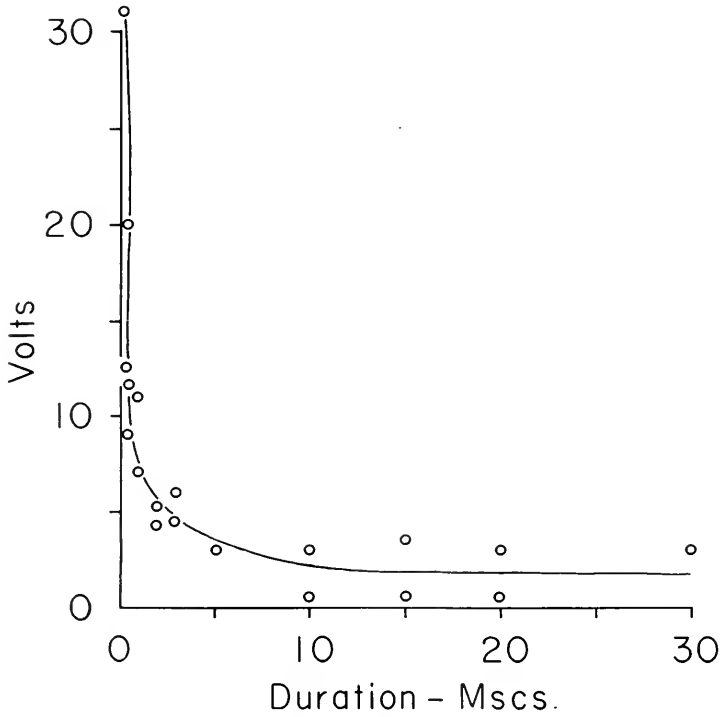


FIGURE 16. Representative strength-duration curve for minimum visually detectable response induced by direct stimulation of lantern of a decapitated Woods Hole *Photuris* male.

dominal stimulation. In the observations described below, induced responses, unless specifically excepted, were recorded with electrodes either in abdominal segment 6 at the level of the last ganglionic mass of the cord, or actually inserted in lantern tissue. In both instances excitation is presumably *via* peripheral nerve.

In connection with changes in flash characteristics, it is important to avoid being misled by differences due simply to magnitude. In Figure 39, for example, the weakest and strongest flashes might be thought to be quite different in form, whereas in fact the relative rates of rise and fall in light intensity are the same in both, and the two flashes could be made to coincide by equalizing their peaks.

3. *Threshold*

The isolated light organ, 15 minutes after preparation, gives reproducible threshold values apparently not much affected by unavoidable variations in tissue-electrode contact. Using this preparation, the interrelations of stimulus voltage and duration for minimum detectible response were studied. The results (Fig. 16) show strength-duration relations similar to insect nerve-muscle. In seven males of *Photuris* with electrodes in the lantern the average rheobase was 2.1 volts and the chronaxie was 3.9 mscs. Minimum values were 0.7 volts and 1.7 mscs., respectively. Values for the female of *Photuris* and for males of *Photinus pyralis* and *P. punctulatus* were in the same range as for the male of *Photuris*.

Threshold for direct current stimulation is relatively uniform. In *Photinus pyralis*, for example, the values in three individuals were 5.5–6.0, 3.5–4.0 and 7.5–8.0 volts.

Threshold is much increased at lower temperatures. In typical measurements on *Photuris* a 10 mscs./6 v. shock elicited a flash of good intensity at 25°, whereas, in the same individual, 50–60 volts at the same duration were required in the 9–12° range to obtain even a much weaker response.

4. *Factors affecting flash intensity*

a. *Stimulus strength and duration.* Within limits, flash intensity varies with voltage of single shocks of given duration (Figs. 8, 17). The same effect is seen during stimulation imposed upon D.C. fields (Fig. 10). In certain species, particularly if the lantern still has central connections, increasing strength of stimulus may induce multiple flashes (Fig. 12). These probably autoexcitatory responses will be considered in detail in the second paper of this series.

As would be expected from the strength-duration findings, variation in stimulus duration alone also affects flash intensity. These effects are considered in the section on flash form.

b. *Effects of stimulation frequency on flash intensity: facilitation.* With subliminal stimulation, the adult firefly lantern exhibits typical summation, response first occurring after several shocks and then growing (Figs. 13, 44). Additive effects occurring with supra-threshold stimuli are well displayed by paired shocks (Figs. 14, 18). The minimum delay which produced detectible summation (*i.e.*, the absolute refractory period) was not investigated in detail, but is clearly less than 5 mscs. in *Photuris* and is apparently between 6 and 8 mscs. in *Photinus pyralis*, using near-threshold stimuli in both instances. Above these minima, facilitation

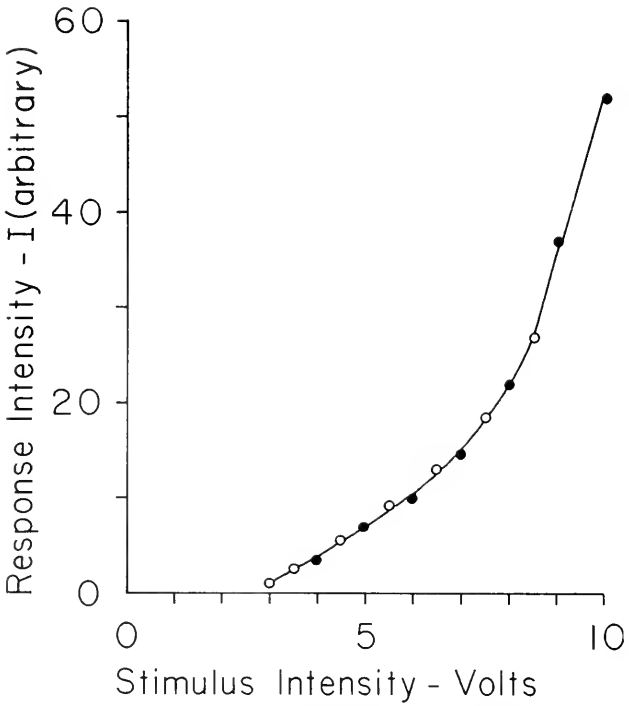


FIGURE 17. Relationship between stimulus strength and flash intensity at constant 10 msecs. stimulus duration. Open circles represent ascending voltage series, closed represent descending series. Woods Hole *Photuris* male, isolated lantern.

increases rapidly to a maximum that shows considerable variation both intra- and inter-specifically. In six specimens of *Photuris* maximum facilitation occurred with a stimulus interval of 10–20 msecs. (Fig. 18), but in two other individuals the most effective intershock interval seemed to be at least 30 msecs. In most specimens of *Photinus pyralis* the buildup and decay of facilitation was more gradual, the most effective delay lying between 50 and 100 msecs.

After maximum facilitation is attained, flash intensity declines sharply with increasing delay. In *Photuris* the facilitating effect of the second shock is practically dissipated by the time the delay reaches 60 msecs.

Concurrently with changes in flash intensity, increasing intershock delay causes changes in flash form, manifested by a slowed rate of rise without change in latency —*i.e.*, a shift in peak position toward later time (Fig. 14, first *vs.* fifth frames), relegation of the original flash to a shoulder on the rise phase of the second response (Fig. 14, sixth and seventh frames), and finally by a splitting of the flash into two peaks representing the separate responses to the two shocks (Fig. 14, eighth and ninth frames). In *Photuris* the separation is complete at an inter-stimulus interval of approximately 85 msecs: in *Photinus pyralis* not until 300 msecs.

At the interval for which separate responses to paired shocks are first detectible, the first flash is sometimes the more intense. However, as the peaks draw apart

the second usually becomes larger than the first (Fig. 14, frame seven *et seq.*). In both *Photuris* and *Photinus pyralis* this apparent facilitation of one response by the preceding one persists, in some instances, for more than a second (Fig. 15).

Facilitation is also shown by the *Photuris* larva, according to data collected by Dr. Albert Carlson (Fig. 19). Here the maximal summation effect of the second stimulus on the response to the first is reached only after 50–60 mscs., and persists at least 500 mscs., a much longer time than in the adult. The augmentation of the second response of a pair as a function of the first response was not investigated in larvae, but, if comparable to other adult/larval response rate ratios, would be expected to persist at least 10 seconds.

When repetitive stimulation is extended from paired shocks to trains, response varies widely depending on stimulus frequency and voltage, temperature, degree of neural integrity, degree of facilitation, and species. Figures 13 and 22 illustrate the rather stable sort of response seen typically with moderate stimulation, in which each flash is independent and the responses increase progressively in intensity and duration for several successive stimulation intervals, then reaching a plateau. As might be expected, the gain in flash intensity is usually more rapid the higher the rate of stimulation (Figs. 20, 21), and in species or individuals producing double flashes, both flash components augment (Fig. 22).

The stimulation frequency limit for production of completely or partially independent responses of course varies with flash duration, among other factors. In

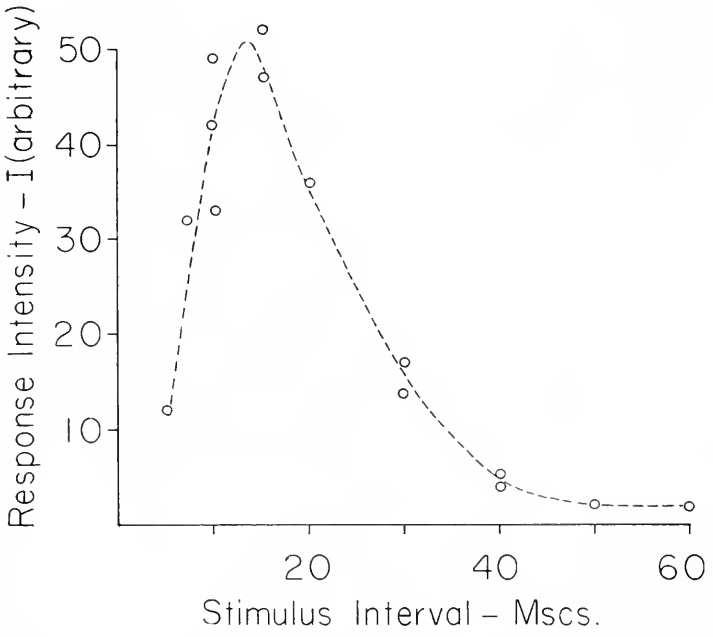


FIGURE 18. Temporal facilitation of electrically induced flashes. Abscissa represents interval from first to second stimulus of a pair, in all instances 3 mscs./5 v. Woods Hole *Photuris* female, isolated lantern. Typical of five other specimens.

Photuris, completely separate responses to each stimulus of a train can usually be elicited at frequencies of at least 10/sec. (Fig. 13), whereas in *Photinus pyralis* any frequency higher than about one per second prevents the luminescence from falling to baseline before the next response. As stimulation frequency is still further increased the individual flashes necessarily begin to merge, the fusion giving a maintained luminescence that changes more or less in proportion to flash intensity, and, in combination with facilitation, may produce responses quite similar to the familiar staircase and tetany of striated muscle (Figs. 23, 24). However, it is not uncommon, when close to the limit of 1 : 1 response to continued serial stimulation, to find the average response intensity falling off markedly after a brief rise (Figs. 25, 27, 30, 33).

Species differences are strikingly illustrated in ability to follow the stimulation frequency. The Woods Hole photurid, for example, is able to respond 1 : 1 up to about 20 shocks per second (Fig. 27). The Iowa photurid fails at between 10 and

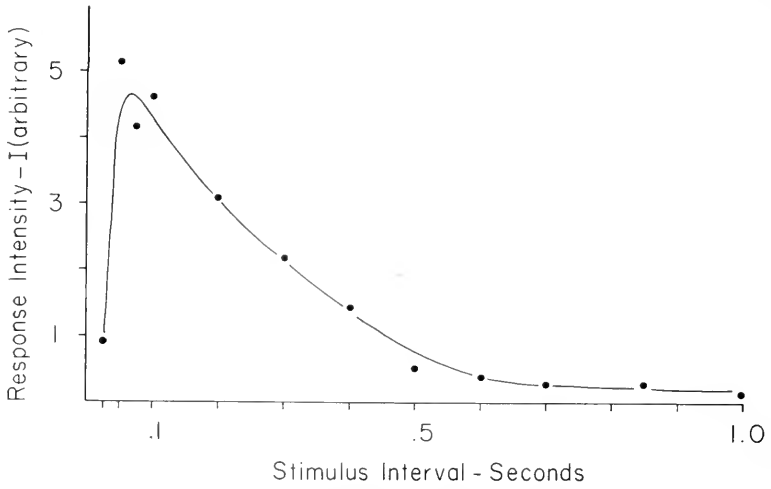


FIGURE 19. Temporal facilitation of electrically induced flashes in the larval firefly. Abscissa as in Figure 18. Stimulus 10 mscs./4 v. Iowa *Photuris* larva, isolated light organ.

15 per second (Fig. 33), *Photinus punctulatus* reaches its limit at about 10/sec. (Fig. 23), the Iowa *P. pyralis* (Fig. 31) and *P. marginellus* at 7/sec., while the Maryland *P. pyralis* barely follows at 4/sec. even with intense stimuli.

Up to the species limit, the ability to respond 1 : 1 to a particular frequency of stimulation increases with either increasing voltage or increasing stimulus duration. The actual frequency limit shows a definite relation to the strength-duration product in that 1 : 1 response can be maintained by a variety of duration-voltage combinations. In *P. marginellus,* for example, the frequency limit of about 7/sec. applies at stimuli ranging from 50 mscs./3 v. to 3 mscs./50 v. However, neither near the limit of 1 : 1 response nor at more moderate frequencies does there appear to be a true reciprocity in the response itself, *i.e.,* in mean flash intensity (Fig. 32).

An effect commonly seen at the frequency response limit is a rather regular alternation in flash intensity (Figs. 28, 30, 33). At stimulation frequencies exceed-

ing the limit of 1 : 1 response, the response differs somewhat depending on species, on stimulus parameters, and on whether or not the lantern is deganglionated, but generally resembles either a more or less typical tetany (Figs. 24, 25) or consists of serial responses to separate stimuli at some exact fraction of the actual stimulation frequency (Figs. 31, 34, 35).

d. *Adaptation and fatigue.* Free-flying fireflies can produce many dozens of consecutive flashes at the species-characteristic frequency without apparent fatigue. Even when flashing excitedly in the laboratory at much higher than normal frequencies, little decrement in either frequency or brightness of spontaneous flashes can be detected (there is about a 20% falloff in mean peak height and a 6% lengthening of interflash interval between the two parts of Figure 1). Repetitive driven flashing may, depending on the conditions of stimulation, either continue practically unchanged for long periods (Fig. 13), go through cycles of varying excitability (Figs. 21, 26, 32), or cease relatively quickly. As an example of one extreme, a female *Photuris* stimulated once per second flashed for more than 70 minutes with only slowly decreasing vigor (over 4000 successive flashes). At the other extreme

TABLE I

Stimulus-response interval at 25° C. Two to six measurements per individual

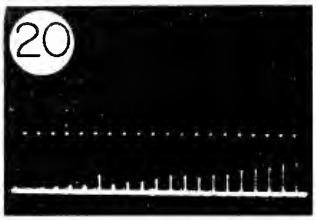
Species	No. individs.	Latency (milliseconds)	
		Average	Range
Photuris	6	86	55–101
Photinus pyralis			
Maryland	4	194	160–206
Iowa	4	166	144–190
Photinus punctulatus, male	10	67	50–85
Photinus punctulatus, female	2		125, 125
Photinus marginellus	2		26, 27
Photinus consanguineus	8	Approx. 100, 175, 250 (3 elements)	
Photuris, larva	1	800	

are records of the sort illustrated in Figures 29, 30 and 38 in which the response decreased greatly or even failed after a few stimuli. Such damping is particularly common when stimulation frequency exceeds the limit of 1 : 1 response (Fig. 34). Rapid degradation in flash intensity in 1 : 1 response series can probably be ascribed to junctional fatigue when shock voltage is high, and to adaptation when stimulus strength is little above threshold.

In addition to frequency effects already described, it was noted that the first response to a train was sometimes conspicuously more intense than the succeeding flashes (Figs. 29, 36, 37, 63, 64).

5. *Latency*

Table I gives a sample of stimulus-response delays from among some hundreds of flashes of good intensity measured in adult fireflies of several species at about 22° C. under comparable conditions of stimulation. These data show that there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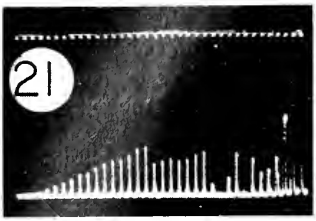
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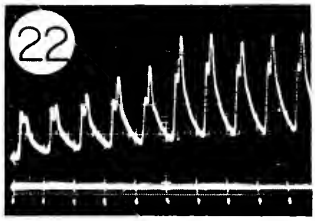
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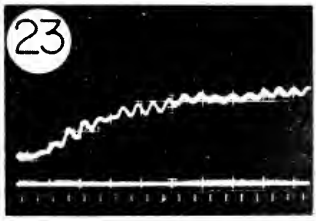
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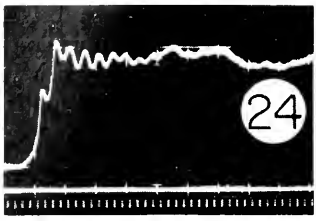
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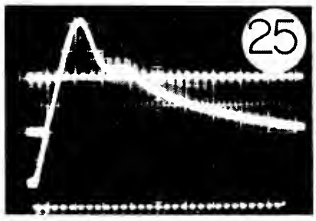
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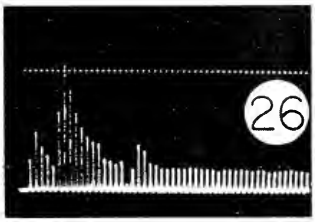
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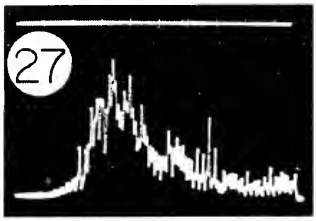
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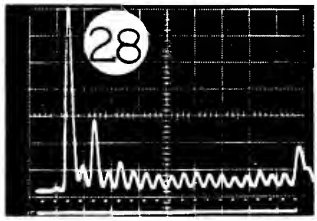
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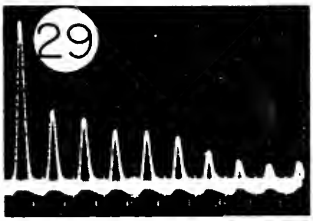
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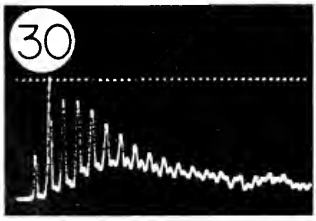
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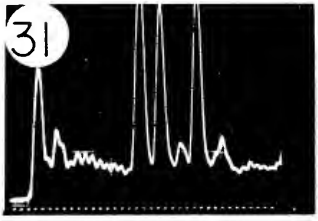
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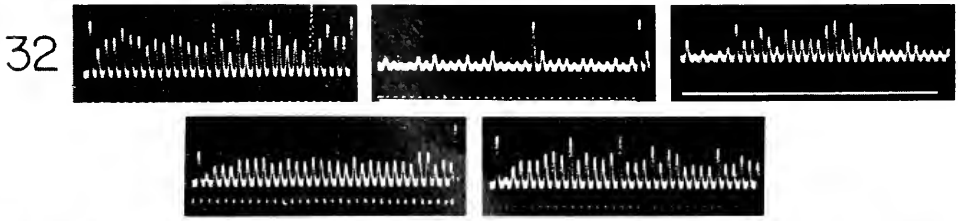
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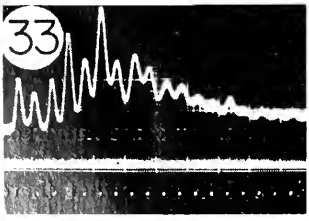
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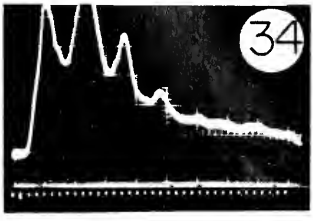
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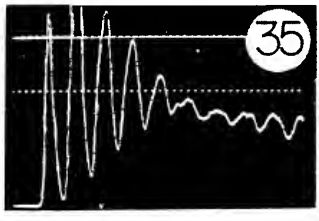
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may be significant and characteristic latency differences between species, or even possibly between what appear to be regional variants of one species (*P. pyralis*). The considerable latency difference between the sexes of *P. punctulatus* was not found in *Photuris*, and females of the other species are so seldom found that no measurements were obtained.

Considering the probable multiplicity of the excitation pathways and the number of response units in the lantern, response latency within individuals is remarkably constant. Serial responses of comparable intensity given in response to regularly repeated identical stimuli may vary less than 5% of their mean value, particularly in deganglionated lanterns.

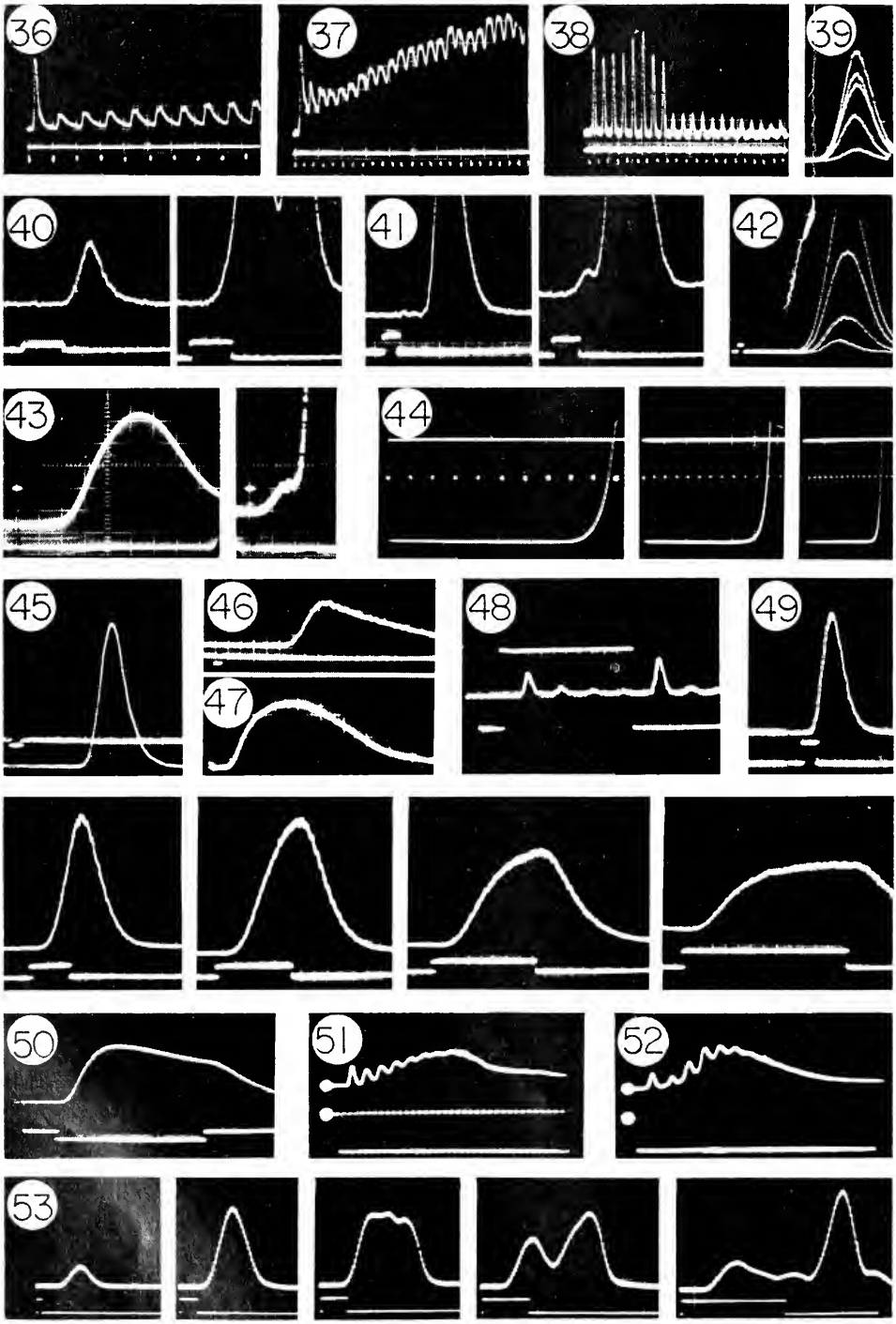
a. *Effects of temperature on latency.* In *Photuris* and in *Photinus pyralis*, our most used species, 8° C. seems to be about the limit to which temperature can be lowered and still permit induced flashing, and even this cannot be accomplished without strong stimulation (see Threshold), change in form (see below), and much prolonged latency (Fig. 54). Above this temperature, latency decreases, at first rapidly. Between 32° C. and 35° C. in *Photuris* there is some indication of an inflection point. In a male studied (not plotted) it was particularly striking. Below this point the temperature-induced changes in latency are fully reversible, but above it permanent damage seems to occur, as indicated by failure of the latency curve to retrace its path as temperature is lowered again.

Since overall latency reflects a complex of conduction and excitation steps, mostly unknown, Q_{10} values are of uncertain worth; however, they lie within the usual range for neuroeffector systems (*ca.* 1.5 at 30° C., and over 2.0 at 20° C.). Temperature characteristics are of even more tenuous significance, but the data appear to yield linear Arrhenius plots up to 30° C.

Though measurements of latency changes of the larval "flash" with temperature are available only for the 14°–26° C. range, differences from the adult pattern are suggested (Fig. 55). First, the trend seems to be linear rather than inflected. Second, the Q_{10} is about 3.0, or about 50% higher than the value for the adult in the corresponding range.

FIGURES 20–31. (20) Woods Hole *Photuris*, male, decapitated, electrodes on prothoracic ganglion. Responses to 10 mscs./15 v. at 2 per second. S = 11 secs. (21) Same as Figure 20 except stimulus frequency 7 per second. S = 5.5 secs. (22) *Photinus punctulatus*, male, isolated lantern. Responses to 2 mscs./10 v., at 2 per second. S = 5 secs. (23) Same as Figure 22 except stimulus frequency 10 per second. S = 2 secs. (24) Same as Figure 22 except stimulus frequency 20 per second. S = 2.1 secs. (25) Iowa *Photinus pyralis*, male, decapitated, electrodes in lantern. Responses to 40 mscs./150 v. at 15 per second. S = 2.5 secs. (26) Woods Hole *Photuris*, male, decapitated, electrodes on prothoracic ganglion. Stimulus 5 mscs./15 v. at 10 per second. S = 5.7 secs. (27) Same as Figure 26 except stimulus frequency 20 per second. S = 5.5 secs. (28) Iowa *Photinus pyratis*, male, decapitated, electrodes in lantern. Stimulus 5 mscs./10 v. at 4 per second. S = 5.5 secs. (29) Iowa *Photuris*, male, decapitated, electrodes in lantern. Stimulus 1 msc./6 v. at 5 per second. S = 2 secs. (30) Woods Hole *Photuris*, male, decapitated, electrodes on prothoracic ganglion. Stimulus 5 mscs./15 v. at 20 per second. S = 2.1 secs. (31) Same as Figure 28 except stimulus frequency 7 per second. S = 5.5 secs.

FIGURES 32–35. (32) *Photinus marginellus*, male, decapitated, electrodes in lantern. Responses to five series of stimuli at 3 per second: 12 mscs./5 v., 20 mscs./3 v., 6 mscs./10 v., 4 mscs./15 v., and 2 mscs./30 v. S = 1 sec. (33) Iowa *Photuris*, male, isolated lantern. Stimulus 2 mscs./20 v. at 10 per second. S = 2 secs. (34) Same as Figure 33 except stimulus frequency 20 per second. S = 2 secs. (35) Woods Hole *Photuris*, male, decapitated, electrodes on prothoracic ganglion. Stimulus 5 mscs./15 v. at 40 per second. S = 2.1 secs.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b. *Effects upon latency of stimulus strength, duration and frequency.* Latency rather frequently appears to decrease progressively with increasing strength of stimulus (Figs. 8, 40). In many instances these effects can be ascribed to the magnitude artifact already mentioned, and this is probably also the explanation of the apparent latency changes seen in responses facilitated in serial stimulation (Figs. 39, 42). Latency may also appear to decrease stepwise due to the becoming visible of an additional response of shorter latency. Such early shoulders on the main flash may appear simply as a consequence of increased amplification of the photomultiplier trace (Fig. 43), or be invoked by increasing stimulus strength (Fig. 40) or duration (Fig. 41).

Striking differences in ostensible latency may occur with trains of subliminal stimuli which exceed the capacity of the lantern for 1:1 response (Fig. 44), although there is ambiguity as to which is the first effective stimulus.

With strong stimulation, some instances of shortened latency are due to the evocation of a separate and qualitatively different sort of early response ("quick flash"—Case and Buck, 1958; 1959b) that is considered in detail in the third paper of this series.

6. *Flash form*

Flash form—the variation in light intensity with time—is subject to so many internal and external influences that few generalizations are possible. We have already alluded to changes in form involved in the transition from single to double flashes (Fig. 14), and similar changes are seen in the evocation of multiple responses by increased stimulus voltage (Fig. 12) and duration (Fig. 53), as well as in spontaneous flashing (Fig. 7). Some factors influence differently the rates of

FIGURES 36–53. (36) *Photinus punctulatus*, male, isolated lantern. Stimulus 2 msecs./10 v. at 2 per second. S = 5 secs. (37) Same as Figure 36 except stimulus frequency 5 per second. S = 5 secs. (38) Iowa *Photuris*, male, isolated lantern. Stimulus 1 msec./5 v. at 5 per second. S = 5.5 secs. (39) Woods Hole *Photuris*, female, decapitated, electrodes immediately anterior and posterior to lantern. Five superimposed responses to 6 msecs./20 v. Temperature 17.9° C. S = 80 msecs. (40) Woods Hole *Photuris*, male, decapitated, electrodes in lantern. Stimulus 60 msecs./4 v. and 60 msecs./15 v. S = 250 msecs. (41) Same as Figure 40 except stimuli 20 msecs./15 v. and 40 msecs./15 v. S = 250 msecs. (42) Iowa *Photuris*, male, isolated lantern. Stimulus 6 msecs./20 v. Temperature 26.7° C. S = 190 msecs. (43) *Photinus consanguineus*, male, isolated lantern with intact ganglia. Two responses to 10 msecs./15 v., the second at 12.5 times the amplification of the first. S = 480, 230 msecs. (44) Maryland *Photinus pyralis*, male, decapitated, electrodes on prothoracic ganglion. Responses to 2 msecs./9 v. at 10, 20 and 40 per second. S = 1100, 650, 440 msecs. (45) Woods Hole *Photuris*, female, decapitated, electrodes in lantern. Stimulus 10 msecs./6 v. Temperature 25.5° C. S = 160 msecs. (46) Same as Figure 45 except stimulus 10 msecs./80 v. Temperature 12° C. S = 600 msecs. (47) Woods Hole *Photuris*, female, decapitated, electrodes in lantern. Stimulus 10 msecs./40 v. Temperature 39.2° C. S = 500 msecs. (48) Woods Hole *Photuris*, male, isolated lantern. Stimulus 400 msecs./8 v. S = 860 msecs. (49) *Photinus consanguineus*, male, isolated lantern. Stimuli 30 msecs./15 v.; 100 msecs./10 v.; 200 msecs./9.5 v.; 300 msecs./9.5 v.; 500 msecs./12 v. S = 250, 306, 340, 410, 390 msecs. (50) *Photinus marginellus*, male, isolated lantern. Stimulus 500 msecs./90 v. S = 850 msecs. (51) Woods Hole *Photuris*, female, decapitated, electrodes in thorax. Stimulus 20 msecs./15 v. at 30 per second. S = 1.4 secs. (52) Same as Figure 51 except stimulus 12 v. D.C. beginning with deflection of lower trace. S = 1.4 secs. (53) *Photinus marginellus*, male, decapitated, electrodes in light organ. Stimuli 15 v. for 20, 80, 100, 200, 500 msecs. S = 800, 650, 700, 950, 1100 msecs.

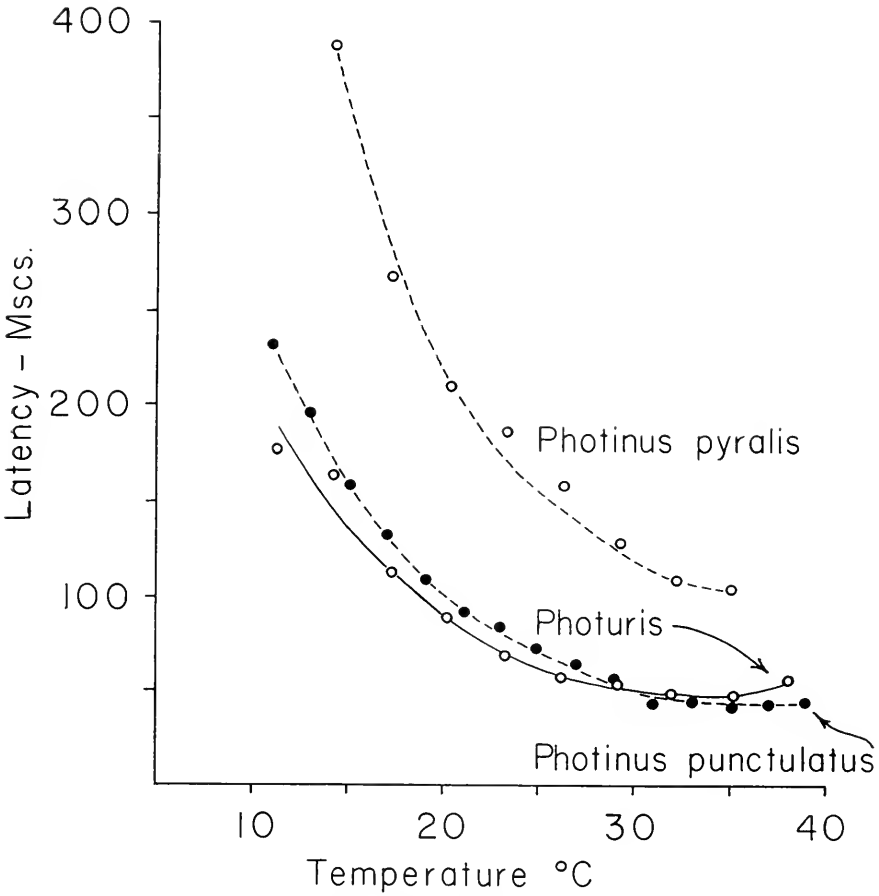


FIGURE 54. Stimulus to response latency in relation to temperature for adults of three species of fireflies. Stimulus varied as function of temperature in order to produce similar magnitude flashes. Points represent means of measurements on 5 male *P. pyralis,* 10 female Woods Hole *Photuris,* 6 male *Photinus punctulatus.* Direct excitation of lantern.

accretion and decay of light intensity. One of the most predictable of these is temperature. Between approximately 15° and 30° C. flash form remains relatively constant (Fig. 56), but temperatures outside this range usually produce a preferential slowing of the decay phase of the flash (Fig. 45 *vs.* Fig. 46). This slowing is usually progressively greater the more extreme the temperature, but in some series there appears to be a relatively sudden form change at around 15° C. The changes toward the ends of the range are difficult to evaluate quantitatively because of the developing steady glow (see below) and the necessarily concomitant changes in stimulus strength. The low temperature skewing seems to be completely reversible, but the slowed decay at high temperature (Fig. 47) persists after return of the animal to normal temperatures, suggesting irreversible damage.

Flash form is of course affected by long or high frequency stimuli that prolong

luminescence. Several such deviations can be induced. A curious effect of lengthening shock duration is a slowed rise demonstrable in flashes of comparable peak intensity given by deganglionated lanterns (Fig. 49). There is also often a perceptible "off effect," of the same latency as the primary rise (Figs. 48, 53). However, it is also clear that the lantern can be stimulated throughout the duration of long stimuli, since a flash of many times normal duration can be produced (Figs. 49, 50). Presumably the same explanation applies to the fact that trains of stimuli above the frequency limit of 1 : 1 response (Fig. 51) may produce the same effect as the continuous passage of current (Fig. 52).

There seem clearly to be elements of both facilitation and of fatigue or adaptation in artificially lengthened flashes. Depending on whether the voltage is low or high, shocks of equal duration may induce either flashes which increase in intensity up to the break point (Fig. 49, fifth frame) or which start high and decline steadily (Fig. 50).

Customarily the peak positions in a homologous series of flashes coincide rather closely (Figs. 39, 57), indicating that the *rate* of rise of light intensity is independent of total light production. However, in some series the peak positions of

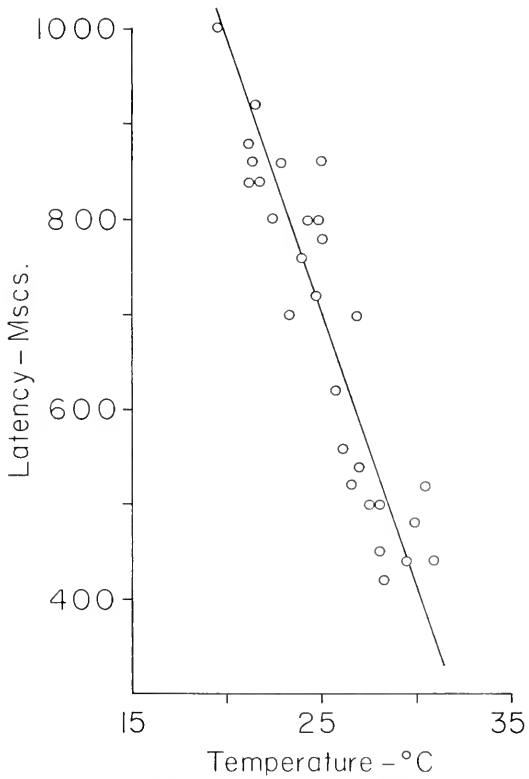


FIGURE 55. Stimulus to response latency for Iowa *Photuris* larvae. Stimulus strength varied with temperature to produce similar magnitude flashes.

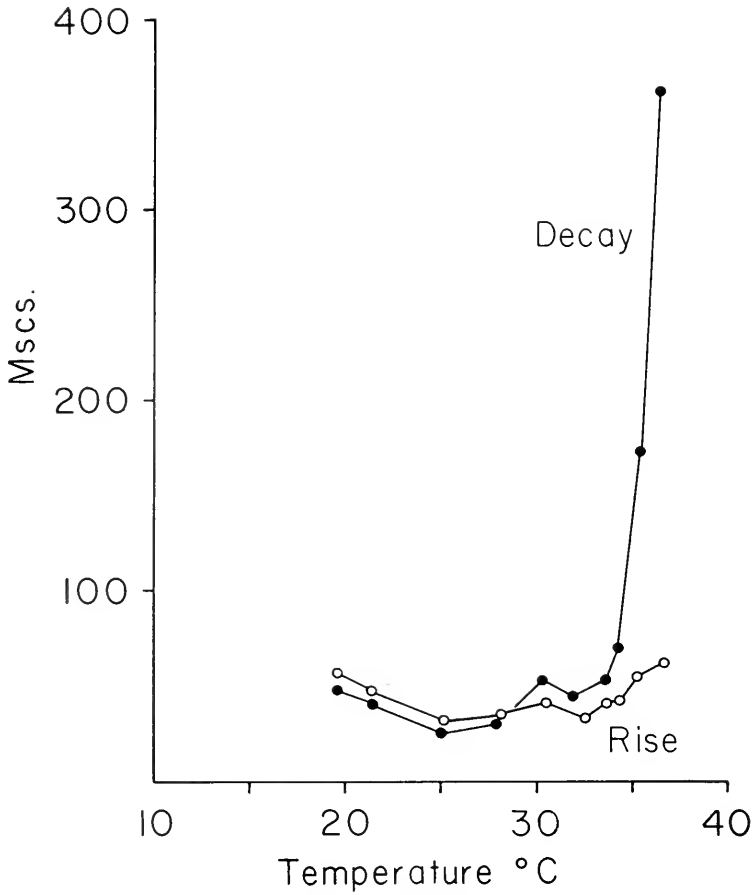


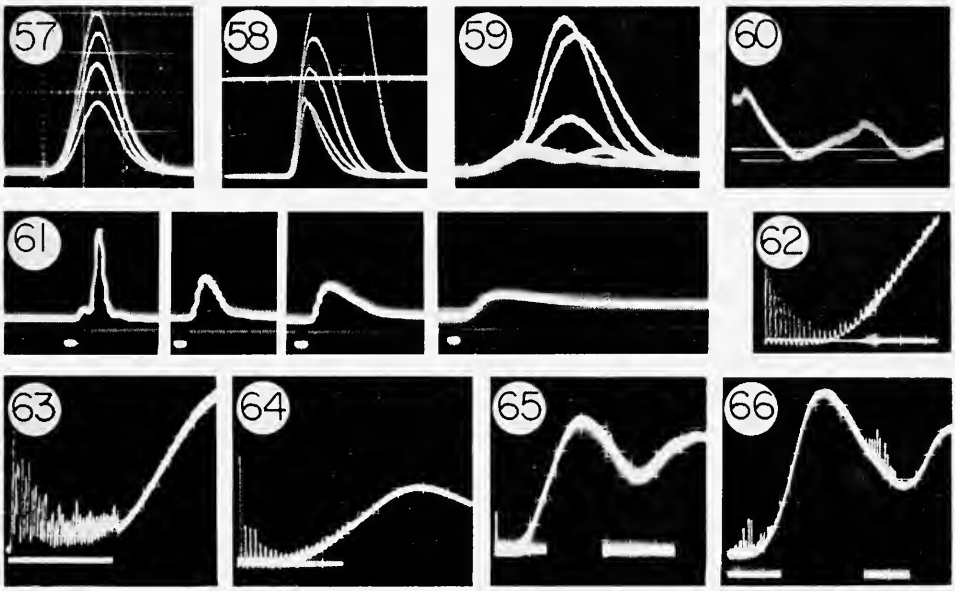
FIGURE 56. Influence of temperature on flash form as indicated by variation in half-rise and half-decay times of flashes induced by electrical excitation of lantern. Stimulus strength varied with temperature in order to produce as nearly equal magnitude flashes as possible. Iowa *Photuris* male.

the low intensity flashes are variable, usually, but not always, in the direction of early peaking (Figs. 58, 59).

Numerous other interesting alterations in flash form of even more obscure causation have been observed. When, for example, a firefly is stimulated every few seconds in decreasing ambient oxygen concentration the decay phase of the flash sometimes tends to be slowed much more than the rise phase as hypoxia deepens (Fig. 61).

7. Glow

In addition to the various types of more or less brief flash, all species of firefly may show moderate to long-continuing steady luminescences which vary widely in



FIGURES 57–66. (57) Woods Hole *Photuris*, female, decapitated, electrodes in prothoracic ganglion. Four superimposed responses to 10 mscs./10 v. S = 100 mscs. (58) Woods Hole *Photuris*, female, intact, with electrodes on prothoracic ganglion. Stimulus 1 msc./7 v. Temperature 22° C. S = 470 mscs. (59) Maryland *Photinus pyralis*, male, decapitated, electrodes in lantern. Five superimposed responses to 5 mscs./48 v. Temperature 13° C. S = 1 sec. (60) Iowa *Photuris*, male, isolated lantern. Response to two trains of 10 mscs./50 v. at 30 per second. S = 45 secs. (61) *Photinus punctulatus*, male, decapitated, electrodes in prothorax. Electrically excited flashes at 0, 18, 34, and 46 secs. after start of exposure to N_2. S = 760, 520, 680, 1220 mscs. (62) Iowa *Photuris*, male, isolated lantern. Stimulus 1 msc./6 v. at 5 per second for duration of sweep. S = 8 secs. Note that in Figures 63–66 stimulation is indicated by unblanked portion of signal trace. (63) Same as Figure 62 except stimulus 1 msc./5 v. at 15 per second. S = 4.2 secs. (64) Same as Figure 62 except stimulus 1 msc./6 v. at 5 per second. S = 9.5 secs. (65) Same as Figure 62 except stimulus frequency 10 per second. S = 9 secs. (66) Same as Figure 62 except stimulus 1 msc./8 v. at 15 per second. S = 9 secs.

causation, intensity, area of lantern affected, and rates of accretion and decay. Though these are not well understood, they belong in any inventory of the photogenic potentialities of the lantern.

Glowing is the normal mode of luminescence of the larva, and occasionally the larval light organs persist through the pupal stage and are functional in the adult (in the eighth sternite) in addition to the normal adult lanterns in segments 6 and 7. The female of *Photuris* rather typically emits a protracted glow from her adult lantern while in flight, and apparently normal specimens of other species are occasionally seen to emit similar glows. It is not possible to distinguish visually between such normal glows and the continuous luminescence that commonly develops in strong repetitive stimulation and which presumably represents flash fusion (Figs. 23–25, 27, 30, 31, 34, 35, 37 etc.).

In instances where the photomultiplier records flashes superimposed on glow (Figs. 62, 64, 66) it is generally found upon microscopic examination that glowing

regions do not flash, and vice versa. The relations of glow and flash are in fact complex and enigmatical. Glowing may sometimes be suppressed (Fig. 63) or reduced (Fig. 60) by high frequency electrical stimulation, thereafter rising spontaneously though temporarily. It may also apparently be enhanced by repetitive stimulation, after a lag (Figs. 65, 66), with or without flashing.

Sustained glowing is also often engendered by non-physiological conditions, such as excess heat or cold, injection of many materials into the hemocoel, toxic vapors, high or low pO_2, death, etc., and it seems probable that there are several types of "glow," perhaps none of which is analogous to the normal glows of larva and adult, or to the transitory glowing associated with vigorous stimulation.

Microscopic examination of the lantern surface contributes relatively little to our understanding of glows. In most instances, as indeed in flashing, the tissue seems to be uniformly alight, and no fine detail can be made out. The impression is often given that the active tissue is deep in the organ, but there is no histological ground for such a distinction (Buck, 1948) nor is it really possible to distinguish between self-luminosity in surface tissue and secondary glowing due to light diffusing through from a deeper source.

Discussion

Different species of firefly have long been known to differ characteristically in color and intensity of light produced and in flash pattern and duration. The present findings give quantitative expression to the differences in flash duration and form, and show that interspecific distinctions extend to such details of excitation as latency and frequency response limit. No correlations are yet apparent between response characteristics and other species differences such as behavior, body size or lantern structure, except in the instance of the larva, where sluggishness of response is associated with a minute and primitively constructed photogenic organ.

From the electrophysiological standpoint the flash response has so many similarities with conventional neuroeffector excitation systems that direct neural control can scarcely longer be doubted. In regard to the specific analogy with muscle (Chang, 1956), the flash is leisurely and its response latency long compared with a striated muscle twitch, but there is rather good qualitative correspondence between the two systems in regard to strength-duration relations, temperature effects, the effects of changing stimulus voltage, duration and frequency, and the courses of fatigue, adaptation and facilitation. Although we are no nearer to having a concrete picture of how nerve and photocyte are associated, the evidence is strong that the association has many of the properties of a conventional neuroeffector junction.

The remarkable species-specific constancy in normal light emission is coupled with an equally remarkable lability to experimental influences, and it is this capacity for varied response that shows that we are very far from being able to define the basic excitatory event. *A priori*, flash form should be of special interest because of the possibility that it might yield kinetic information. However, in view of the facts that the lantern is estimated to contain of the order of 600,000 photocytes (Buck, 1948) which are controlled in localized groups by peripheral nerve twigs (Hanson, 1961), any hope that the flash mirrors directly the time course of either the basic chemiluminescence or the excitation process is illusory. It has in fact been argued (Buck, 1955) that when light from numerous unresolved foci is de-

tected by an integrating device such as eye or photomultiplier tube, symmetrical, skewed or multiple flashes of any desired form can theoretically be produced merely by varying the gross sequence and spread of excitation. Therefore, although it is possible to give plausible explanations of many of the luminescent phenomena here recorded in terms of conventional interplay between excitation and response mechanisms, it seems preferable to defer most such attempts until conductional, junctional and effector processes can be separated clearly, and the experimental preparation can be limited, if not to single units, at least to constant effector populations.

Nevertheless, the flash is not totally devoid of information. The preferential slowing of the decay phase of the flash at low temperatures (Fig. 46) and in hypoxia (Fig. 61) indicates that the rise and extinction processes are qualitatively different, rather than being merely the two phases of a reversible process. The lability and variability of decay also suggest that it reflects some sort of active control process rather than, for example, dieaway of the ultimate chemiluminescence. The slowing effect of hypoxia on decay of luminescence might be similarly interpreted as interference with a metabolic extinction process. It could also reflect direct oxygen-limitation of the chemiluminescence; however, the virtual absence of fatigue in moderate repetitive stimulation indicates that actual exhaustion of some reactant in light production is unlikely ever to become rate-limiting in the normal over-all response.

Even in the mass flash of the whole lantern there are sometimes clear indications of functional heterogeneity. The flashing of *Photinus consanguineus* is especially interesting in this respect since it normally shows three peaks which have constant latencies (Table I) but independently modulated amplitudes (Fig. 7). The fixed sequence of excitation, shown by both intact and decapitated specimens—but not, significantly, by deganglionated lanterns (Fig. 49)—undoubtedly reflects central nervous programming, while the variable intensities of the three peaks suggest effector populations of variable sizes. Similarly, the early shoulders evoked by intensified stimulation (Figs. 40, 41) probably reflect recruitment of additional groups of effector units with higher threshold and shorter latencies.

A second point of interest about the spontaneous flashing of *P. consanguineus* (Fig. 7) is that whereas each of the three peaks varies continually and markedly in intensity, the total light emitted in each of the last four over-all flashes has an extreme range of variation of only 8% of the mean value, and the first flash is only 25% low. This raises the interesting possibility that the effector units are excited in relays so that total light per over-all flash is kept constant without all photocytes having to participate in each flash. In single-flashing fireflies the maintenance of a uniform flash presents no particular problem, assuming uniform excitation, but the regulation apparently practiced by *P. consanguineus* would appear to require some sort of sensory feedback.

In the pair-shock series shown in Figure 14, both stimuli occur in advance of the response (as long as it is single), so the changes in rise slope and peak position seem reasonably explicable on the basis of conventional facilitation. In the stimulus duration series shown in Figure 49, however, the lantern has no way of knowing, at the time its response begins, how much longer the current will flow—and yet the rise slope progressively flattens and the peak shifts to the right as the stimulus lengthens. Broadening of the flash seems scarcely ascribable to developing inhibi-

tion or to a rescheduling of the firing times of the effector units, because total light increases. It seems, therefore, that although direct current flow may stimulate continuously, producing a response analogous to constant-current contracture of muscle (Fig. 49, last frame; Fig. 50), there may also occur interaction of on and off effects of the pulse. When the pulse is short (Fig. 49, first and second frames) the interaction resembles facilitation and increases the flash height, whereas when start and finish of the pulse are widely separated the effect is seen mainly in a much-prolonged decay phase. Lack of the break effect might also explain why the D.C. threshold is higher than that determined with pulses. In some circumstances, possibly when deganglionation has removed the potential of continuous neural re-excitation, the make and break effects of long pulses occur separately (Fig. 48).

In repetitive spontaneous flashing it is of some interest to inquire how successive flashes are interrelated in magnitude and timing. If we assume a neural pacemaker of not absolute regularity it would be expected that, because of persisting facilitation, shorter interflash intervals would be correlated with more intense succeeding flashes and longer interflash intervals with smaller succeeding flashes. An alternative possibility would be that flash intensity is a function of concentration of substrate available for luminescence—in which case big flashes (using more substrate) should tend to be followed by small, and big flashes should tend to be preceded by longer than average interflash intervals (needed for substrate replenishment). An analysis of the 80 flashes and 79 intervals in the series illustrated in Figure 1 revealed that (a) the flashes following short intervals were 5% more intense than average, (b) the flashes following long intervals were 18% smaller than average, (c) the flashes following big flashes were 7% larger than average, and (d) the flashes following small flashes were 11% smaller than average. We construe these findings to indicate that flash intensity reflects degree of neural facilitation rather than substrate concentration.

The phenomenon or phenomena of induced glowing raises questions of great potential interest, but in view of the paucity of definite information it need only be said that (a) the irreversible, homogeneous, unresponsive, oxygen-sensitive, unchanging glows that are induced by various mechanical or chemical injuries probably represent the uncontrolled luminescing of the substrate stored in the photogenic tissue and are different from either the transitory decay tails of some flashes or the reversible glows associated with high frequency stimulation, (b) the sluggish rate of change of glows, even those responsive to stimulation, cautions against considering them neurally-mediated responses in any usual sense, (c) glows that decay either spontaneously or under stimulation, or glows that augment with stimulation, have some counterparts in flashing behavior; but glows that augment in the absence of stimulation (*e.g.*, Fig. 63) seem to pose an especially interesting problem for the future.

The glow of the firefly larva presumably belongs in a separate category since it is the normal mode of lighting and since it is responsive to quite modest stimulation intensities. In view of the long-known absence from the larval lantern of the tracheal end cells or end organs that form such a characteristic feature of the adult lantern, it seems reasonable to regard the tracheal end cells as being somehow associated with the ability to delimit luminescence sharply. It does not follow, however, that glowing in the adult necessarily means end cell inactivation.

Summary

1. Records are presented of normal spontaneous flashes and of flashes induced by a variety of electrical stimuli at a variety of anatomical sites in several species of lampyrid firefly.

2. The flashes of adult firefly lanterns have long response latencies (25 to 250 msecs. at 25° C. in different species) and durations (100 to 1000 msecs.) and can be repeated many hundred times with only slight fatigue. The response itself shows strength-duration relations and frequency responses (summation, treppe, tetany) which are similar to those of more conventional neuroeffector systems. A striking long-lasting neuroeffector facilitation is also evident.

3. Response latency lengthens with falling temperature, Q_{10} values for the 10°–30° range varying from about 2.4 to 1.4. Extreme temperatures slow the decay phase of luminescence preferentially, as does hypoxia.

4. The flashes of most species differ characteristically in time course, response latency and other electrophysiological properties.

5. The responses of the *Photuris* larva are roughly similar to those of the adult, but slower by a factor of about 10.

6. The time course of light intensity change during flashes induced under various conditions is discussed in possible relation to underlying excitation and effector mechanisms. Long lasting glows are also considered in this context.

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VARIATIONS IN THE DORSAL PATTERN OF CYATHURA POLITA (STIMPSON) FROM ESTUARIES ALONG THE COASTS OF EASTERN UNITED STATES AND THE GULF OF MEXICO[1]

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It has been established that the isopod which has been collected in coastal estuaries and tidal marshes of the United States from Louisiana to Maine belongs to a single species, *Cyathura polita* (Stimpson) (Miller and Burbanck, 1961). Any species having such an extensive distribution might be expected to show some morphological variation, and if such variation were inherited, geographical races within the species might be recognizable. Differences in pattern and coloration are genetically controlled in certain crustaceans. In *Tisbe reticulata* (Bocquet, 1951; Battaglia, 1958), there is inheritance both of color and of the distribution of the violet, rose, and light brown pigments within the sectors of a dorsal, hypodermal network. *Sphaeroma serratum* (Bocquet, Lévi and Teissier, 1951) also has distinct colored forms which can be classified and for which the mode of inheritance can be determined. The presence or absence of chromatophores form inheritable patterns in *Jaera marina* (Bocquet, 1953, 1954) and *Cambarellus shufeldtii* (Volpe and Penn, 1957). The forms of *Jaera* were distinguished chiefly on the basis of colored and uncolored bands which corresponded to the segments of the body. There was also a pattern of small white dots, usually elliptical in shape, the "impressions musculaires," which was evident on pigmented segments, but this pattern remained essentially the same in all phenotypes recognized. *Cyathura polita* has an intricate and distinct dorsal pattern formed by light areas from which chromatophores are absent alternating with areas filled by dark chromatophores (Fig. 1). These light areas appear to correspond to the less extensive "impressions musculaires" of *Jaera marina* and to be areas of attachment of muscles.

Casual examination of cyathurans shows a marked similarity of pattern design in all the individuals. During the course of extensive studies of *Cyathura polita* (Burbanck, 1959; Miller and Burbanck, 1961; Burbanck, 1961a; Burbanck, 1961b), it was noted that the pattern was most clearly defined on the head and on the first two thoracic segments. When it was noticed that the pattern of animals collected from the Ashepoo River, South Carolina, looked "different," the arrangement of chromatophores in the anterior segments was compared with that of cyathurans collected in other areas, and it was decided to make a comparative study of the central portion of the pattern of the first thoracic segment. This configuration of chromatophores was chosen because it showed two types of variation which could be used in a comparative study, *viz.*, a variable number of uncolored areas and differences in their orientation.

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FIGURE 1. Female *Cyathura polita* from Silver Glen Springs, Florida, 19 mm. in length. Photograph courtesy of Dr. E. L. Hunt.

MATERIALS AND METHODS

To determine if there was a recognizable pattern difference between geographically separated populations, animals from two areas from which large samples had been obtained were compared. Ninety-five animals collected in the Silver Glen-Juniper Springs area of Lake George, Florida, in 1959 and 1960 were compared with a total of 117 animals collected from the Pocasset River, Massachusetts, in May and June of 1960. In addition to these large collections, specimens from the entire range of *Cyathura polita* were examined. These included the animals collected by W. D. Burbanck and reported on in 1959, and more recent collections from the Gulf of Mexico, the Hudson River, the coasts of Connecticut and Rhode Island, and Cape Cod, Massachusetts.

Cyathurans were preserved in 70% alcohol and glycerine; after five years in this preservative, the chromatophore pattern is still distinct. Photographs, camera lucida drawings, sketches, and diagrams were made of the central part of the pattern of the first thoracic segment. This central unit, roughly bilaterally symmetrical as is the whole dorsal pattern, was usually composed of 5 or 6 uncolored areas or "parts"—two larger parts each with a vertical portion (V in Fig. 2) and a divergent arm (A in Fig. 2) and 2–4 smaller, basal parts (B in Fig. 2) arranged in various ways with respect to the larger parts. When analyzing large numbers of animals, it was convenient to use diagrams of the pattern. Thus, Figure 2 is a diagrammatic representation of the pattern photographed in Figure 3.

FIGURE 2. Diagram of central part of pattern on first thoracic segment of a cyathuran from Pocasset River, Massachusetts. V = vertical portion of large part; A = divergent arm; B = basal part.

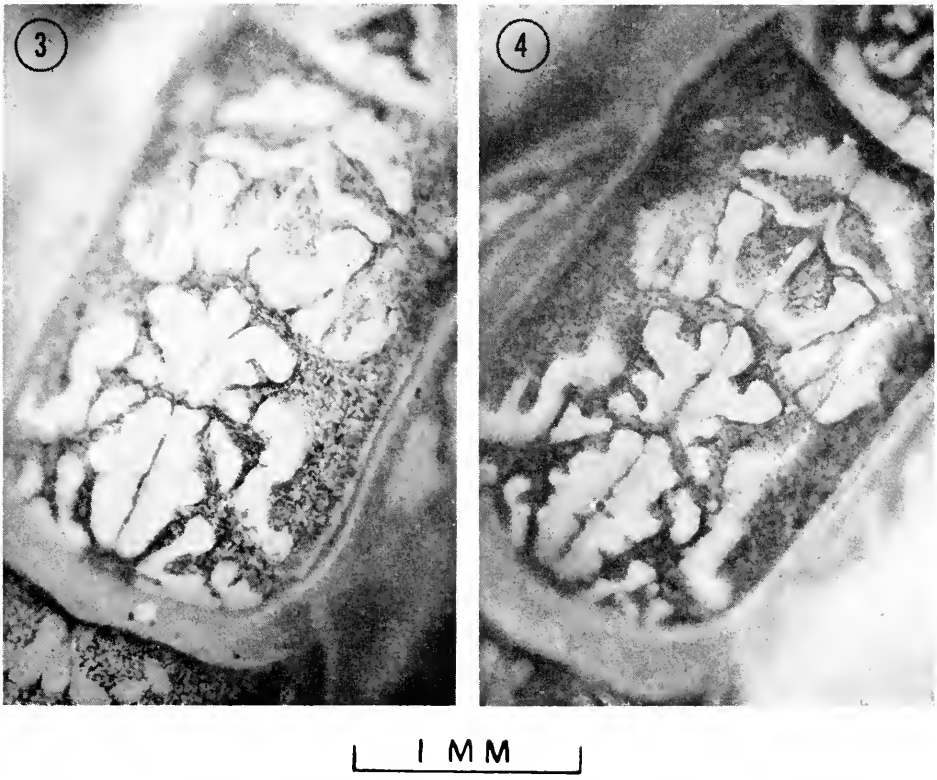


FIGURE 3. First thoracic segment of male collected at Pocasset River, Massachusetts, May 15, 1960.

FIGURE 4. First thoracic segment of a female collected at Silver Glen Springs, Jan. 2, 1960.

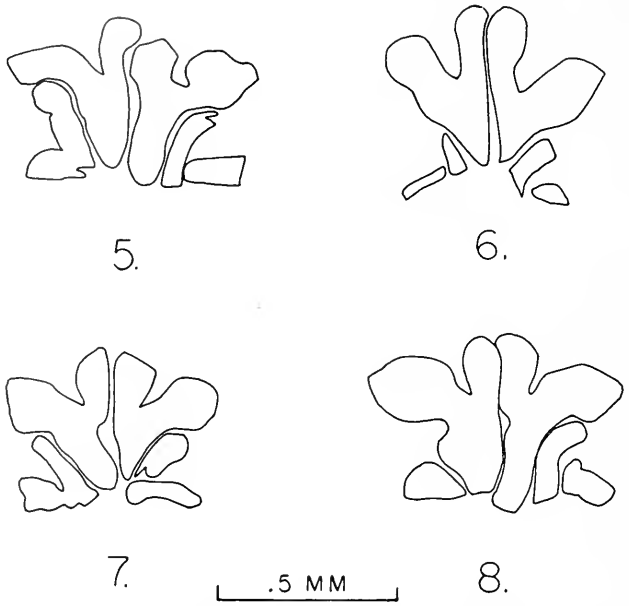
Juveniles about 8 mm. in length and larger showed as clear and characteristic a pattern as did adult animals. The dorsal patterns of very small animals had an "unformed" appearance and were not included in this study.

OBSERVATIONS AND RESULTS

An analysis of the patterns of the Lake George, Florida, and the Pocasset River, Massachusetts, animals revealed that there was a basic difference in the orientation of the parts of the pattern studied. In the southern animals, the arms of the pattern are oriented upward (Figs. 4 and 6), and in the northern animals one or both of the arms tend to angle horizontally or downward (Figs. 3 and 5). This difference is not absolute. Of the 95 Lake George animals examined, 82 animals showed the upward orientation; 11 were of the northern type, two with both arms horizontal and 9 with one arm oriented upward and the other horizontally; and in two animals the pattern was abnormal. A pattern was considered abnormal if it was fragmented into 10 or more parts or if it was fused and distorted from the usual shape. If there was abnormality in the part under analysis for this study, there was usually distor-

tion of the pattern of the whole animal. Of the 116 animals from Pocasset River, 93 showed the northern type of orientation, 65 with both arms horizontal and 28 with only one arm horizontal; 22 were of the southern type; and 2 were abnormal. Thus, 86% of the animals from Lake George exhibited the southern type of pattern and 79% of the Pocasset River animals were of the northern type.

The number of component parts of this central figure of the pattern of the first thoracic segment varies from 8 to a fusion into one single mass. The usual range is from 4–6 parts with the mode for the northern animals at 5 and for the southern animals at 6. This difference is not so striking, however, if the average number of parts is compared—4.93 for Pocasset animals, 5.40 for Lake George animals.



FIGURES 5–8. Camera lucida drawings of central part of pattern of first thoracic segments. Figure 5: Female from Pocasset River, Massachusetts, June 12, 1960. Figure 6: Female from Silver Glen Springs, Florida, Jan. 2, 1960. Figure 7: Female from upper reaches of Bass River, Massachusetts, Oct. 26, 1960. Figure 8: Male from Stony Brook, Massachusetts, Oct. 12, 1960.

In addition to the number of parts, the arrangement of the parts tends to differ with the geographical origin of the animals. In the Pocasset River animals, the part of the pattern studied tends to be "squared off" at the base; the two large parts extend down to a hypothetical base line and the small parts on either side rest on this "base line." (See Figs. 3, 5, 7, 8.) If both arms are horizontal with the upper edges roughly parallel with this base line, the whole effect is one of rectangularity. The pattern of Lake George animals tends to be elongated rather than squared. As already noted, the arms reach upward, often with a widely flared V between the arm and the central vertical part (Fig. 6). Instead of the small pieces being arranged at the base beside the vertical part, the lower end of the vertical part may be truncated and the small pieces arranged as a downward but interrupted continua-

tion of the vertical segment (Fig. 6), flaring out to the side but not lined up and squared off as in many of the Pocasset River animals. This difference in the arrangement of the parts of the pattern cannot be analyzed as precisely as the orientation of the arms or the number of the fragments but is recognizable in a majority of the specimens.

Two additional sizeable collections of *Cyathura polita* were made in the fall of 1960 in connection with other aspects of cyathuran research and were available for pattern analysis. The patterns of 51 animals collected by dredging the upper three-fourths of the Pocasset River substantiated the characterization of the Pocasset population as to the orientation of the arms and the squaring off of the basal parts, but patterns containing 5 and 6 parts occurred in about equal numbers. Forty-six cyathurans collected late in October in one sample from Stepping Stone Creek, north of Chatham on Cape Cod, were almost entirely northern as to orientation and arrangement of the pattern but again the number of parts did not exhibit a pronounced mode of five as did the spring collection of Pocasset animals.

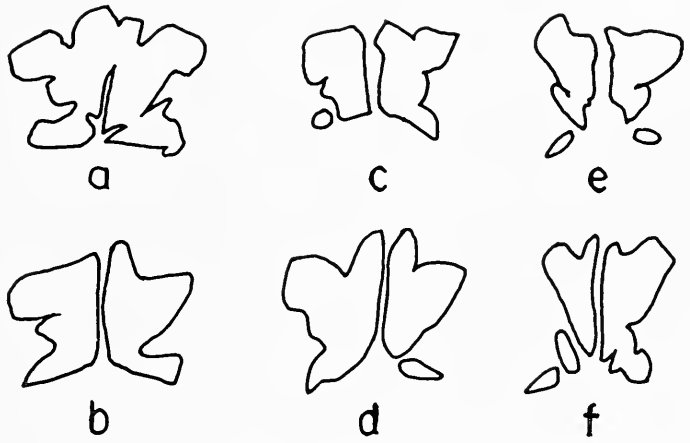


FIGURE 9. Sketches of patterns from animals from the extremes of the known range of *Cyathura polita*. a: Cider Hill Creek, Maine. b: Lake Pontchartrain, Louisiana. c and d: St. Louis Bay, Mississippi. e and f: Perdido Bay, Alabama.

A survey of animals collected along the Atlantic Coast of the United States corroborated the division of *Cyathura polita* into two groups on the basis of dorsal pattern. Although the samples examined were relatively small (1–15 animals with the exception of larger special collections from Cape Cod, Mass., and 26 animals from Beacon, N. Y.), animals from the Hudson River northward were chiefly of the northern type, and animals from New Jersey to Florida were predominantly of the southern type.

There was a tendency for animals at the extremes of the known geographical range of *C. polita* to show a modification of the basic design of the pattern. At the northern extreme in Maine the general rectangular, northern type of outline remained the same, but there was frequently a fusion of parts of the pattern even across the midline. In samples of only ten animals, there would be one animal in

which the part of the pattern being studied was fused into one piece (Fig. 9a). At the southwestern extreme along the Gulf of Mexico in Alabama, Mississippi, and Louisiana, there was a similar modification in that the number of parts was reduced and the upward orientation of the arms was less pronounced than in the Lake George animals. The pattern of animals from Lake Pontchartrain, Louisiana (Fig. 9b), was usually made up of two halves which were not further subdivided into parts and which were more suggestive in outline of the squared-off northern pattern than of the southern pattern as typified by the Lake George animals. Eastward from Louisiana, animals from Mississippi and Alabama showed patterns intermediate between the Louisiana and Lake George, Florida, types. The Mississippi animals were very similar to the Louisiana ones but half of them had the basal part of one side of the pattern separate from the main part (Figs. 9c, 9d). The Alabama animals were intermediate between the Florida type and the Mississippi and Louisiana patterns; in Figure 9e, the arms are suggestive of the northern and Louisiana type but the orientation of the parts at the base is as in Florida animals. Other Alabama animals were as in Figure 9f, where the outline is that of the southern type and the number of parts is less than is usual for the southern type of pattern but more than for Louisiana and Mississippi animals. The maximum number of parts (except for one abnormal pattern which had 6) for the ten Alabama animals was four, shown by five animals.

FIGURE 10. Sketches of patterns of three animals from Ashepoo River, South Carolina.

It was the observation of a fusion of parts similar to that seen at the extremes of the range of *Cyathura polita* which was noted in the animals from the Ashepoo River, South Carolina, and which initiated this study (Fig. 10). Comparison of the pattern of animals from the Ashepoo River with Maine, Pocasset River, Lake George, and Gulf of Mexico animals reveals that the orientation of the arms is that of the southern type and the fusion of the parts is similar to that of the Gulf and Maine animals with the additional feature of a high proportion of animals with the pattern fused across the midline.

Late in 1960, the opportunity arose to examine about 500 cyathurans collected in 34 estuaries of Cape Cod, Massachusetts, in connection with a study of the distribution of *Cyathura* in the Cape Cod area (Burbanck, 1961b). There were usually about 10 animals per sample, but in two instances there were about 50 and in three other collections there were 29–30 animals each. The northern type of pattern was present in 93% of these animals, and the average number of parts was 5.0. The general aspect of the majority of the patterns was rectangular as may be considered characteristic of the northern type of pattern, but there was a variation recognizable in enough instances to suggest that it might be a Cape Cod variant, a modified northern type. Particularly in the collections from two relatively isolated

populations, the upper reaches of Bass River (29 animals) and Stony Brook (11 animals), there were animals with a pattern different from the typical Pocasset River animals (Figs. 7, 8). Of the 29 animals collected from the run-off from Mill Pond in the upper reaches of Bass River, only in the patterns of 9 animals were both arms directed horizontally. In 16 animals one arm was horizontal with roughly parallel sides but the other was neither directed upward as in the southern type nor horizontally or downward with parallel sides as in the typically northern pattern. Not only was this an unusually high percentage of animals with a half-and-half orientation of the arms, but the one northern type arm was usually not as extended as in half-and-half animals from Pocasset River (compare Figures 5 and 7).

Collections from nearby Lake Tashmoo and Lagoon Pond on Martha's Vineyard Island, Massachusetts, made in 1959 and 1960 were also of the northern type. The patterns of those from Lagoon Pond resembled those from the extremes of the range in that the number of parts was reduced, and in one collection of 12 animals, the pattern was fused across the midline in 7 of them.

Whenever collections were made from the same locality over a period of several years, the patterns did not vary appreciably. Animals collected from the Silver Glen Spring area of Lake George, Florida, in 1956, 1959, and 1960 all exhibit patterns which are predominantly of the southern type with very little variation. Collections from Pocasset River have been made yearly since 1955 and the pattern of all animals examined has been consistent within a normal range of variation regardless of the year of collection. Even some of the peculiarities of pattern observed in certain Cape Cod animals collected in 1960 were present in earlier collections from the same places.

Discussion

The persistence over a period of years of a recognizable difference in pattern suggests that the pattern design may be an inherited character. With such a wide range of climatic conditions from Louisiana, around Florida, and north to Maine, it was anticipated that the chromatophore pattern might vary and that there might be populations which could be considered distinct races within the species. Specimens of *C. polita* from its whole known geographical range have been examined and found to be similar enough morphologically to be considered one species (Miller and Burbanck, 1961). The variability of the chromatophore pattern, however, and the correlation of two distinct pattern types with geographical distribution, raises the question whether there may be ecotypes within the species *Cyathura polita.*

Three of the four levels of variability postulated by Clausen and Hiesey (1958) in their analysis of ecological races in the *Potentilla glandulosa* complex are recognizable in *Cyathura polita.* There is variation of pattern within the local populations which is the first level of variability according to Clausen and Hiesey. The second level is that of varying, isolated, local populations, within the same climatic range and under similar edaphic conditions. The populations of *Cyathura* along the Gulf coast were distinct as to pattern design but living under similar environmental conditions. The variation noted on Cape Cod may also be at this level as may be the population from Lagoon Pond, Martha's Vineyard Island. The

third level of variability is defined as that distinguishing populations in climatically or edaphically different habitats. In the case of *Potentilla glandulosa* they were ecotypes with physiological differences sometimes accompanied by morphological differences. The existence of a northern type pattern and a southern type in *C. polita* is suggestive of this level of variability. Further investigation needs to be made of other morphological variations, such as the anatomy of the appendix masculinum (Miller and Burbanck, 1961) and chromosome numbers, and physiological studies will be needed of the tolerances of the animal before the hypothesis of the existence of ecotypes within the species can be verified. The fourth level of variation distinguished in *Potentilla* is the establishment of morpologically and physiologically distinct taxonomic subspecies. On the basis of present knowledge, there is no evidence that *Cyathura polita* has reached this level of variability and differentiation.

Until more is known about the breeding habits of *C. polita* and animals can be successfully kept under laboratory conditions for longer periods of time than at present, a genetic analysis of the inheritance of pattern types cannot be made. The present picture is suggestive of a wide-ranging, interbreeding (?) population within which have evolved two types of dorsal pattern under two different types of climate, northern and southern. The pattern types may form a graded series or cline, but to confirm this it would be necessary to examine larger collections of animals from the populations between Cape Cod and Lake George as well as from the extremes of the range. There is evidence of specialization of the pattern at the extremes of the range and in isolated populations of both the northern and southern type animals. The regional peculiarity of pattern noted in the cyathurans from the Ashepoo River, South Carolina, was an example of the development of variation in an isolated population, a population which disappeared after a hurricane struck this area in the fall of 1959 (Burbanck, 1961).

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EFFECTS OF NEURAL ACTIVITY ON THE FIREFLY PSEUDOFLASH[1,2]

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The pseudoflash of the adult firefly, first recorded by Snell in 1932, is produced by subjecting the animal to a sudden increase in oxygen concentration after anoxia. A dull, hypoxic glow develops during anoxia and spreads over the entire organ, gains in intensity, then slowly declines to extinction. If air is readmitted at any time during the hypoxic glow, there occurs a brilliant pseudoflash of one second or longer. According to Snell and later, Alexander (1943), the pseudoflash reflects the unimpeded entry of oxygen into the luminous tissue through supposedly valvular tracheal end cells (Dahlgren, 1917), the valves being temporarily inactivated by hypoxia. This theory implies that firefly luminescence is normally oxygen-limited and that the pseudoflash is independent of neural activity.

McElroy and his associates (summarized in McElroy and Hastings, 1955) suggest the possibility of luminescence control by chemical reactions not directly involving oxygen, so that the light organ may be oxygenated at all times. Further, these workers have proposed a flash-generating mechanism by which the nerve impulse brings about release of pyrophosphate which in turn stimulates the light-yielding reaction.

Hastings and Buck (1956) concluded from experiments on decapitated adults, isolated light organ segments and excised photogenic organs, that nerve impulses originating in the central nervous system played no role in the photogenic response to hypoxia since in all these preparations an hypoxic glow developed in low oxygen concentrations and a pseudoflash resulted with readmission of air. They further noted, as had others previously, that pseudoflash and hypoxic glow involved only one or a portion of one segment in some cases, that some animals failed to give either response, and that considerable variation in both responses occurred even within one individual.

The variations of hypoxic response of individual fireflies, ranging from no response during quiescent periods to complete, two-segment responses in actively flashing animals, indicate that factors besides hypoxia play a role in pseudoflash production. A number of observations, previously reported in abstract form (Carlson, 1959), strongly implicate the nervous system. The purpose of this

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[3] The research reported here was completed in the Department of Zoology, State University of Iowa, and at The Marine Biological Laboratory, Woods Hole, Massachusetts.

report is to present in detail these observations concerning the role of the innervation in pseudoflash and hypoxic glow.

Materials and Methods

Seventy-four adults of two firefly species, *Photinus pyralis* Linn. and *Photuris* sp., were the subjects of this study. Fireflies were exposed to various concentrations of oxygen in nitrogen and to pure nitrogen. The experimental animal was secured ventral side up on a narrow glass spatula provided with platinum stimulating electrodes. The spatula was placed immediately next to the gas inlet in a glass tube one foot long and one centimeter in diameter. A photomultiplier tube (RCA 931-A) and dissecting microscope were positioned above the gas chamber and both were shielded by black cloth from stray light. Gases entered the chamber through a Y-tube provided with stopcocks arranged to permit rapid alternation of two gas mixtures. Gases were prepared from commercial compressed nitrogen and oxygen metered through two-stage reduction valves and calibrated Fischer-Porter flow meters. Mixture composition was checked by gas analysis with a Scholander 0.5-cc. analyser.

In preparation for a pseudoflash one valve was rotated 180° to shunt off the oxygen and admit either pure nitrogen or a nitrogen-oxygen mixture containing

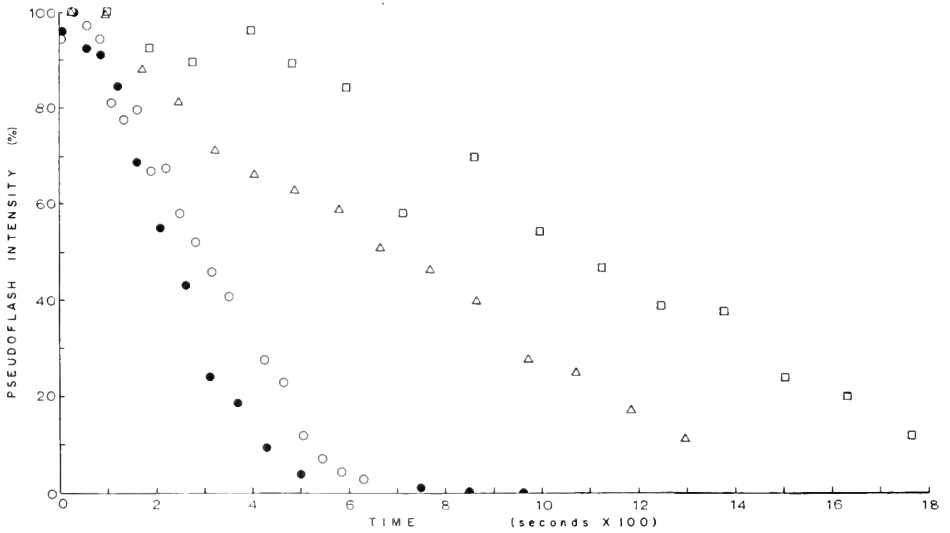

Figure 1. Decrease of pseudoflash intensity in successive pseudoflashes induced at hypoxic glow maximum with recovery periods of constant duration in adult males, *Photinus pyralis*. Ordinate: Pseudoflash intensity in per cent of most intense pseudoflash in each series.

In this and following graphs open circles denote successive pseudoflashes with a constant air recovery period of 10 seconds. Closed circles denote a second run with the same animal immediately after inducing the animal to flash and with a constant air recovery duration of 11 seconds. Squares denote successive pseudoflashes in another animal with a constant air recovery duration of 60 seconds. Triangles denote successive pseudoflashes in a third animal with a constant air recovery duration of 30 seconds. Pseudoflashes in all cases were induced with oxygen concentration of 17.25%. Oxygen concentration during hypoxia was 0.5%.

less than 2.5% oxygen. Rotation of this stopcock also opened a signal circuit. At an appropriate time the same valve was then rotated back 180° which allowed a higher concentration of oxygen to reach the animal suddenly and also closed the signal circuit. The pseudoflash was detected by the photomultiplier, and its output was led to one or both channels of an Offner Dynograph.

Nerve action potentials in the adult light organ during pseudoflash generation were recorded with the animal in a chamber with inlet and outlet tubes and covered with a transparent plastic sheet. Uninsulated platinum recording electrodes were placed on the light organ surface after cuticle removal. Action potentials were led through an AC amplifier to one beam of an oscilloscope. The other beam recorded the photomultiplier output.

Results

1. *Induction of pseudoflashes in Photinus adults*

In actively flashing animals hypoxia initially produces a dark period of five seconds or less, followed by an hypoxic glow, which begins suddenly as a dull glow over the entire lantern, then gradually and uniformly brightens. Pseudoflashes induced after the hypoxic glow develops are of high intensity. In quiescent, non-flashing adults either no hypoxic glow and pseudoflash can be induced even with prolonged hypoxia, or a very dull, spotty, hypoxic glow develops after a dark period lasting for minutes. The subsequent pseudoflash is greatly reduced as compared with those induced in individuals with bright hypoxic glows. If the quiescent animal is first stimulated to flash, its capacity for developing a bright hypoxic glow and pseudoflash is greatly enhanced.

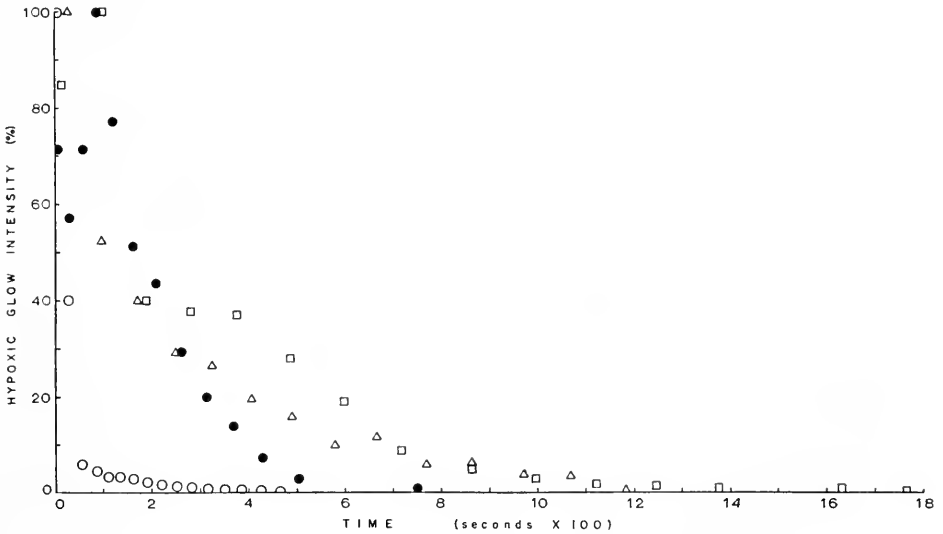

Figure 2. Decrease of hypoxic glow maximum intensity with successive pseudoflashes and with recovery periods of constant duration in adult males, *Photinus pyralis*. Ordinate: Hypoxic glow intensity in per cent of most intense hypoxic glow in each series.

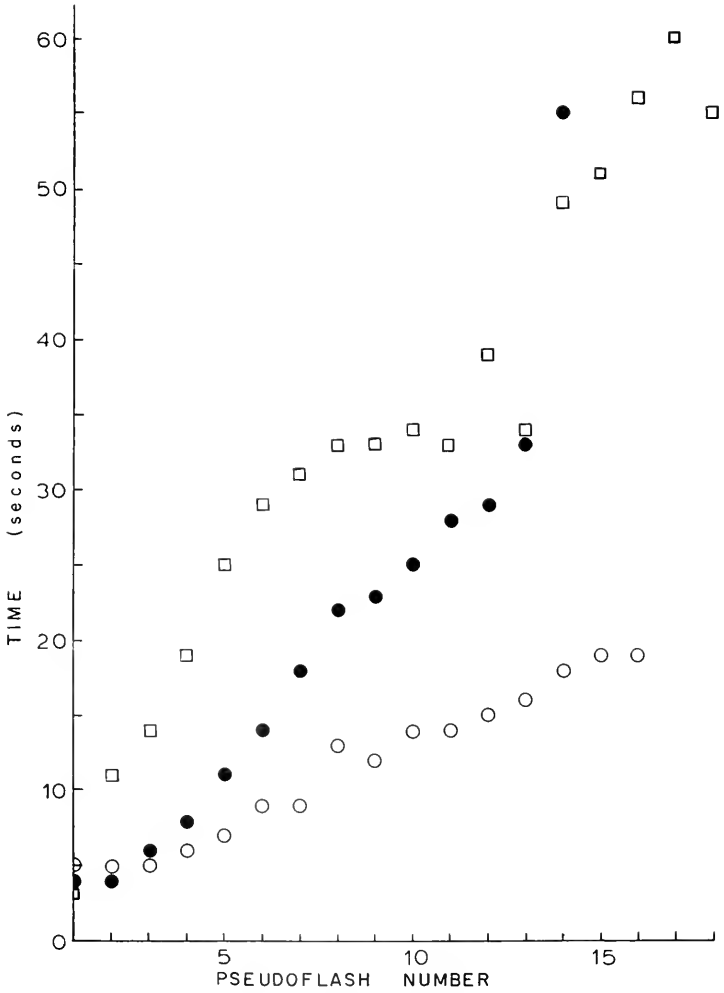

FIGURE 3. Increase in duration of hypoxia prior to the onset of the hypoxic glow in successive pseudoflashes in adult males, *Photinus pyralis*. Ordinate: Hypoxic duration prior to hypoxic glow onset in seconds.

In spontaneously flashing *Photinus* adults a large number of successive pseudoflashes of high intensity can be induced. However, quiescent animals exhibit a characteristic series of changes during successive pseudoflashes which strongly suggest neural involvement. If such animals are first induced to flash spontaneously, then exposed to a given hypoxic oxygen concentration during attainment of hypoxic glow maximum, alternating with fixed periods of exposure to air, the pseudoflashes produced go through the following progressive changes:

(1) Pseudoflash intensity slowly declines (Fig. 1). (2) Hypoxic glow intensity rapidly declines initially, then declines at a slower rate (Fig. 2). (3) The

time required from onset of hypoxia to onset of the hypoxic glow increases (Fig. 3). (4) The time from onset of hypoxia to the attainment of maximal hypoxic glow increases (Fig. 4). (5) Animals that have undergone these changes can be restored to their original condition of intense hypoxic glow and pseudoflash by stimulating mechanically or electrically until they begin to flash spontaneously (Figures 1, 3 and 4; open and closed circles showing two successive runs with the same animal). The rate at which these progressive changes occur is highly variable not only among different individuals but also within the same individual at different times. The graphs indicate the range of effects observed in a total of 30 runs on 13 individuals studied.

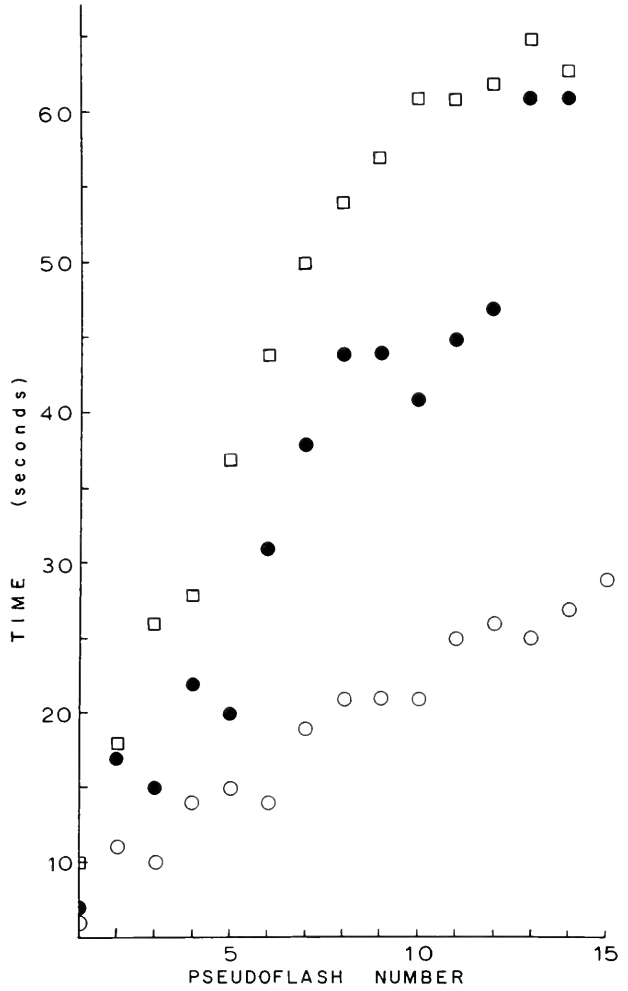

FIGURE 4. Increase in duration of hypoxia prior to attainment of maximum hypoxic glow intensity with successive pseudoflashes in adult males, *Photinus pyralis*. Ordinate: Hypoxic duration prior to hypoxic glow maximum intensity in seconds.

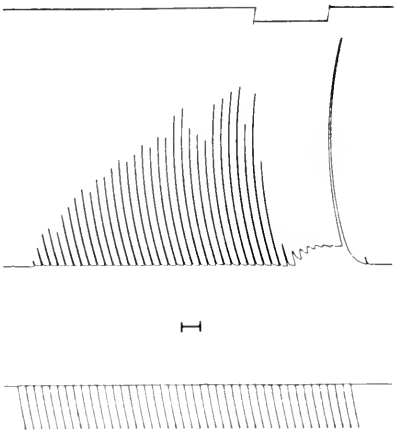

FIGURE 5. Electrically excited neural flashes in air and during hypoxia, followed by a single pseudoflash in an adult male, *Photinus pyralis*. Time axis reads from left to right. Top trace: up, 17.9% oxygen; down, .065% oxygen. Middle trace: light intensity. Bottom trace: stimulus signal. One stimulating electrode in anterior abdomen and other dorsal to light organ through anus. Stimulation: 8 volts, 50-msec. duration, frequency 0.5 per second. Heavy horizontal line represents 5 seconds on this and all subsequent figures except where otherwise noted.

2. *Effects of electrical stimulation on hypoxic glow and pseudoflash*

Electrical stimulation of a firefly in air normally produces a facilitating series of flashes. When stimulation continues into the hypoxic period, flashes diminish and a high intensity hypoxic glow appears (Fig. 5). A pseudoflash can be induced

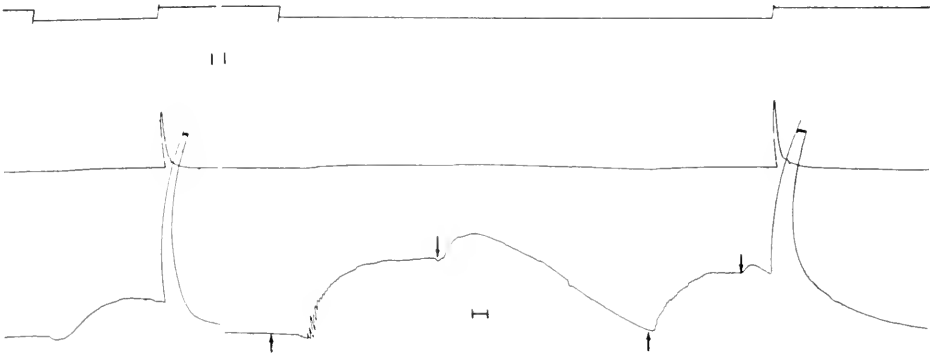

FIGURE 6. Effect of electrical stimulation during hypoxia upon hypoxic glow in an adult male, *Photinus pyralis*. Top trace: up, 17.25% oxygen; down, .05% oxygen. The middle and bottom traces both indicate light intensity, the bottom trace at 10 times the amplification of the middle trace. Stimulating electrode pair in abdomen dorsal to light organ. Stimulation: 5 volts, 50-msec. duration, frequency 1 per second. Short parallel lines indicate a break in the record. In this figure and subsequent figures where used, arrows pointing up indicate stimulus onset and arrows pointing down indicate stimulus cessation. The figure shows first an anoxic sequence without electrical excitation followed by two stimulus trains during hypoxia. In last two episodes glow rose temporarily after stimulation ceased.

as soon as electrically excited flashes undergo an increase in duration, which appears to be characteristic of the hypoxic state. Electrical stimulation during hypoxia increases the rate of hypoxic glow rise and intensity of glow attained (Fig. 6). It will also be noted that when stimulation ceases a rapid brightening usually occurs followed by a decline, an effect which is typically observed. The hypoxic glow was maintained in one animal for 20 minutes by periodic stimulation in 0.6% oxygen; without stimulation it might be expected to persist less than two minutes.

Although prolonged stimulation during hypoxia favors a pseudoflash of increased duration, stimulation, when continued during readmission of air, superimposes no discrete flashes on the pseudoflash rise. However, this may occur during pseudoflash decay as illustrated in Figure 7. Electrical stimulation commonly augments the hypoxic glow by recruiting new, discrete luminescing areas

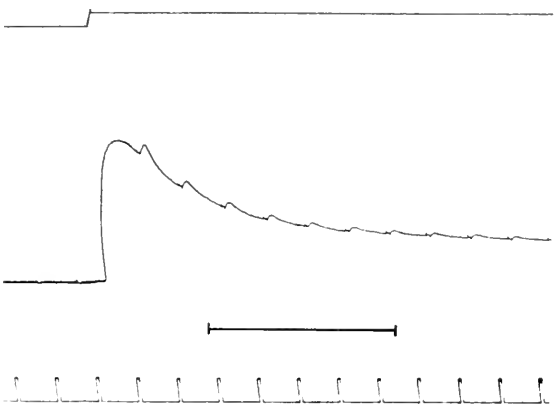

FIGURE 7. Electrical stimulation during readmission of 17.75% oxygen and consequent pseudoflash in an adult male, *Photinus pyralis*. Top trace: up, 17.75% oxygen; down, .05% oxygen. Middle trace: light intensity. Bottom trace: stimulus signal. Stimulating electrode pair placed in sides of abdomen dorsal to light organ. Stimulation: 50 volts, 40-msec. duration, frequency 1 per second. Tiny marks on middle trace most apparent during hypoxia are stimulus artifacts via ground circuit, not light emission.

of the lantern in serial fashion, one or more areas per pulse. The effect is illustrated in Figure 8; each pulse initiates luminescence which covers one additional area completely.

3. *Effects of an anticholinesterase and of denervation on hypoxic glow and pseudoflash*

In order to examine further the role of possible neural activity on the pseudoflash, the effects of injection of an anticholinesterase and of nerve transection on the hypoxic glow and pseudoflash were studied. Eserine, 10^{-4} *M*, in Roeder's saline (Roeder, 1953), injected into the body cavity of the decapitated adult, after removing the tip of the abdomen to permit flow of the solution through the body,

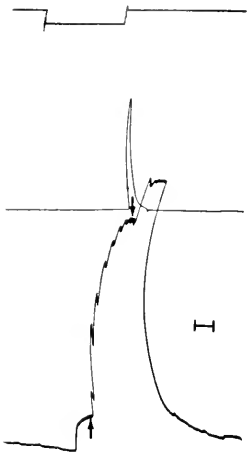

FIGURE 8. Effects of electrical stimulation during rising phase of hypoxic glow in an adult male, *Photinus pyralis*. With each shock hypoxic glow augmented as new areas begin to glow and remain glowing. Same effect can be noted in Figure 6, after first arrow. Top trace: up, 17.25% oxygen; down, .05% oxygen. Middle and bottom traces: light intensity, amplification of bottom trace being 1000 times middle trace. Stimulating electrode pair on right and left sides of seventh abdominal segment dorsal to light organ. Stimulation: 10 volts, 50-msec. duration, frequency 1 per second.

produces nearly continual high intensity scintillation and flashing in air (Case and Buck, 1959). Under these conditions the rate of rise and maximal intensity of hypoxic glow are increased greatly. Pseudoflashes of normal characteristics can be induced after as little as 5 seconds of hypoxia (Fig. 9).

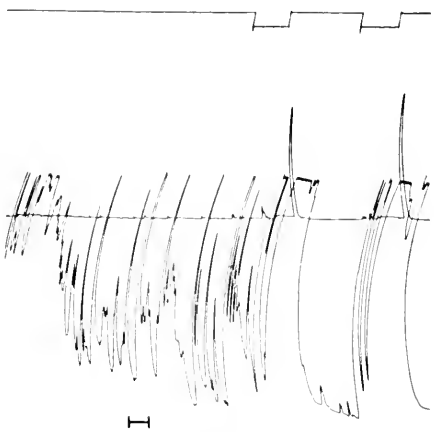

FIGURE 9. Effect of perfusion of 10^{-4} *M* eserine through a decapitated adult male, *Photinus pyralis*. Note the continual scintillation and rapid onset of hypoxic glow. Top trace: up, 17.25% oxygen; down, .05% oxygen. Middle and bottom traces: light intensity, amplification of bottom trace being 200 times middle trace.

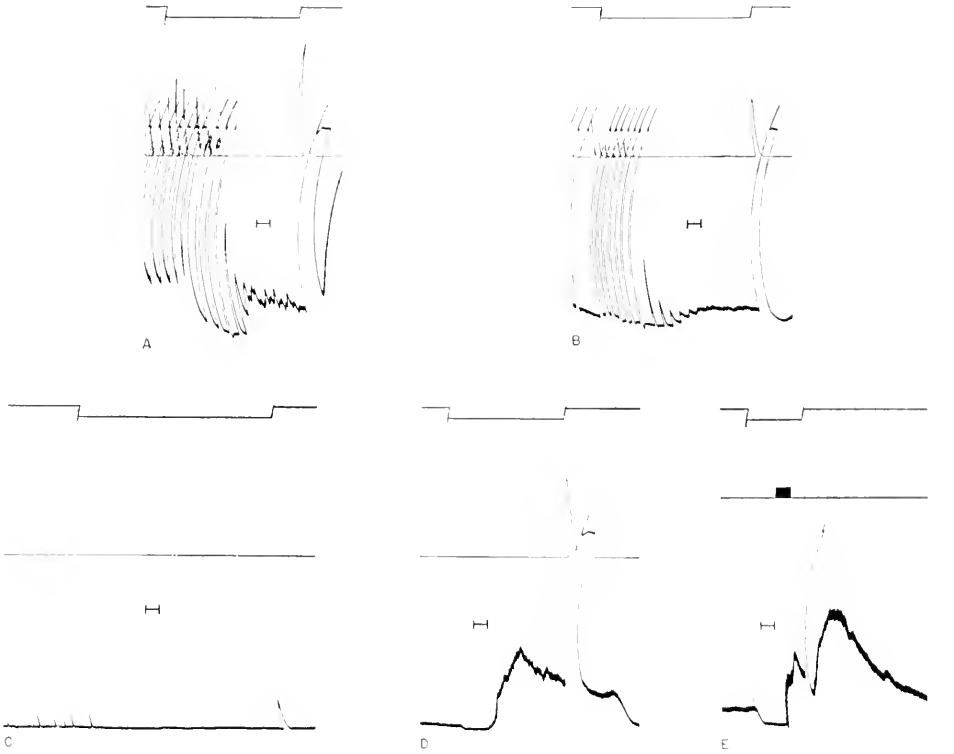

FIGURE 10. Effect of transection of the ventral nerve cord between the sixth and seventh abdominal segments of a female, *Photuris sp.* Except where noted otherwise the following conditions were maintained. Top trace: up, 21% oxygen; down, .05% oxygen. Middle and bottom traces: light intensity except where otherwise noted, amplification of bottom trace being 500 times middle trace. (A) Normal pseudoflash, both sixth and seventh abdominal segments give a total flash. Spontaneous flashing is seen in this figure, as well as in B, during onset of hypoxia. (B) Normal pseudoflash, sixth segment masked. Note the reduction in intensity of the pseudoflash since only the seventh segment is being recorded. (C) Inability to induce pseudoflash in segment 7, 8 minutes after transection, sixth segment masked. Top trace: up, 21.4% oxygen; down, 0.4% oxygen. Light leak from sixth segment appears as small flashes on left, bottom trace. (D) Pseudoflash 20 minutes after transection, seventh segment masked, showing response in sixth segment still normal. Amplification of light signal in bottom trace, 1000 times middle trace. (E) Pseudoflash 45 minutes after transection with stimulation, sixth segment masked. Stimulating electrode pair inserted laterally into the seventh segment dorsal to the light organ. Middle trace: stimulus signal. Bottom trace: light intensity with same amplification as before. Stimulation: 15 volts, 20-msec. duration, frequency 20 per second.

In some instances, transection of nerves supplying the light organ of abdominal segment 7, at the level of the intersegmental membrane between segments 6 and 7, abolishes spontaneous flashing in the seventh segment and leaves unaffected the light organ of segment 6. Gradual reduction of hypoxic glow and pseudoflash intensity occurs in segment 7 until both are completely abolished after two hours, while the response in segment 6 can be maintained by mechanical irritation, eliciting

spontaneous flashes. In other cases, particularly in *Photinus,* the pseudoflash is immediately abolished in segment 7, except in one or two tiny spots which gradually lose the ability to generate pseudoflashes. Stimulation with electrodes placed dorsally in the seventh (denervated) segment partially restores the pseudoflash and hypoxic glow. The hypoxic glow can also be intensified by stimulation during hypoxia as in the normal light organ (Fig. 10).

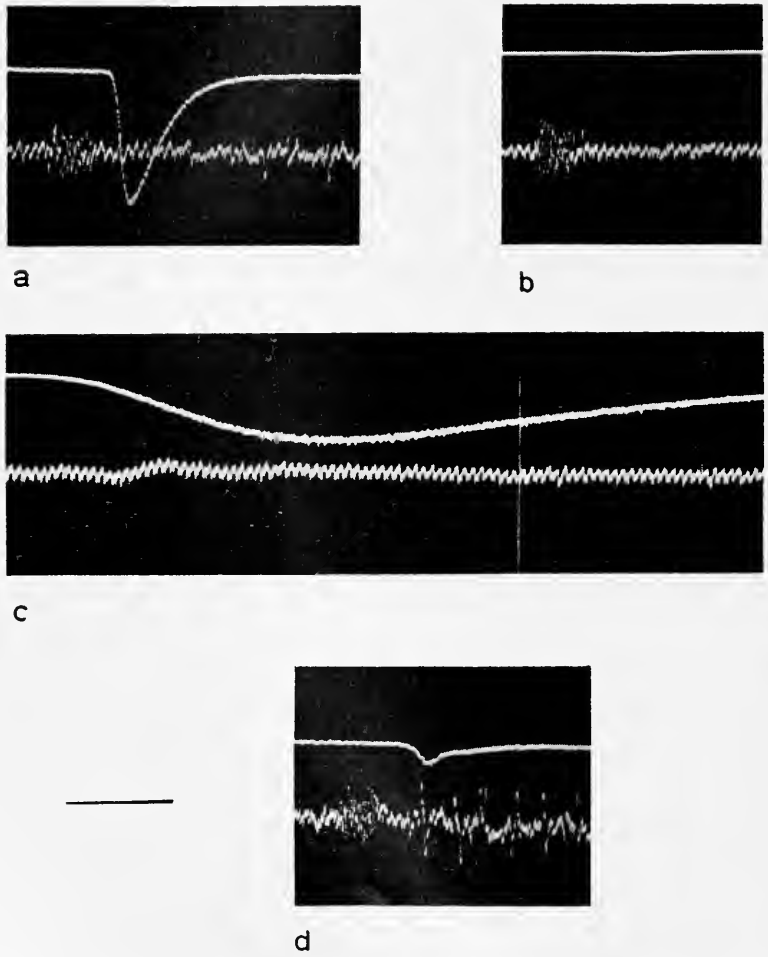

FIGURE 11. Recording of neural action potentials, spontaneous flashes, and pseudoflash in the lantern of an adult male, *Photuris pennsylvanica.* Photomultiplier output on upper trace (increasing intensity down). Action potentials on lower trace. Time base indicated by horizontal, dark line which represents 100 milliseconds. Time base reads from left to right. (a) Spontaneous flash and generating neural burst prior to hypoxia. (b) Extremely reduced spontaneous flash and preceding neural burst after hypoxia onset. (c) Pseudoflash induced with air. Neither neural nor muscle action potentials are seen. (d) Spontaneous flash and preceding neural burst following pseudoflash in air recovery period.

4. *Demonstration of lack of spontaneous neural activity during pseudoflash*

In order to further define the role of the nerves in pseudoflash generation, nerve action potentials were sought during the hypoxic period and pseudoflash by placing recording electrodes on the exposed ventral surface of the light organ. The results are illustrated in Figure 11. The animal was flashing spontaneously prior to the onset of hypoxia and before each flash a discrete burst of neural activity was noted (Fig. 11a). Immediately after onset of hypoxia two flashes of reduced magnitude were observed, each associated with an undiminished train of action potentials (Fig. 11b). Following these flashes no bursts or even isolated nerve impulses could be observed. Although no hypoxic glow was observed, a pseudoflash was produced without associated action potentials (Fig. 11c). Spontaneous flashes and associated neural bursts resumed immediately following the pseudoflash in air (Fig. 11d).

Discussion

These observations on active, quiescent, electrically stimulated and eserine-injected animals, and the results of nerve transection, strongly implicate the innervation in the pseudoflash. Our inability to detect nerve impulses during the hypoxic glow period, however, indicates that individual pseudoflashes in the intact animal can be induced independently of neural activity. One supposition seemingly warranted by the decline of successive pseudoflashes in quiescent animals is that previous neural activity serves as a priming mechanism. A priming activity with regard to spontaneous activity has been suggested by Case and Buck (1959).

The priming mechanism of neural activity on hypoxic glow and pseudoflash can be explained on the basis of the scheme of firefly luminescence developed by McElroy and his associates. Hypoxia may allow substrate accumulation from precursors in the intact animal just as it does *in vitro* as shown by Hastings, McElroy and Coulombre (1953), who were able repeatedly to induce flashes similar to pseudoflashes by alternating hypoxic and aerobic conditions until surface denaturation of the enzyme occurred. The reduction of intensity in successive pseudoflashes in our experiments would represent, according to this scheme, accumulation of an inhibitory complex. When pseudoflashes can no longer be induced, presumably all the enzyme is inhibited. As McElroy and Hastings (1955) speculated, one result of neural stimulation might be release of inorganic pyrophosphate which on the basis of *in vitro* observations would free the active enzyme and result in the ability to again induce high intensity pseudoflashes.

If nerves operate in fact as a priming mechanism by mediating destruction of an inhibitor, a number of observations of previous investigators could be explained. This action might explain why it is difficult or impossible to elicit pseudoflashes in quiescent animals since in them the active enzyme would be completely inhibited. This suggests that during the daily non-flashing periods the light organ would contain large amounts of this inhibitor, and neural activity would be low or absent. The great deal of areal variation observed in the pseudoflash response may simply be the result of spotty neural priming. This is supported by the observation that a total organ pseudoflash is usually obtained in actively flashing animals and by the observations that electrical stimulation during

hypoxia in quiescent animals commonly augments the hypoxic glow by recruiting new, discrete luminescing areas (Fig. 8).

Summary

1. Hypoxic glows and pseudoflashes were studied in adults of the lampyrid fireflies, *Photinus pyralis* Linn. and *Photuris* sp.

2. In spontaneously flashing *Photinus* adults a large number of successive pseudoflashes of high intensity can be induced. Induction of pseudoflashes in quiescent animals results in decline of successive pseudoflash and hypoxic glow intensities, and increase in the time required not only for hypoxic glow onset but also for development of maximal hypoxic glow intensity.

3. Electrical stimulation during hypoxia increases the rate of hypoxic glow rise, commonly by recruiting new luminescing areas within the lantern, and can maintain the hypoxic glow beyond the normal period. Electrical stimulation when continued during readmission of air superimposes flashes on the pseudoflash decay phase only.

4. Perfusion of the body cavity with 10^{-4} *M* eserine enhances the ability to induce high intensity pseudoflashes and hypoxic glows.

5. Transection of the nerves supplying the seventh abdominal segment reduces the ability of that segment to produce pseudoflashes, while pseudoflashes can be obtained from that portion of the lantern occupying the sixth segment. Electrical stimulation of the seventh segment after nerve transection can partially restore its pseudoflash capability.

6. No nerve impulses were observed during the hypoxic interval immediately prior to and during induction of the pseudoflash although neural bursts and their associated flashes were noted before and after hypoxia and during its early period.

7. The observations implicate the innervation in the pseudoflash. A possibility suggested is that previous neural activity serves as a priming mechanism by mediating the removal of a chemical inhibitor.

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EUGLENA GRACILIS IN SYNCHRONOUS DIVISION. II. BIOSYNTHETIC RATES OVER THE LIFE CYCLE

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Mapping of the major metabolic and synthetic events occurring over the course of the life cycle of single cells forms one of the principal endeavors of current physiological efforts, and very refined (albeit tedious) techniques have permitted notable successes in this area. However, the small size of growing cells imposes serious quantitative—and qualitative—limitations on the information to be gleaned from this approach, since many of the coarser techniques require masses several orders of magnitude above that provided by the single cell. Furthermore, natural variability of cells grown under identical conditions—best exemplified perhaps in the scatter of individual generation times (Powell, 1955; Prescott, 1959)—can often be extensive. The use of synchronously-dividing populations as a model of the single cell promises to surmount some of these difficulties, though new ones may well be introduced.

That populations of cells can be induced to divide rhythmically has been known for some time (von Denffer, 1949), but a clear appreciation of the possibilities offered by such systems appeared only within the last decade (Hotchkiss, 1954; James, 1954; Scherbaum and Zeuthen, 1954). It has already been made the subject of several review articles (Scherbaum, 1960; Zeuthen, 1958). Increased interest in this field has resulted in the elaboration of certain obvious restrictions which should be satisfied by the synchronously-dividing population before it may fairly be viewed as a working model of the single cell. For example, the time consumed in the burst of division activity should be relatively short compared to the over-all generation time; and the increase in population number occurring in each burst should be commensurate with the normal division behavior of member cells, if the division burst is to be equated to division of the single cell. These are but two of the more obvious parameters which characterize synchronous cell division. More subtle restrictions include continued and repetitive division bursts, *i.e.,* populations in which synchrony can be maintained indefinitely (as long as the environmental stimulus causing synchrony is continued) (Padilla and James, 1960). A first corollary of continued division synchrony is the "balanced growth" of all member cells, in the usage of Barner and Cohen (1956). A further restriction is always implied, namely that growth of member cells in the population be

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at least as synchronous as their division. Unfortunately, none of the cell types thus far synchronized can completely satisfy all these restrictions; as noted by Zeuthen (1958), however, even a modest degree of synchrony can be useful.

It is possible to synchronize division activity of free-living photosynthetic cells, at a constant temperature, merely by growing the cells autotrophically on light-dark cycles (Sweeney and Hastings, 1958: Tamiya *et al.*, 1953). If the light and dark periods are of appropriate lengths, the average cell grows uninterruptedly over one whole generation, and stores sufficient energy in the light period to satisfy demands for those processes culminating in cell division (Bernstein, 1960; Cook and James, 1960). In all photosynthetic systems so grown, the "burst" of division activity is confined for the most part to the dark period, so that growth and division are truly separated.

In populations of *Euglena gracilis*, the percentage of cells completing division in successive bursts gradually decreases with increased population density, presumably because mutual shading limits effective utilization of the photosynthetic apparatus. At population densities below about 5000 cells per ml., 90–95% of the cells complete division at each burst; if the culture is diluted once in each generation with fresh salt medium (at the temperature of the culture), so that the population density never approaches levels of the stationary phase, division synchrony can be maintained without abatement for long periods of time, perhaps indefinitely (see Figure 1). It can be seen from Figure 1 that divisions commence

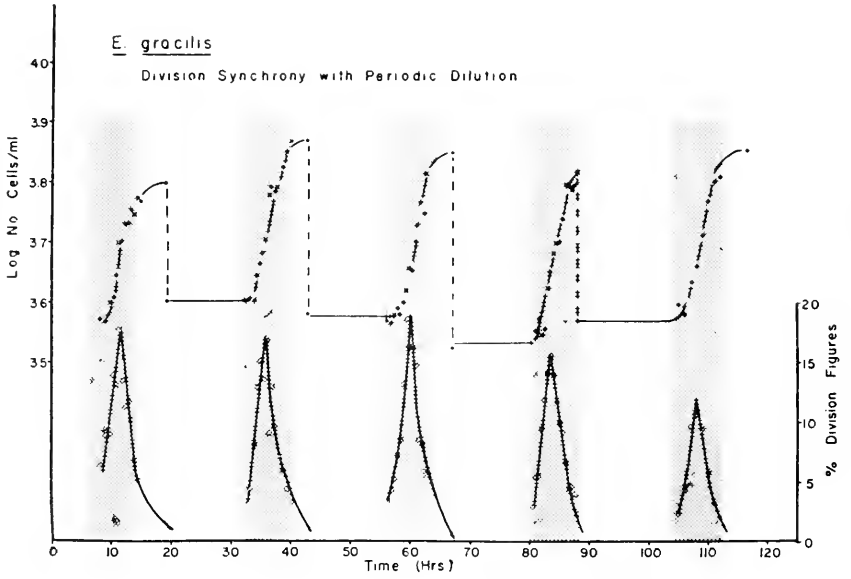

FIGURE 1. Light-induced division synchrony of *Euglena gracilis*. The upper curve is a plot of the logarithm (to the base 10) of the population density. The 8-hour dark periods, to which nearly all of the cell divisions are confined, are represented by the shaded areas. The light periods are of 16 hours duration. The broken lines indicate dilution with fresh salt medium at the temperature of the culture (20° C.). The lower curve shows the proportion of cells in recognizable fission.

at about the third hour of each dark period, and are terminated some two or three hours after the onset of the subsequent light period. Except for this very early portion, no cell divisions occur in the light period, which has a total duration of 16 hours. Of those cells completing division, 95% do so within a 6-hour span of time (James, 1960). Thus, about 75% of the total generation time is devoid of cell divisions, and most of this fraction is spent in the light, where major biosynthetic activities of *Euglena* should occur. Since the division synchrony of *Euglena gracilis* is well-defined and repetitive, the metabolic regulation of synthetic rates must be in fine adjustment at all ages, especially since the energy supplied (as light) is just that needed for growth and division of the average cell over one life cycle. Characterization of growth patterns in synchronized *Euglena* is therefore profitably made, and cultivation on a near-basal medium, where the photosynthetic apparatus itself must be duplicated, perhaps renders such a study all the more meaningful. Moreover, synchronous growth at a constant temperature obviates difficulties associated with the interpretation of temperature-sensitive rate processes, and thus circumvents some of the criticisms which have been leveled against other methods of synchronizing cell division (Abbo and Pardee, 1960; Mitchison and Walker, 1959; Prescott, 1960).

Mass increase and the distribution of volumes in member cells of synchronized *Euglena* populations have been reviewed in a previous paper, and the analogy of the population as a single cell discussed in some greater detail (Cook, 1961). While a wide range of cell volumes exists at any time, the (weighted mean) volume and dry mass exhibit a linear increase, to a doubling, over the whole of the 16-hour light period. The average *Euglena* in synchronous populations, then, is to a first approximation a successful working model of the single cell. The present report will describe growth patterns of other important cell parameters over the life cycle.

Methods and Materials

Culture methods have been described in detail previously (Cook and James, 1960). A constant-temperature water bath (American Instrument Co.) circulated water continuously through a thin transparent plastic jacket surrounding the cylindrical culture vessel, so that the culture was maintained at 20° C. An in-line reservoir of fresh medium, also kept at 20°, was used to dilute the culture once in each generation (see Figure 1), so that the population density at harvest always lay between 3000 and 5000 cells per ml., well below levels of the stationary phase. While this approach is only quasi-chemostatic, it is not likely that the medium ever became limiting for growth of *Euglena;* and in addition, changing characteristics of the average cell as a function of population density were avoided (Summers, 1960).

The medium was that of Cramer and Myers (1952), slightly modified (Padilla and James, 1960): $(NH_4)_2HPO_4$, 1.0 gm./L.; KH_2PO_4, 1.0 gm./L.; $MgSO_4 \cdot xH_2O$, 0.2 gm./L.; $CaCl_2$, 0.02 gm./L.; $Fe_2(SO_4)_3 \cdot xH_2O$, 3.0 mg./L.; $MnCl_2 \cdot 4H_2O$, 1.8 mg./L.; $CoSO_4 \cdot 7H_2O$, 1.5 mg./L.; $ZnSO_4$, 0.4 mg./L.; $Na_2MO_4 \cdot 2H_2O$, 0.2 mg./L.; $CuSO_4 \cdot 5H_2O$, 0.02 mg./L. These salts are chelated by sodium citrate (0.8 gm./L.), which is not used as a carbon source by *Euglena.* The vitamins B_1 (0.1 mg./L.) and B_{12} (0.0005 mg./L.) are required by *Euglena,* and were added aseptically to the autoclaved salt-citrate solution after sterilization by filtration. The phosphates buffer the medium to a pH of 6.8. In this medium, $(NH_4)_2HPO_4$

is the sole nitrogen source. However, small amounts of the sulfhydryl-bearing amino acids methionine (10^{-5} *M*) and cysteine hydrochloride (6.4×10^{-4} *M*) were routinely added to the medium (after sterilization by filteration). While these concentrations do not support growth, they can effectively improve division characteristics of *Euglena* in synchronous culture (Cook and James, 1960; James, 1960). Incandescent lamps (General Electric Co.), with an incident intensity of 130 foot-candles, were the sole energy source, and nutrition, save for catalytic amounts of the vitamins and two amino acids, was strictly autotrophic. In continuous light at 20° C., this intensity is saturating for growth of *Euglena*.

Cultures were continuously stirred, by means of a teflon-covered magnetic impeller bar, to ensure even illumination and rapid exchange of gases. Harvests were made by means of a gravity-flow siphon, and stirring further served to randomize the distribution of cells within the culture. After the syphon was flushed, all aliquots were representative of the population as a whole.

Cultures were axenic, and were kept on the light-dark cycle from inoculation. Dilution of the medium was initiated when the appropriate population density was reached, and repeated once in each generation; harvests of any given culture were deferred until at least five light-dark cycles had elapsed after the first dilution. Generally, two light-dark cycles suffice for the induction of division synchrony, and initial harvests were delayed only as a precaution against the possible tardy stabilization of other cellular parameters.

All cell counts were made with the Coulter cell counter (Coulter Co.). Details of this procedure can be found elsewhere (Mattern *et al.*, 1957); the mean values of replicate counts made with this instrument are within 2% of the true value when no correction for coincidence is necessary. For the present case, aliquots were always diluted to cell densities yielding a linear counting rate, so that coincidence was negligible.

At harvest, cells were first packed by gentle centrifugation, and then resuspended to densities appropriate to the analytical procedure being followed. Representative aliquots of this concentrated suspension were then diluted with the salt medium for cell counts.

Total protein content of the average cell was estimated by the Folin-Ciocalteau phenol test as modified by Lowry *et al.* (1951), after prior extraction of pigments and other soluble fractions with 90% aqueous acetone. Bovine serum albumin in known concentrations was simultaneously run as a standard, and protein levels in *Euglena* are expressed in these equivalents.

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted according to the method of Schmidt and Thannhauser (1945). Optical densities of the hydrolyzed DNA and RNA (in 5% perchloric acid) were measured at 260 and 315 mμ with the Beckman DU spectrophotometer. Regression curves obtained with serial concentrations of commercial DNA and RNA permitted conversion of the observed difference reading ($O.D._{260} - O.D._{315}$) into absolute amounts per cell.

Pigments of the photosynthetic apparatus were estimated according to the method of Richards with Thompson (1952). After overnight extraction of a known number of cells in 90% aqueous acetone at 15° C., the initial extract was made up to volume (10 ml.) with two washes. Optical densities were determined in the Beckman DU spectrophotometer at wave-lengths of 750, 665, 645, 630, 510,

and 480 mμ, with 90% acetone as a blank. Pigments in *Euglena* include the chlorophylls *a* and *b*, and several carotenoids, of which beta-carotene is the most important in terms of absolute amounts (Fogg, 1953). None of these absorb appreciably at 750 mμ, and readings at this wave-length (usually negligible) were routinely subtracted from those at the other wave-lengths as a correction for turbidity. Simultaneous equations developed by Richards with Thompson (1952) permit calculation of pigment concentrations from the observed optical densities. For the chlorophylls, concentrations in absolute amounts are obtained. The method serves as a satisfactory measure of chlorophyll *a* in *Euglena*. Chlorophyll *b* is present in much smaller amounts, however (about 10% that of chlorophyll *a*). This pigment is therefore largely masked by the other pigments, and accurate measurements are not feasible by this method. Since the several carotenoids are estimated as a mixture, concentrations must be expressed in arbitrary units. Richards with Thompson have proposed the Standard Pigment Unit (SPU) for this measure, and this unit is retained here. Their equations are satisfactory for estimates of carotenoid content in *Euglena* (Cook, 1960).

The method of cultivating cells which was adopted for these studies limited the working volume of the culture to about one liter per day, *i.e.*, a yield of no more than 5×10^6 cells in each generation. Since some of the procedures required almost this many cells for a single determination, a complete picture of synthetic rates could

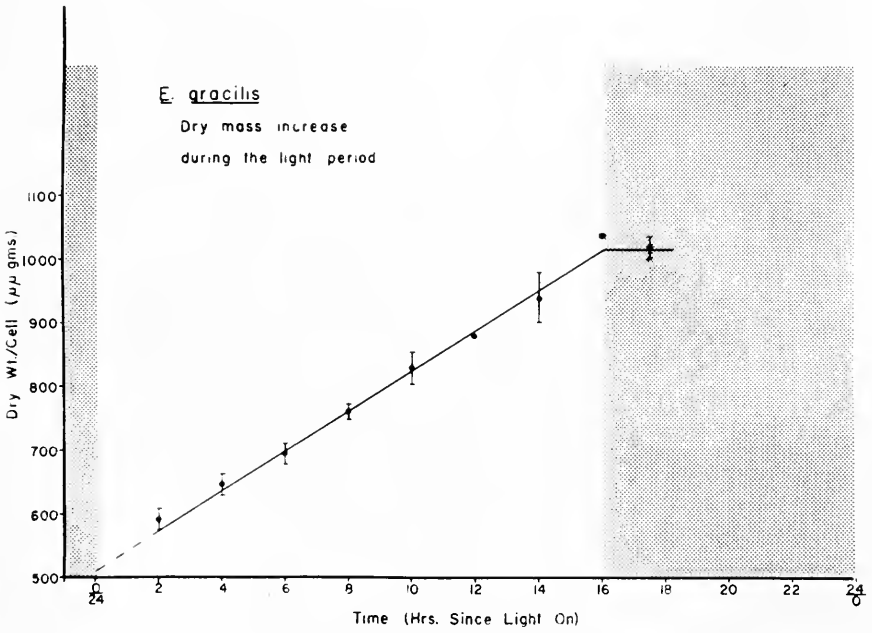

FIGURE 2. Dry mass of the average cell at different times during any given light-dark cycle. The abscissa is the time scale for one complete cycle, beginning arbitrarily with the onset of the 16-hour light period. The shaded area at the left represents the end of the preceding dark period, and that at the right the whole 8-hour dark period for the cycle shown. Limits of one standard deviation are indicated.

be compiled only by pooling data from many different light-dark cycles. Continued synchronous culture by periodic dilution, as illustrated in Figure 1, thus served practical as well as theoretical reasons. In the following, statistical estimates of experimental error are shown as the standard deviation about the mean, and represent both errors of analysis and of cell counts.

Unfortunately, the cell used in these studies was previously referred to as *Euglena gracilis* var. *bacillaris* (Cook and James, 1960). Certain characteristics, not discovered until after that paper had been accepted for publication, made it clear that the cell was not in fact *E. gracilis* var. *bacillaris,* but another strain of the species. Pending positive identification, the cell is called merely *Euglena gracilis.*

Results

Figure 2 shows the dry weight of the average *Euglena,* measured at different times during the light-dark cycle. These data have been reviewed before (Cook, 1961), and are shown here for reference. The dark period is represented by the shaded portion, and the abscissa is the time-scale for one complete cycle—24 hours—beginning arbitrarily with the onset of the light period. The rate of dry mass increase is clearly linear over the whole of the light period, and is terminated when the cells enter the dark period. Since cell divisions are still occurring in the first two hours of the light period, meaningful mass measurements on a per cell basis are not easily made at this time. It seems fair to estimate mass changes in the early hours of the light period by extrapolation from the rate observed after cell divisions are complete. The extrapolated portion of the rate curve in Figure 2 is indicated by the broken line. (This treatment ignores the possibility of a brief lag in mass accumulation very early in the light period.) Since *Euglena* essentially doubles in number at each burst of division activity, continued balanced growth demands a duplication of major cell constituents over the life cycle. Figure 2 indicates that this is indeed the case for the dry weight of *Euglena;* the average filial cell has a dry mass of about 500 $\mu\mu$grams, and the average parent cell 1000 $\mu\mu$grams. Mass loss in the 8-hour dark period is negligible.

Synthetic rates for total protein are not so straightforward. As can be seen from Figure 3, protein synthesis is terminated (after a doubling) at the tenth or eleventh hour of the light period, at a level which remains constant thereafter. The filial cells contain about 200 $\mu\mu$grams protein, and the parent cells 400. The protein : dry mass ratio is 0.4 both at the beginning and end of the light period, but goes through a slight maximum (0.5) in the interim.

Nucleic acid levels in the average cell were determined less frequently. Figure 4 shows the RNA content at four different ages. If the rate curve is again extrapolated to the beginning of the light period, it can be seen that a doubling occurs linearly during the entire light period. The filial cell contains 23 $\mu\mu$grams, and the parent cell 46 $\mu\mu$grams. Less confidence can be placed in the DNA data, and this study will bear repetition with culture conditions yielding larger numbers of cells for this analysis. However, Figure 5 indicates that DNA is probably doubled in a linear fashion throughout the light period.

Duplication of chlorophyll *a* is completed by the fourteenth hour of the light period, after which no further increase occurs (Fig. 6). Similarly, carotenoid synthesis (in *Euglena,* mostly beta-carotene) is terminated about two hours before

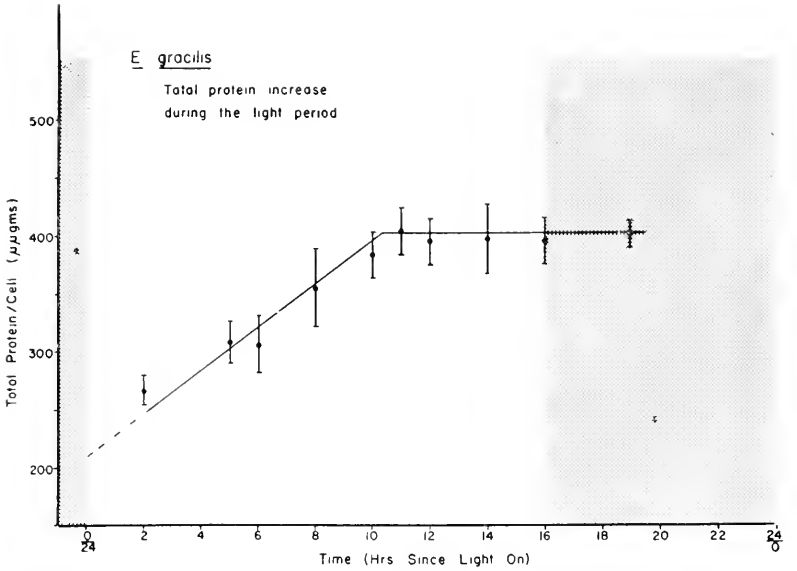

FIGURE 3. Total protein of the average *Euglena* at different times during any given light-dark cycle.

the onset of the dark period (Fig. 7). The ratio carotenoids : chlorophyll *a* is thus constant throughout the life-cycle of the average cell. Chlorophyll *a* contributes about 3% to the total dry weight of *Euglena,* although this value goes through a slight maximum at the fourteenth hour of the light period. From data of Goodwin and Jamikorn (1954), it can be calculated that the carotenoids in synchronized

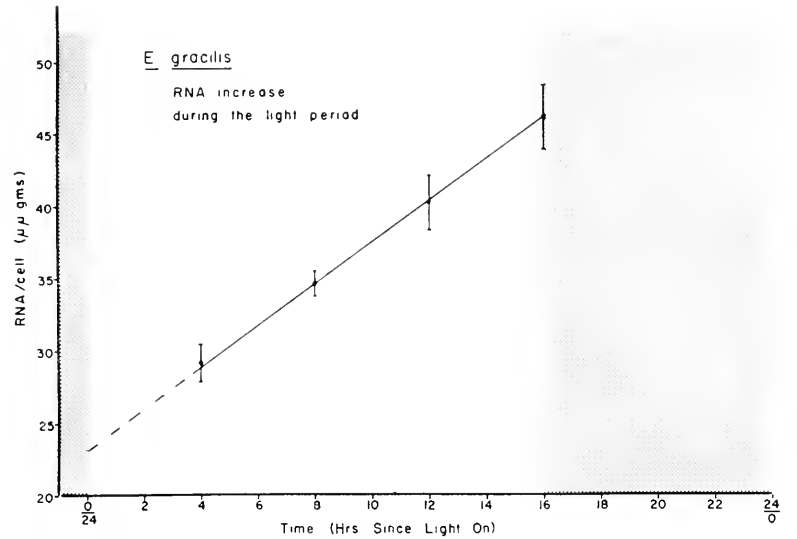

FIGURE 4. RNA synthesis by the average cell during the light period.

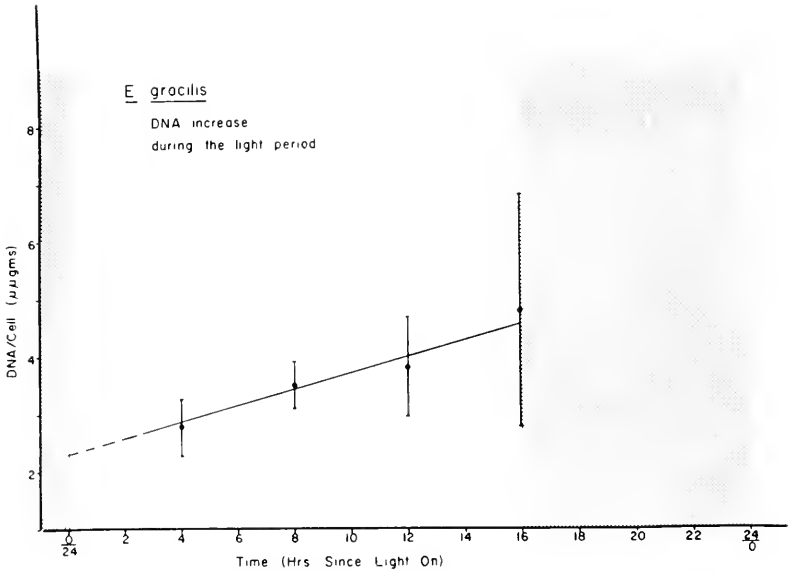

FIGURE 5. DNA content of the average *Euglena* during the light period.

Euglena constitute one to two per cent of the total dry weight (Cook, 1960), and this, too, goes through a slight maximum in the light period.

Table I lists the concentrations of chlorophyll *b* calculated for different ages. While there is considerable error involved in estimates of this pigment by the method used here, the expected two-fold difference in chlorophyll *b* levels should at least

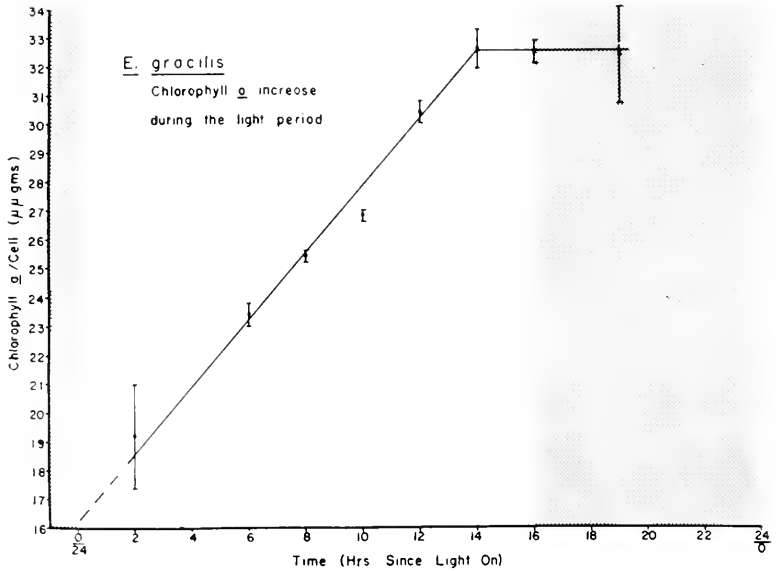

FIGURE 6. Chlorophyll *a* content of the average cell during any given light-dark cycle.

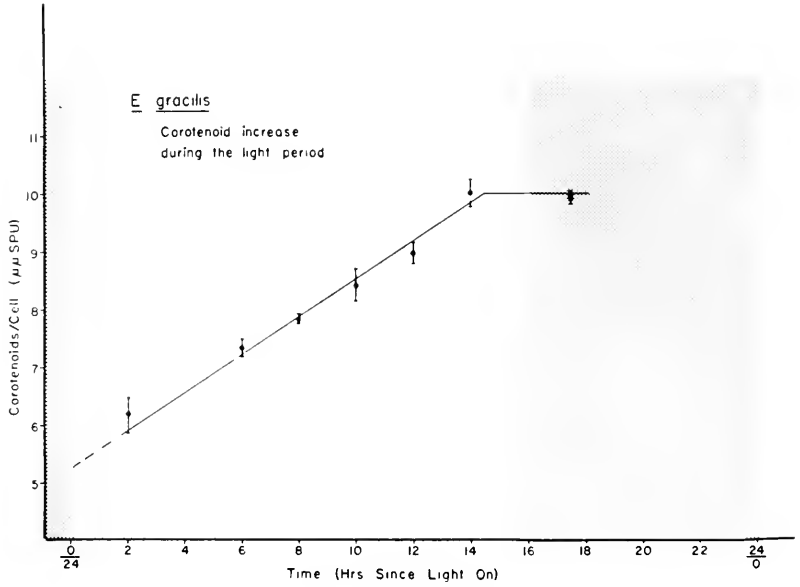

FIGURE 7. Carotenoid synthesis by the average *Euglena*.

be indicated; that is to say, even rough approximations should reveal some tendency toward increasing amounts if chlorophyll *b* is indeed duplicated in the light period. No such differences are seen over the major portion of the light period, however; at the sixth hour, the average cell has 2.60 ± 0.38 $\mu\mu$grams chlorophyll *b*, and at the onset of the dark period, 2.67 ± 0.24 $\mu\mu$grams. It is not at all unlikely that chlorophyll *b* is duplicated in the very early portion of the light period. The uncertain role of chlorophyll *b* in the family of photosynthetic pigments need not after all demand a constant ratio of chlorophyll *a*:chlorophyll *b*. Thus, Smith (1949) and Koski (1950) have shown that the biosynthesis of chlorophylls *a* and *b* in etiolated seedlings proceeds at different and apparently unrelated rates. For *Euglena*, a more sensitive analysis than that used here must be adopted before accurate rate measurements of chlorophyll *b* synthesis can be known. The possibility that a continuously changing ratio of photosynthetic pigments occurs over the life cycle of *Euglena* implies a regulation of metabolism which could be of first importance to the over-all balanced growth of the cell.

TABLE I

Chlorophyll b *content of* Euglena gracilis *in synchronous culture, calculated according to the equations of Richards with Thompson (1952). The numbers following "Age" represent the time in hours following onset of the 16-hour light period.*

Age	2	6	8	10	12	14	16	17.5	19
Chlorophyll *b* ($\mu\mu$gm./cell)	1.87 ±.42	2.60 ±.38	2.53 ±.08	2.44 ±.06	2.79 ±.22	3.21 ±.21	2.67 ±.24	2.86 ±.14	3.11 ±.45

Discussion

The energy supply supporting cell division, an event which comes in the dark period in synchronous populations of *Euglena gracilis,* as well as maintenance of the cell in this period, must ultimately be derived from photosynthetic products elaborated in excess of immediate needs for incremental growth, and stored up during the light period against future requirements of the dark period. In point of fact, an estimate of the quantity of this stored energy was the basis leading to the particular light-dark cycle on which *Euglena* is grown (Cook and James, 1960). One might therefore have expected the total dry mass of the average cell to somewhat more than double during active synthesis in the light period. Figure 2 indicates that this is not the case, however. Each parent cell of 1000 $\mu\mu$grams dry weight yields two filial cells of 500 $\mu\mu$grams (assuming equal division), and each of these enters the subsequent light period with just this weight. Thus, parent and filial cells traverse the entire 8-hour dark period with no significant reduction of mass.

Cramer and Myers (1952) have estimated that *Euglena gracilis* consumes twice its own volume of oxygen every hour. While respiratory rates of *Euglena* in synchronous culture have not yet been determined, it is probable that similar rates will be found. With this assumption, and with the further assumption that an RQ of 1 obtains, it can be calculated that the total carbon lost (as CO_2) in the dark period is only 35 $\mu\mu$grams for the average parent and generated filial cells. This is less than 4% of the total dry weight, not likely to be detected by a technique which has a probable error of about 5%.

In most euglenoid flagellates, protein constitutes about 30–40% of the total dry weight (Cook, 1960). In autotrophic strains, photosynthetic pigments contribute about 5%, and the nucleic acids 4 or 5%. Most of the remaining dry mass, some 50%, will be made up of storage products—lipids and paramylum, the characteristic polysaccharide of these cells (Kudo, 1954). Although these storage products were not measured over the life cycle of *Euglena,* it is inferred that their accumulation must complement protein synthesis in such a way that the over-all increase in dry mass, of which these fractions make up the bulk, follows the linear rate shown in Figure 2. The ratio of storage products to dry mass therefore probably passes through a minimum at the same time of the light period as the protein : dry weight ratio exhibits a maximum.

Major synthetic patterns of the average cell in synchronized populations of *Euglena gracilis,* growing autotrophically on a simple, near-basal salt medium at 20° C., are summarized in Figure 8. The ordinate is in relative units, from 1 to 2, the range of increase in these parameters expected for the single cell over its life cycle. The time scale on the abscissa is shifted somewhat from that seen in the earlier graphs, with respect to the light-dark cycle, to more nearly accommodate the actual age of the average cell. This cell has its inception, by division of the parent, some two hours before the onset of the light period, and 24 hours later is itself the parent of two filial cells, an event which occurs at the sixth hour of the subsequent dark period, being preceded by a mitosis which is probably of three or four hours duration (Leedale, 1958). Most of the interphase is spent in the light, where the cell completes its physical growth and duplicates most (if not all) of its principal biochemical constituents. Increase in dry mass is limited to the light period, and follows a linear rate over the entire 16 hours, as does the cell volume (Cook, 1961).

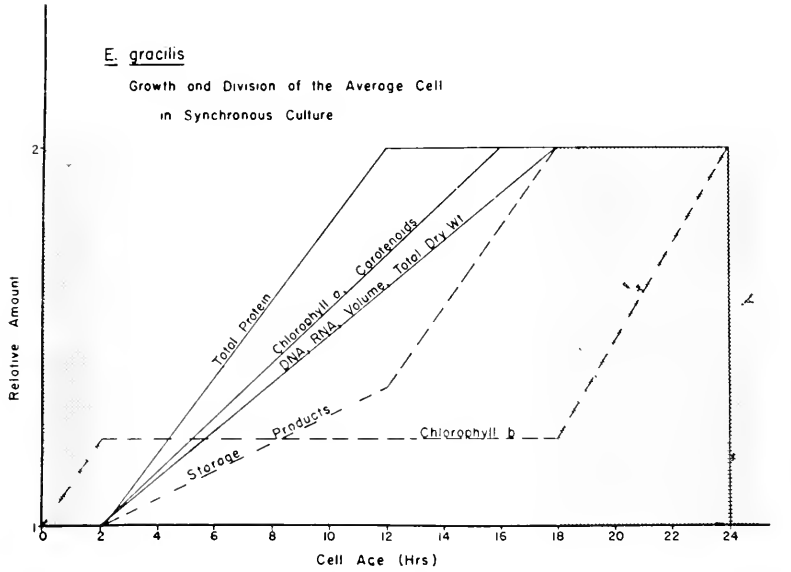

FIGURE 8. Summary of biosynthetic rates of major constituents in the average *Euglena* during any given light-dark cycle. The ordinate shows the relative content, the range of values observed in duplication of the various quantities listed (Figs. 2–7). The broken lines are inferred rates (see text). The average cell is formed by division of a parent about two hours before the onset of the light period (age 0) and divides at about the sixth hour of the subsequent dark period (age 24) to yield two filial cells.

RNA is similarly doubled, and probably also DNA. Protein synthesis, though limited to the light period, is complete by the twelfth or thirteenth hour of the cell's life; storage products, which make up most of the remaining dry mass, are most likely accumulated so as to complement protein synthesis (Fig. 8, broken line). Chlorophyll *a* and the several carotenoids are synthesized only in the light, and are duplicated by the fourteenth hour of this period, when the cell is 16 hours old. Chlorophyll *b* synthesis (Fig. 8, broken line) may occur in the dark period, but the possibility of duplication very early in the light period is not to be excluded. Certainly, most of the cell's growth is confined to the light period.

Division of the average cell, on the other hand, comes in the dark. With well-defined synchronous systems, the average cell is not likely to divide before growth has been completed, and for the present case this must of necessity follow the light period. Moreover, it has been shown that visible light can inhibit cell division in the euglenoid flagellates (Cook, 1960; Leedale, 1959), and division of even mature euglenoid cells can be delayed by light for as much as 20% of their normal generation time. A repetitive cycling of metabolic events, such as the growth patterns described in this paper, coupled with a periodic inhibition of cell division, are doubtless additive in their effect on the continued synchronous divison of *Euglena gracilis*. While photosynthetic cells can of course grow and multiply in continuous light, it is likely that light can be used more effectively for growth (and division) when punctuated periodically by dark periods. Sorokin and Krauss (1959), for example, have reported that *Chlorella* has a higher growth rate with intermittent periods of

dark than in continuous light, and Warburg (1957) has commented that culture of autotrophic cells in continuous light is contrary to conditions demanded by their biochemical evolution. Implicit in all this is the possibility that synthesis of minor cell fractions in synchronized *Euglena,* not necessarily of importance in terms of absolute mass, but yet critical for most effective growth, might well occur with a greater probability in the dark period. It is not unreasonable to imagine that activation energies for such syntheses are reduced in the dark; and culture on a repetitive light-dark cycle would then soon confine these events to the dark period. The varying ratios of the major metabolic constituents of *Euglena,* as seen in Figure 8, further emphasize that metabolic processes need be integrated only with reference to the whole life-cycle of the cell, and not necessarily with any particular portion thereof.

The author wishes to acknowledge the continued encouragement of Dr. T. W. James throughout the course of this work.

Summary

1. When grown autotrophically on an appropriate light-dark regimen, populations of the flagellate *Euglena gracilis* divide synchronously, an approximate doubling of cell number occurring in each dark period.

2. Growth of the average cell during the light period, which is essentially devoid of cell divisions, is described in terms of dry mass, total protein, DNA, RNA, chlorophyll *a,* and the carotenoids. The average *Euglena* duplicates all of these constituents in each light period.

3. Dry mass, volume, RNA, and probably DNA of the average cell increase to a doubling in a linear fashion over the whole of the 16-hour light period.

4. The photosynthetic pigments chlorophyll *a* and the carotenoids (beta-carotene) increase at rates which do not deviate greatly from linearity.

5. Synthesis of total protein is completed 5 or 6 hours before termination of the 16-hour light period. It is inferred that storage products are synthesized at rates complementing protein synthesis in such a way that dry mass increases linearly.

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OSMOTIC TOLERANCE AND REGULATION IN CRABS FROM A HYPERSALINE LAGOON

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It has been well established that the semi-terrestrial crab *Pachygrapsus crassipes* can osmo-regulate strongly in both dilute and concentrated sea water (Jones, 1941; Prosser *et al.*, 1955; Gross, 1955). On the other hand *Hemigrapsus oregonensis,* which is a less terrestrial crab than *Pachygrapsus,* was shown by Jones (1941) to be a strong hyper-osmotic regulator (maintenance of blood concentration above the concentration of the external medium), but was incapable of hypo-osmotic regulation in concentrated sea water after an immersion period of 72 hours. Gross (1957a), however, demonstrated hypo-osmotic regulation for *Hemigrapsus* in 150% sea water for a period of about 20 hours. The osmotic behavior of these two crabs fits well into the correlation between terrestrial behavior and hypo-osmotic regulation first indicated by Jones (1941) and expanded by Gross (1955). That is, crabs tending toward life on land are able to regulate in concentrated sea water and this in turn is accompanied by the additional ability to regulate in dilute sea water.

The adaptive significance of osmotic regulation in terrestrial and semi-terrestrial crabs was questioned by Gross (1955), inasmuch as adult land crabs will drown when completely immersed. Also, the semi-terrestrial *Pachygrapsus* is but rarely found in salinities deviating much from normal and, in fact, shows a precise preference for normal sea water. Thus, this highly active crab will avoid osmotic stress and is capable of seeking the normal salinities of the open sea (Gross, 1957b).

In January of 1959, Los Penasquitos lagoon, near San Diego, California, became isolated from the open sea by a sand bar. In early summer it was observed that large populations of *Pachygrapsus crassipes* and *Hemigrapsus oregonensis* and a relatively small population of the fiddler crab, *Uca crenulata,* were trapped but thriving in the lagoon where salinities were in excess of 150% sea water (5.3% salt). This was the first situation in which the author had found *Pachygrapsus* in the field where it was exposed to osmotic stress yet could not reach normal sea water. Although a complete field study of the environmental salinities of *Uca crenulata* has not been made, it is known to be a strong hypo-regulator (Jones, 1941) and is commonly known to live in back-bay areas where high salinities would be expected. On the other hand, the presence of *Hemigrapsus* in a hyper-saline lagoon was indeed surprising and suggested that contrary to laboratory findings was probably demonstrating hypo-regulation of significant magnitude and duration.

This investigation is concerned with ionic and osmotic regulation in *Hemigrapsus* and *Pachygrapsus* collected from Los Penasquitos lagoon during the period from July 1, 1959, to December 2, 1960, when the lagoon remained isolated from the open

sea. Physical and other biological aspects of the lagoon during parts of this period are presented by Carpelan (1961).

Materials and Methods

Blood and urine were sampled from the crabs as previously described (Gross, 1959). In the field blood and urine were taken from the animals immediately after capture. The samples were then placed into cold vials and quick-frozen on dry-ice. Also, sea water samples were taken at the site of capture and from different depths as a check for stratification. All samples were then returned to the laboratory for analysis. Salinity was determined by conductivity, using a 1000-cycle bridge. Na and K were determined by flame photometry; Ca and Mg by titration with ethylene diamine tetra acetic acid (EDTA) as previously described by Gross (1959). Melting points of blood and urine were made by the method of Gross (1954). Salinity preference was determined by means of a selectivity chamber (Gross, 1957b).

Results

Figure 1 presents the blood concentrations of both *Pachygrapsus* and *Hemigrapsus* sampled in the field at various dates and salinities. Blood concentrations (% sea water) are represented by: (a) total cations (mM/l.) (Na, K, Ca and Mg) relative to those found in normal sea water, and (b) by melting points. For seventeen crabs on which both blood melting point and total cations were determined, the ratio, $\frac{\text{melting point (\% sea water)}}{\text{total cations (\% sea water)}} = 0.98$, S.D. = 0.006. Thus, total cations (mM/l.) can be converted with sufficient precision to melting point and either type of determination will show the degree of osmotic regulation demonstrated by the crabs. Although the salinities of the lagoon water differed from site to site, those indicated in Figure 1 represent the media from which the crabs were actually collected.

It thus can be seen that *Hemigrapsus* (indicated by circles) was indeed regulating osmotically in the hypersaline water. Regulation was particularly strong in 160% sea water, but some *Hemigrapsus* were maintaining the blood concentration considerably below the ambient medium of 175% sea water (August 25, 1959). When the lagoon water reached approximately 180% sea water in mid-September, 1959, hypo-osmotic regulation in *Hemigrapsus* had essentially broken down; only one crab could be considered to be regulating with any strength. It also should be noted that the blood of several crabs was above the concentration of the indicated medium. This perhaps can be explained by the fact that most crabs observed (both species) at this time were piled up at the interphase between air and water, having migrated out of the depths of the lagoon. It is, therefore, likely that crabs captured when immersed had been out of the water and exposed to evaporation which had in turn elevated the blood osmotic pressure. While there were many dead and moribund *Hemigrapsus* at the edges of the lagoon at this time, only active specimens were sampled.

When the lagoon water reached a concentration of about 190% sea water no living *Hemigrapsus* could be found, although *Pachygrapsus* (indicated by triangles) were still surviving and active in these salinities. It cannot be concluded from these

data, however, that the upper limits of salinity in which *Hemigrapsus* can survive in nature lie somewhere between 180% and 190% sea water because there are many other physical and biological factors involved.

As was expected some *Pachygrapsus* were demonstrating strong hypo-regulation in all the salinities indicated in Figure 1.

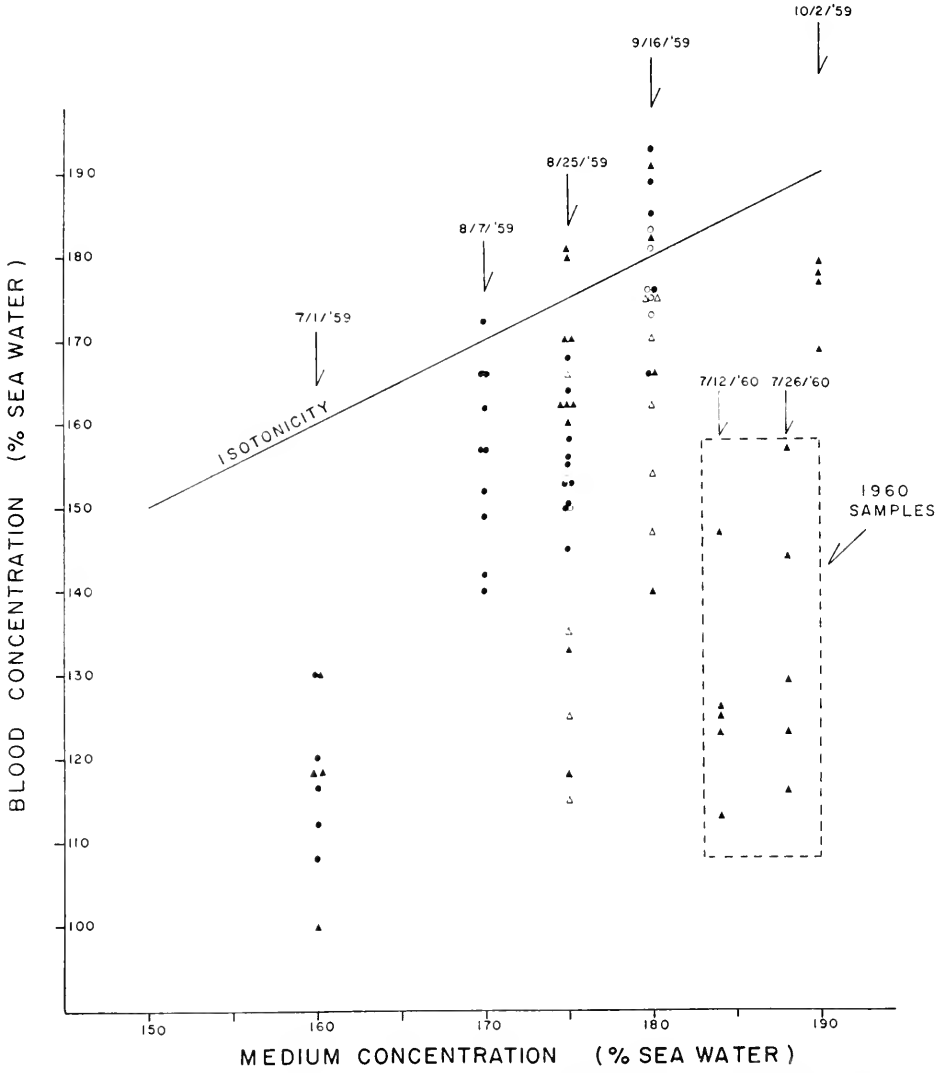

FIGURE 1. Osmotic regulation and tolerance by *Pachygrapsus* and *Hemigrapsus* in Los Penasquitos lagoon. Blood concentrations for individual crabs are represented by circles for *Hemigrapsus;* by triangles for *Pachygrapsus.* Each point represents a different individual. Blood concentrations by melting point determination are indicated by open points; blood concentrations by total cations (mM/l.) are indicated by solid points. Dates of sampling are indicated by arrows. The 1960 samples are enclosed by broken line.

An interesting phenomenon demonstrated by both species in Figure 1 is their ability to tolerate high blood concentrations. Thus, active *Hemigrapsus* and *Pachygrapsus* had blood concentrations which were close to isotonic or hypertonic to 180% sea water.

In December, 1959, the lagoon was filled by the high tides and the salinity dropped to 109% sea water although the isolating bar remained intact. However, no living *Pachygrapsus* or *Hemigrapsus* could be found. Nevertheless, in the summer of 1960, both *Pachygrapsus* and *Hemigrapsus* were found in relatively small numbers in the isolated lagoon which again had become hypersaline. The specimens of *Hemigrapsus* were sufficiently small to have washed over the bar during the winter high tides in the larval stage and developed in the lagoon. Most *Pachygrapsus* were mature and therefore either entered the lagoon across the bar in the adult stage from a sandy beach not likely to be inhabited by this species or were remnants from the 1959 population. Blood concentrations of the 1960 *Pachygrapsus* samples (enclosed by broken line in Figure 1) show that hypo-osmotic regulation was stronger in the 1960 crabs than in the 1959 crabs. The combined 1960 blood samples taken from animals immersed in salinities greater than 180% sea water concentrations were lower in concentration on the average than the 1959 samples taken from crabs immersed in 175% sea water, $P < 0.01$. This suggests that rigorous selection had taken place which favored hypo-regulation in *Pachygrapsus,* and that the 1960 specimens were survivors of this selection from the 1959 population. As indicated above, the salinity of the lagoon water in early 1960 was close to normal. Thus, the 1960 crabs were not regulating more strongly than those collected in 1959 because of long-term acclimatization, although two successive seasons of exposure to increasing salinities might have had the same effect.

While immature *Hemigrapsus* were captured from the lagoon in 1960 when the maximum salinity was 188% sea water, samples of sea water from the sites of capture were not taken. Thus, the degree of regulation for this species cannot be told for 1960. It is interesting, however, that two pooled samples of blood from these small specimens were approximately isotonic to the maximum salinity found in the lagoon at that time (188% sea water).

It is apparent from the data in Figure 1 that contrary to laboratory finding (Jones, 1941; Gross, 1957a) *Hemigrapsus* in nature is quite capable of strong hypo-osmotic regulation. The contradiction between laboratory studies and the field studies of the present investigation could be caused by two factors: (a) prolonged exposure to increasingly high salinities for a period of months, permitted physiological acclimatization, or (b) the increasing salinities obliterated the non-regulating crabs, thus selecting for strong regulators which were the only crabs remaining to be observed. Of course, both factors may be involved. In order to test these two possibilities a group of *Hemigrapsus* collected from Los Penasquitos lagoon when the salinity was in excess of 160% sea water was back-acclimated by gradual steps to 100% sea water. After a period of about two months in 100% sea water the crabs were immersed directly into 150% sea water for a period of 72 hours. The blood was then sampled and analyzed for total cations. Of seven surviving specimens thus treated only one demonstrated significant hypo-osmotic regulation. The blood of this one crab was equivalent in concentration to 134% sea water which is not as strong regulation as the weakest regulator observed in a

field situation where the lagoon water was 160% sea water (Fig. 1). Nevertheless, this is better regulation than has been reported for *Hemigrapsus* previous to the present investigation.

The results of this experiment are difficult to interpret because the loss of regulation may be a matter of physiological breakdown caused by the prolonged laboratory conditions. The experiment might be interpreted to mean that sudden transfer from normal to 150% sea water did not permit time for physiological acclimation which would occur in an isolated lagoon. Also, the fact that one specimen of this group was regulating in 150% sea water for a period of 72 hours, an ability not shown for *Hemigrapsus* either by Jones (1941) or Gross (1957a), suggests that some selection for hypo-regulation had taken place in the lagoon. Evidence was produced above that such selection had occurred in the case of *Pachygrapsus*. It seems reasonable that both selection and physiological acclimation were involved.

An attempt was made to acclimate *Hemigrapsus* gradually from 100% sea water to increasing salinities in order to demonstrate physiological acclimation, but the experiment failed because of a high mortality rate under the prolonged laboratory conditions.

Table I compares the blood ion concentrations of the two species of crab immersed in 100% sea water and 175% sea water. It should be pointed out that the

TABLE I

Ionic concentration in the blood of Hemigrapsus *and* Pachygrapsus

		100% sea water* (laboratory conditions)			175% sea water (lagoon field samples)		
		Mean	S.D.	No.	Mean	S.D.	No.
Na (meq/l.)	P	483	17.3	36	802	105	10
	H	452	10.3	6	726	39.3	10
	M	464			801		
K (meq/l.)	P	7.36	1.4	36	14.1	2.2	10
	H	8.13	1.1	6	12.5	1.9	10
	M	9.8			15.0		
Ca (meq/l.)	P	29.6	5.9	44	43.8	5.0	10
	H	37.7	5.3	5	51.0	10.3	10
	M	20.0			35.9		
Mg (meq/l.)	P	20.0	6.1	44	36.4	14.7	10
	H	70.0	13.3	5	135	31.4	10
	M	104			191		
Total (meq/l.)	P	540			896		
	H	568			925		
	M	598			1043		

P = *Pachygrapsus*
H = *Hemigrapsus*
M = Medium
* = Blood ion concentrations for *Pachygrapsus* in 100% sea water were reported previously (Gross, 1959).

values for crabs immersed in 175% sea water were determined from samples taken in the field. The values for normal sea water were for laboratory conditions for both species. However, the *Hemigrapsus* in this case were collected from Los Penasquitos lagoon returned to the laboratory and back-acclimated gradually to 100% sea water. Thus, the values for *Hemigrapsus* blood following immersion in normal sea water are subject to some question. Nevertheless, differences in the regulation of ions between *Pachygrapsus* and *Hemigrapsus* are salient in both salinities. In the first place, Na is regulated more strongly by *Hemigrapsus* than by *Pachygrapsus*. That is, the blood Na is significantly less concentrated in *Hemigrapsus* in both the normal and concentrated sea water than it is in *Pachygrapsus*, $P < 0.05$. On the other hand, blood Mg concentrations in *Hemigrapsus* are more than three times as high as they are in *Pachygrapsus* for both salinities. The regulation of K and Ca does not differ dramatically between the two species.

It is difficult to say on the basis of means that *Pachygrapsus* is the stronger hypo-osmotic regulator of the two species. Yet an examination of Figure 1 reveals that in the highest salinities where both species were studied, the strongest regulators, by far, were *Pachygrapsus* and the evidence is strong that *Pachygrapsus* survived *Hemigrapsus* at the extreme salinities. In view of these observations and the findings of earlier investigations (Jones, 1941; Gross, 1957a), it seems fair to consider *Pachygrapsus* the stronger regulating species in concentrated sea water. Yet it is suggested in Table I that the difference in osmo-regulatory ability is merely a difference in the ability to regulate Mg. That is, *Pachygrapsus* is a far stronger regulator of Mg than is *Hemigrapsus*.

Table II presents urine ion concentrations for the two crabs collected from 175% sea water. Since these samples were taken in the field, under conditions where contamination of urine samples with blood were more likely than under better controlled laboratory conditions, great precision cannot be placed on the concentration values. The mean urine Na for *Pachygrapsus* (572 meq/l.) is not significantly less than the mean urine Na for *Hemigrapsus* (687 meq/l.) although these concentrations would correlate well with the respective concentrations of Na in the blood of the two species. It is particularly interesting that the Mg concentration in the urine of the two species is about equal because as indicated above *Pachygrapsus* is a much stronger regulator of Mg than is *Hemigrapsus*. Values for urine Mg are smaller in *Pachygrapsus* than previously reported for lower salinities (Prosser *et al.*, 1955; Gross, 1959) which suggests that contamination with the blood had occurred. However, urine samples are more difficult to take from *Hemigrapsus* than *Pachygrapsus* and it is therefore likely that contamination of urine in *Hemigrapsus* also took place. It is concluded that while the values for urine Mg given in Table II are not precise, they strongly suggest that the antennary glands of both species are capable of concentrating Mg about equally.

Inspection of the total cation concentrations in urine and blood for both species (Table I and Table II) shows higher total ions in the urine than in the blood (meq/l.), but not knowing the anion constituents, this cannot be interpreted to mean that the urine is hypertonic to the blood. The blood and urine were compared by melting point determinations on six individual *Pachygrapsus* removed from the lagoon when the salinity was about 180% sea water. The average ratio, urine concentration/blood concentration, was 1.02 (range = 1.00 to 1.06). Thus, if the

TABLE II

Ionic concentration in field samples of urine taken from Hemigrapsus *and* Pachygrapsus *collected from 175% sea water*

		Mean	S.D.	No.
Na (meq/l.)	P	572	137	5
	H	687	84.8	8
K (meq/l.)	P	16.4	3.3	5
	H	12.0	1.9	8
Ca (meq/l.)	P	46.0	9.9	5
	H	56.9	14.9	8
Mg (meq/l.)	P	353	87.6	5
	H	364	195	8
Total (meq/l.)	P	987		
	H	1120		

P = *Pachygrapsus*
H = *Hemigrapsus*

urine is slightly more concentrated than the blood, it is not sufficiently so for the antennary glands to be effective as osmotic regulators.

Salinity preference was measured for *Pachygrapsus* collected from Los Penasquitos lagoon when the salinities were high at least six months after its isolation from the open sea. Nine crabs collected from the lagoon were individually placed in a salinity selectivity chamber for at least 36 hours where they could choose between 50%, 100%, 130% or lagoon water from which the particular crab had been collected (160%–180% sea water). A record was, therefore, obtained on the salinities visited and on the time spent in each. Adequate precautions were taken against choice for position rather than salinity. The procedure and apparatus are discussed by Gross (1957b). Thus, of the nine crabs, five preferred 100% sea water, two preferred 50% sea water, one preferred 130% sea water and only one demonstrated a preference for the salinity from which it had been collected. The mean time spent by all the crabs in the different salinities (% total time in the box) were: 50% sea water, 21.6; 100% sea water, 32.3; 130% sea water, 12.8 and lagoon water only 6.5% of the total time. It, therefore, seems likely that the preference for normal sea water previously described for *Pachygrapsus* persists even though the animal has been removed from normal sea water for months. It is even more apparent that it avoids the high salinities in which it had been living.

It has been reported by Gross and Marshall (1960) that the tissues of *Pachygrapsus* (as suggested by muscle) increase in water content when the animal is transferred from normal to dilute sea water and decrease in water content when the animal is transferred from normal to concentrated sea water, the resulting volume changes in the tissue taking place at the expense of the blood space. The above authors point out that such alterations in the anatomy of the blood space likely would affect the efficiency of the vascular system and consequently the adaptiveness of the animal. They suggested that volume control in the tissue might occur after

a prolonged exposure to osmotic stress, thus permitting the vascular system to return to a normal anatomy.

Ten *Pachygrapsus* collected from Los Penasquitos lagoon on July 12, 1960, were returned to the laboratory in Riverside where they were immersed in lagoon water (183% sea water) for an additional 24 hours. Samples of muscle tissue were removed from the legs of each crab, blotted uniformly, weighed, and dried to constant weight in a drying oven at 95° C. The difference between dry weight and wet weight was considered to be the water content of the tissue. Thus, the mean water content of samples from each of ten crabs was 69.24% wet weight, S.D. = 1.46. Water content of muscle from *Pachygrapsus* immersed in normal sea water was reported as 75.00% wet weight, S.D. = 1.40; from 150% sea water, 71.70% wet weight, S.D. = 1.71 (Gross and Marshall, 1960). All the above values are significantly different; $P < 0.01$. It, therefore, becomes apparent that the muscle of *Pachygrapsus* does not return to normal volume when immersed in concentrated sea water for a period of months and this is probably true for the other formed tissues. Thus, if the volume of the blood space is returned to normal after prolonged exposure to osmotic stress it must be brought about by other means. Burger and Smythe (1953) suggest that the nephridial bladders and stomach of *Homarus* serve such a purpose.

Discussion

The crabs *Pachygrapsus* and *Hemigrapsus* can be adapted to high salinities in two ways: (a) tolerance and (b) hypo-osmotic regulation. While the responses of *Pachygrapsus* to concentrated sea water in the field do not differ greatly from those reported for laboratory studies (Jones, 1941; Prosser *et al.*, 1955; Gross, 1957a), the responses of *Hemigrapsus* are quite different from laboratory findings and point to a case where short-term studies have led to an erroneous conclusion, namely that *Hemigrapsus* cannot hypo-regulate.

Pachygrapsus might be considered the stronger hypo-regulator of the two species and as indicated above, this is likely made possible by its stronger ability to regulate Mg. It is particularly interesting, however, that *Hemigrapsus* should be a weaker regulator of Mg than *Pachygrapsus* because it seems equally capable of concentrating this ion in its urine (Table II).

Now a crab which is regulating in a concentrated medium tends to lose water with the physical gradient and also by way of the urine it excretes. Yet as previously demonstrated (Gross, 1957a) the crab (at least *Pachygrapsus*) retains its normal body volume when immersed in concentrated sea water. This means, of course, that an uptake of water must balance the loss. A highly permeable animal, losing relatively large volumes of water to the external medium by diffusion, could thus exclude only a small fraction of its water intake in the form of urine. Also, if water loss were compensated for by drinking, then a highly permeable animal, experiencing a large water loss, would drink relatively large volumes of the concentrated medium which would mean a large intake of Mg. There is evidence that the exoskeleton of *Hemigrapsus* is more permeable to water than that of *Pachygrapsus* (Gross, 1957a). It may be that *Pachygrapsus* because of its lower permeability can exclude a greater fraction of its water intake in the form of urine, than can *Hemigrapsus* which in turn would mean a greater exclusion of Mg. It might also

be that *Pachygrapsus* having a smaller water turn-over does not drink as much as does *Hemigrapsus* and consequently takes in less of the Mg-rich water.

It was indicated above that the crabs (particularly *Hemigrapsus*) of Los Penasquitos lagoon migrated out of the depths to the shores' edge when the salinities became high, yet it cannot be concluded that salinity *per se* caused this movement for there were other physical and biological factors involved. For example, the oxygen tensions in the depths of the lagoon were extremely low in the summer months and by the end of August, 1959, were nil (Carpelan, 1961). It is believed that the lack of oxygen was the most critical stress forcing the migration. There was some rotenone poisoning in limited areas of the lagoon on several occasions during the summer of 1959 (Carpelan, 1961). However, this is not believed to have influenced the migration because the poison is a highly unstable substance which could be effective only for a short period in the limited areas where used. Besides, rotenone was used in early June and July, yet *Hemigrapsus* did not move into the shallows until August. Surface temperatures for several locations in the lagoon remained between 25° and 29° C. during July, August and September of 1959 (Carpelan, 1961). Thus, it does not seem that temperature could be critical in forcing the shoreward migration.

Los Penasquitos lagoon presents the physical stress which would tend to select for hypo-osmotic regulation, that is, high salinities. The lagoon also becomes stagnant, and in acquiring anaerobic conditions presents a stress which could force animals capable of movement toward the shore and out of the water. Thus, a selective pressure favoring aerial respiration is presented by the lagoon. While there are no available records of the salinities of this lagoon during the rainy season of a wet year, Carpelan (1961) has shown significant decreases in salinity following the light rains of 1959 and it is likely that run-off during heavy rains could dilute the lagoon water considerably.

If dilute as well as hypersaline conditions in coastal lagoons of the past were common, then the correlation between osmotic regulation and terrestrial life among crabs can be explained as a reflection of the history of the animals rather than necessarily a physiological adaptation to habitats in which the animals are typically found today. The adaptive significance of osmotic regulation in semi-terrestrial and terrestrial crabs was questioned by Gross (1955).

It is suggested, therefore, that coastal lagoons might have afforded ideal conditions for the evolution of terrestrial crabs. That is, selective pressures are available in these bodies of water favoring: (1) hyper-osmotic regulation, (2) hypo-osmotic regulation, and (3) aerial respiration, three physiological conditions common to crabs showing degrees of the terrestrial habit.

It probably would have been necessary for the crabs to be trapped as were the Los Penasquitos populations so that obligatory selection would have been imposed upon them. Also it probably would have been necessary that access to the open sea and normal salinities be seasonally available. Inasmuch as ovigerous crabs were not found in Los Penasquitos lagoon when the salinities were high, it is unlikely that reproduction occurred in the lagoon during the stressed conditions. However, this does not mean that such a population would be non-breeding, for if the isolated body of water were opened to the sea periodically, then normal salinities would become available which would permit reproduction and the escape

of free-swimming larvae to the open sea. It may be that the retention of a preference for normal sea water in *Pachygrapsus* after months in hypersalinities is an adaptation which assures reproduction in normal salinities. The periodic availability of normal sea water might explain why the land crabs have not evolved reproductive mechanisms for terrestrial life.

It does not follow that lagoons would have had to dominate the coastal areas where land crabs evolved, although lagoons which are opened intermittently to the sea during high equinoctical tides and rainy seasons are common today on the coast of South Africa, Brazil, Australia and south Texas (Ladd *et al.*, 1957). Neither would it be necessary for the majority of a particular species to inhabit the lagoons. All that would be required would be large absolute populations within the lagoons where they would be rigorously selected for the three physiological features which would have to have hereditary components. It also would be required that the three physiological factors would not be selected against in normal sea water. Thus, adults which were severely selected in an isolated lagoon for the ability to regulate osmotically and to respire in the air would periodically reproduce. Their young would then escape to the open sea and in effect increase the gene frequency for the characters in question within the general population. Given sufficient time, the characters could be common to the species. Of course, once the first step was made toward terrestrial life selective pressures might favor this feature in situations outside of the lagoon.

Edney (1960) emphasizes that the evolutionary route toward land directly across the littoral zone from the sea is more difficult than by way of estuaries and swamps because the problems of terrestrial adaptation are imposed at once. Gislén (1947) considers estuarine mangrove swamps as most likely pathways toward terrestrial life. Hedgepeth (1957) discusses the open marine beach as a direct route for landward migration. Neither of these possibilities, however, explains the common occurrence of both hyper- and hypo-osmotic regulation among terrestrial crabs.

On the other hand, conditions in coastal lagoons such as described above could not only provide the selective pressures favoring both types of osmo-regulation, but also could provide the conditions whereby the initial step from sea to land could be evolved in a gradual, but obligatory manner.

The direct invasion of land across a marine beach would require an initial step where the stresses of aerial life would be imposed suddenly. It would seem that the disadvantage of such a sudden step would outweigh the advantages of becoming land-borne. Perhaps crabs which appear to have become land-borne directly across the littoral zone (*e.g. Ocypode*) have in their history experienced selective pressures favoring such a move in ecological situations (lagoons of varying salinity) which differ from their present typical habitats.

Ferronnière (1901) was one of the earlier workers to point out that animals capable of tolerating low salinities commonly are also capable of tolerating high salinities. He also observed that it is common for species of the supra-littoral zone to inhabit brackish as well as hypersaline waters.

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Summary

1. The crabs *Pachygrapsus crassipes* and *Hemigrapsus oregonsis* were found thriving in an hypersaline lagoon which had been isolated from the sea for months and had attained salinities in excess of 175% of normal.

2. Contrary to previous reports *Hemigrapsus* was found to be a strong hypo-osmotic regulator in concentrations as high as 175% sea water. The discrepancy between previous laboratory findings and the field results of the present investigation are attributed to the prolonged acclimatization period in lagoon water of gradually increasing salinity and to severe selection permitting only hypo-regulators to be sampled.

3. No living *Hemigrapsus* were found in the lagoon when the salinities had exceeded 180% sea water.

4. *Pachygrapsus,* as was expected from previous studies, was regulating osmotically in the lagoon when the concentrations were above 185% sea water.

5. Both *Pachygrapsus* and *Hemigrapsus* can tolerate blood concentrations in excess of 160% sea water and remain active.

6. *Pachygrapsus* is a stronger hypo-osmotic regulator than *Hemigrapsus.* Since it maintains its blood Mg considerably lower than does *Hemigrapsus* in concentrated sea water, the stronger ability to hypo-regulate may be made possible by the greater capacity on the part of *Pachygrapsus* to excrete Mg.

7. The urine Mg for *Hemigrapsus* is about equal in concentration to that of *Pachygrapsus* when the crabs are immersed in 175% sea water, even though the blood Mg concentration is more than three times higher than that of *Pachygrapsus.*

8. *Hemigrapsus* is a stronger regulator of Na in hypersaline water than is *Pachygrapsus.*

9. The antennary glands in both *Pachygrapsus* and *Hemigrapsus* are ineffective as organs of hypo-osmotic regulation.

10. When *Pachygrapsus* is transferred from normal to concentrated sea water the volume of its muscle tissue, as indicated by water content, becomes reduced. This reduction in volume persists in hypersaline waters even after months of immersion. Thus, there is no evidence of volume control for muscle tissue.

11. After months of isolation in a hypersaline lagoon *Pachygrapsus* shows a preference for normal sea water when offered a choice of salinities and will avoid the hypersaline water from which it has been captured.

12. Evidence is produced showing that coastal lagoons present selective pressures favoring: (1) hyper-osmotic regulation, (2) hypo-osmotic regulation, and (3) aerial respiration, three physiological characteristics common to terrestrial and semi-terrestrial crabs. Coastal lagoons are suggested as ideal sites for the evolution of land crabs.

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SOME EVOLUTIONARY PATTERNS IN FISHES' BLOOD[1]

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Electrophoretic analyses of serum protein fractions of some fishes and certain other animals have been presented by Deutsch and Goodloe (1945), Deutsch and McShan (1949), Engle *et al.* (1958), Irisawa and Irisawa (1954), and Moore (1945). In general it was shown that there are characteristic, reproducible, species differences in the plasma proteins and differences between the lower and higher animals. Deutsch and McShan give data on the blood plasmas of 16 fresh-water fishes and Engle *et al.* gave data on 20 species of marine fishes. Both groups of authors reported that gamma globulin fractions in the plasma of teleost fishes were absent or diminished, but Engle *et al.* found them high in the elasmobranchs.

Sulya *et al.* (1961) presented plasma protein data derived by paper electrophoresis from 183 specimens of 26 species of fishes from the Gulf of Mexico, and this is the most extensive series of data on fishes presented so far. In that report the techniques are described, with a discussion of certain physiological and clinical implications.[2] Gamma globulin was found in all teleosts and in some it was quite high. Data were given by species and there was some discussion of differences between the various fishes.

We propose here to call attention to certain facts of evolutionary significance, derived from Sulya *et al.* (1960 and 1961), and to present part of the unpublished data.

The Data

The electrophoretic patterns were illustrated by Sulya *et al.* (1961), but the tables, which list the plasma protein components of each species, were too long for publication. Therefore, those data have been deposited as Document 6454 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. They may be purchased as photoprints or on 35-mm. film by advance payment of $1.25 by check or money order made out to Chief, Photoduplication Service, Library of Congress.

The data presented in Table I are average values for the various Orders of fishes, arranged in a series from the generalized to the more specialized, according to the classification of Berg (1947). In general this arrangement conforms to the sequence from lower to higher fishes, as presently understood. The original data were the mean values for all specimens of the given species. The species were then

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[2] Sulya *et al.* (1961) have been criticized for not offering full information regarding quantification, especially staining of the strips. For that reason we wish to state here, somewhat out of context, that the procedure used was Spinco "Procedure A" in which the dye is bromphenol blue.

TABLE I

Plasma protein distribution in fishes arranged in the order of increasing evolutionary development

Order	Number of species	Total protein gms. %	Albumin gms. %	Globulins gms. %				Other plasma globulins
				α_1	α_2	β_1	γ_1	
Lamniformes (sharks)	3 (4)*	2.18			.695	.805	.539	.143γ_2
Lepidosteiformes (gars)	3 (5)*	2.87		.125	.344	1.386	1.011	
Clupeiformes (herring-like fishes)	4 (63)*	3.59	.082	.988	1.144	.810	.421	.122γ_2
Siluroidea (catfishes)	2 (10)*	2.94	.688	.226	.429	.546	1.159	
Mugiliformes (mullets)	2 (23)*	3.78	.550	.893	1.127	.378	.811	
Perciformes (perch-like fishes)	12 (78)*	3.70	.291	.661	.889	.723	.658	.048α_1(2) .108α_2(2) .041β_2 .230γ_2

* The number in parenthesis represents the number of animals.

all given equal rank, regardless of the numbers of specimens, and the means for the species values are presented as the values for the Orders in the table. Thus 12 values were averaged for the Perciformes and only two for the Mugiliformes.

The writers had hoped to utilize the data on fish plasma previously published, along with our own, so as to have a more extensive series of fishes in the tables. However, previous studies were carried out by the moving boundary electrophoresis while our own were made by paper electrophoretic methods, and there is a basic difference between the patterns obtained by the two methods. In the moving boundary procedure, the pattern is obtained from the refractive index gradient relative to cell level and the pattern represents, therefore, all refracting components irrespective of their chemical composition. On the other hand, the paper electrophoresis method measures only those components which combine with the staining dye. The data obtained by the two methods may be similar, in general, but not necessarily so. In some instances we observed fish plasma cholesterol values that were very high relative to higher vertebrates. In these cases the lipids may be expected to contribute to refraction, thus giving the associated fractions higher values in the moving boundary procedure.

DISCUSSION

Of the four major groups of living fishes (Elasmobranchii, Holocephali, Dipnoi and Teleostomi) we have representatives only of the first and the last.

However, these two Classes are widespread and numerous (which is the reason we have specimens), and the other two are somewhat side-issues to the main stem of evolution in fishes. It should also be recalled that the Teleostomi are divided into two subclasses, the Crossopterygii and the Actinopterygii, the first being represented among living fishes only by *Latimeria,* of South Africa and Madagascar, and we have no specimens. The main line of evolution of the higher vertebrates probably passed through the Crossopterygii, but this group is an evolutionary side issue in fishes and in fact it is virtually extinct. Therefore, the data presented cover representatives along the main stem of evolution in fishes, as represented by living species, somewhat better than might appear at first glance. One large gap concerns the most primitive of the Actinopterygii, the sturgeons and paddlefishes.

The table shows that there is gradual increase in the percentage of total plasma protein from lower to higher fishes. Only the Clupeiformes are out of sequence. In high protein content of the plasma the Clupeiformes are similar to the advanced rather than to the lower fishes. This Order corresponds largely to the Isospondylii of Jordan (1923) and previous workers. The Perciformes (Acanthopterygii, of Jordan and others), the most specialized fishes, have about a 70% higher content of plasma proteins than the elasmobranchs. There is considerable variation between the species and among the Perciformes the range was 1.68 to 6.19 grams of plasma proteins per 100 ml. of plasma. The plasma protein content of fishes is low as compared to mammals.

Irisawa and Irisawa (1954) first showed that albumin was not present in the sera of two elasmobranchs, a skate and a shark. Drilhon and Fine (1959) made similar observations on two species of sharks, both by electrophoretic and reagent methods. Sulya *et al.* (1961) found the same condition in three other species of sharks. Additionally, Table I shows that there was no albumin in the plasma of three species of gars. These are fresh-water fishes which sometimes enter brackish waters, where all of our specimens were caught. Furthermore, there was no albumin in the plasma of two (*Dorosoma cepedianum* and *D. petenense*) of the four clupeid fishes examined. Two of the twelve species of perciform fishes examined (*Cynoscion arenarius* and *Pogonias cromis*) also lacked albumin in the blood plasma. Analbuminemia seems to be the common condition among the lower fishes; it gradually becomes less prevalent as the fishes become more specialized but does not entirely disappear, and a small per cent of the higher fishes lack albumin in the blood plasma. Thus analbuminemia among fishes seems to be a primitive characteristic. However, Deutsch and McShan (1949) reported albumin in the plasma of sturgeons, the most primitive of living Teleostomi, and that group merits further attention.

The table shows that the sharks, gars, catfishes and mullet had four globulin components in the plasma, and the clupeids have five. It is clear that the perciform fishes, with eight types of globulin, have generally more complex plasma proteins than the lower groups.

Species data show that ten of the perciform fishes had four or five plasma globulin fractions. The croaker, *Micropogon undulatus,* had only two fractions, but the speckled squeteague, *Cynoscion nebulosus,* had eight. These two fishes are considered by most authorities to be in different sub-families of the family

Sciaenidae. One of the gars and one of the sharks were found to have only two globulin fractions; the other two species in each group contained three and four fractions.

In terms of average numbers of globulin fractions per species for each Order, there is an increasing complexity up the evolutionary scale, but again the Clupeiformes are out of sequence. The mean numbers of globulin fractions per species are: sharks 3, gars 3, clupeids 4.5, catfishes 3.5, mullets 4, percoids 4.5.

Species differences are clearly reflected in the plasma protein complexes. For instance, *Dorosoma petenense* possesses gamma-2 globulin, but *D. cepedianum* does not, and the latter has over 11 times as much alpha-1 globulin as the former; one of the catfishes has alpha-1 globulin, but the other does not; the striped mullet has ten times as much albumin as the silver mullet and only one-fourth as much gamma-1 globulin. On the other hand, the white squeteague, *Cynoscion arenarius,* and the black drum, *Pogonias cromis,* have remarkably similar plasma proteins.

Berg (1947) separated the Mugiliformes from the Perciformes because they have abdominal pelvic fins, which is a primitive characteristic. In general the blood plasma proteins of the mullet are less complex than in the Perciformes and similar to certain species of the catfishes and clupeids. This gives some corroborative evidence to the correctness of Berg's separation of the Mugiliformes from the Percomorphi. On the other hand, the plasma proteins of the mullets are also similar to certain perciform species of the families Carangidae, Sciaenidae and Scombridae. Thus, the difference between the plasma proteins of lower and higher fishes is a general character of the groups which does not always hold true when certain species are compared.

The Clupeiformes studied here have a higher plasma protein content and greater protein complexity than would be expected from their position on the evolutionary scale. On the other hand, they show primitive affinities in that half of the four species examined possess no albumin, and the other two only have small amounts.

Sulya *et al.* (1960) confirmed previous reports on the high electrolyte content in the blood of marine elasmobranchs. They found that the three major electrolytes, Na, K and Cl, were about 66% higher in the plasma of sharks than in the teleosts. They also found that the total cholesterol, including cholesterol esters, in plasma of sharks and gars is comparable to the levels in higher vertebrates, 173–151 mg.%, respectively, but in all the remaining teleosts it was considerably higher (Clupeiformes 663, Siluroidea 367, Mugiliformes 560 and Perciformes 606). These are mean figures for the Orders and species variations are averaged out. It was pointed out that there seems to be an association of plasma cholesterol content and concentrations of certain plasma proteins. Resemblances of the normal blood in fishes to the blood of the nephrotic rat and of man with biliary cirrhosis were noted. It appears that the normal condition in fishes' blood is comparable in some ways to certain pathological conditions of mammals, including man. This point is significant, not only from the evolutionary standpoint, but as indicating a new line of approach to the understanding of certain types of pathology of the kidney and liver of mammals. In this regard, it should be noted that Baril *et al.* (1961) called attention to resemblances of the serum of young alligators to that of humans with renal disease.

Summary

1. A study was made, by electrophoretic techniques, of the plasma proteins of 26 species of elasmobranch and teleostome fishes from the northern Gulf of Mexico.

2. A general increase was found in the amount of plasma proteins from lower to more specialized fishes. Elasmobranchs lacked albumin in the plasma. This condition of analbuminemia also held true for all the gars and half of the Clupeidae studied; it generally disappeared in the more specialized fishes, but not entirely, and was found in two species of the Perciformes. There was a gradual increase in the number of globulin fractions of the plasma, from lower to higher fishes, among the Orders of fishes, which did not always hold true at species levels.

3. Closely related species could be distinguished by the presence or absence and varying amounts of plasma proteins, but there were exceptions and certain species in the same family could only be differentiated doubtfully on that basis.

4. The plasma proteins of the fishes examined showed a trend of increasing complexity from the generalized to the more specialized fishes.

5. The content of the chief electrolytes, Na, K and Cl, in the plasma of marine sharks was found to be much higher than in teleosts, in confirmation of earlier workers.

6. Total cholesterol in the plasma of sharks and gars was found in the same range as in mammals, but in the remaining teleost fishes it was a great deal higher.

7. Relations between cholesterol and plasma proteins in the blood of fishes resemble pathological conditions in mammals, including man.

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THE DISTRIBUTION OF MINERAL MATERIAL IN THE CALCIFIED CARAPACE AND CLAW SHELL OF THE AMERICAN LOBSTER, HOMARUS AMERICANUS, EVALUATED BY MEANS OF MICROROENTGENOGRAMS [1, 2]

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The histology of the calcified crustacean integument has been investigated by means of the light microscope (Williamson, 1860; Vitzou, 1882; Herrick, 1895; Drach, 1939; Dennell, 1947; Travis, 1955, 1957, 1960) and the polarizing microscope (Prenant, 1927a, 1927b; Dudich, 1931; Drach, 1939). Some aspects of calcification in relation to shell structure have been reviewed by Richards (1951).

The calcium carbonate found in the exoskeleton of the European lobster, *Homarus gammarus*, exists in an amorphous form (Prenant, 1927a). X-ray diffraction patterns obtained from the dry, intact carapace of the American lobster indicate that some calcite is present, but that much of the mineral does not have a distinct crystalline structure (Glimcher, personal communication).

Microroentgenography has been a useful technique in the evaluation of the degree of mineralization in various types of calcified tissue (Engström *et al.*, 1957). This report discusses microroentgenograms of lobster shell which were obtained using comparatively thick sections of calcified integument from the carapace and the claw shell. By exposing the animals to sea water labelled with calcium-45 for a short period of time immediately before sacrifice, labile areas of mineral could be identified.

Materials and Methods

Two male lobsters, 1 (518 grams) and 2 (436 grams), were obtained live from a local market. The lobsters were in intermolt condition, stage C-4 (Drach, 1939). The animals were allowed to remain in a tank of artificial sea water for seven days. They were then placed for two hours in an artificial sea water solution labelled with calcium-45 and strontium-85 which contained 25 mEq./l. calcium. After a two-hour period, the lobsters were removed from the labelled sea water, rinsed

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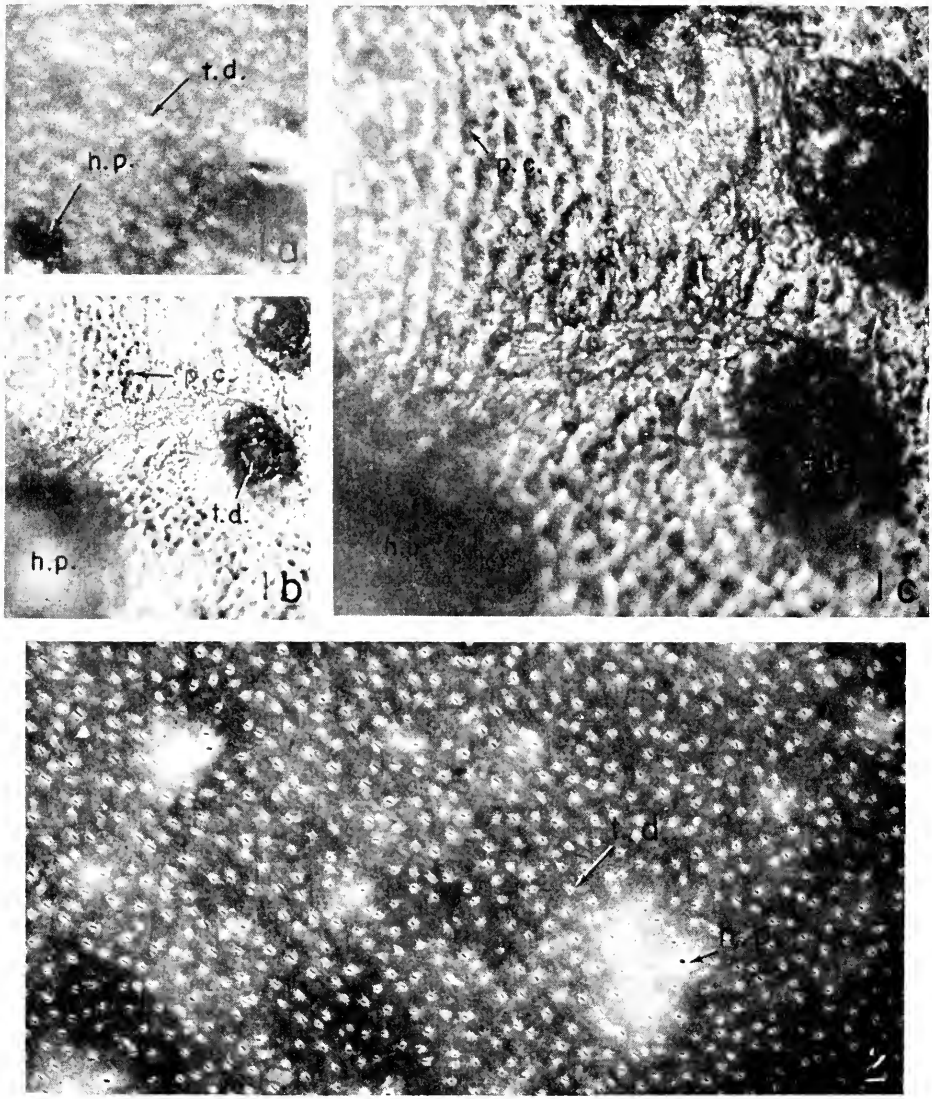


FIGURE 1. Surface view of dorsal claw shell, above muscle attachment.
FIGURE 1a. Obliquely reflected light. 30 × magnification.
FIGURE 1b. Directly reflected light. 250 × magnification.
FIGURE 1c. Directly reflected light. 500 × magnification.
FIGURE 2. Planar section, claw 1, principal calcified zone. Microroentgenogram. Enlarged 42 ×. h.p. = hair pore, t.d. = tegumental gland duct, p.c. = pore canal.

in a bath of distilled water, and blotted with gauze sponges. The posterior half of the carapace and the flat portion of the claw above the point of muscle attachment were removed. Pieces of shell about 20 mm.^{2} were obtained from the flat dorsal surface of the large member of the crusher claw above the point of muscle

attachment and 15 mm. from the tip of the claw. Carapace shell samples of similar size were also obtained at a point 15 mm. from the posterior and from the lateral borders of this tissue. The shells were rinsed briefly with a second jet of distilled water, and the non-calcified membrane was partially stripped off. Shells were dried at 100° C. for 12 hours.

A randomly selected area of dorsal claw shell, above the point of muscle attachment, was photographed at a magnification of 30 × by obliquely reflected light (Fig. 1a). By means of a metallurgical microscope, photographs of the shell by directly reflected light were obtained (Figs. 1 b-c). Dr. M. E. Nicholson of the Metallurgy Department of this University assisted with this procedure.

Irregular pieces of shell, about 16 $mm.^2$, were embedded in methyl methacrylate. One hundred-micron longitudinal and transverse sections were cut as well as one transverse 50-micron section from the carapace. The sections were polished on both sides with emery paper and placed directly upon the emulsion side of Kodak Maximum Resolution plates. Microroentgenograms were prepared according to the technique described by Bergman and Engfeldt (1954) and Engström *et al.* (1957). Target-to-film distance was at least 25 cm. Polychromatic radiation from a Machlett x-ray tube, with a tungsten target and a beryllium filter, with 6 KV tension, were used to obtain the microroentgenograms. The developed microroentgenograms were photographically enlarged. Positive prints of these enlargements are shown in Figures 2–8. In these figures, areas which are white in the microroentgenogram print represent areas of greater mineral concentration than areas with a gray cast.

To obtain photomicrographs of the claw sections, a reflected light source was used, while a transmitted light source was employed in the preparation of the photomicrographs of carapace sections.

Autoradiographs were prepared by placing the tissue sections in contact with the emulsion side of Kodak glass lantern slide plates. The tissues were allowed to remain in contact with the glass plates four months before the plates were developed. Darkening of the photographic film was shown to be due to beta particles from calcium-45 rather than gamma rays from strontium-85. Aliquots of solutions containing either calcium-45 or strontium-85 were pipetted onto blotter paper planchettes, designed so that a planchette of approximately the same size as a shell sample contained approximately the same amount of activity. After the planchettes were dry, they were placed in contact with the photographic plates for two months. No darkening due to strontium-85 gamma rays was observed when the plates were developed after this time.

The calcium content of adjacent fragments of shell was determined by the method of Clark and Collip (1925) after the shells had been ashed at 450–500° C. The calcium concentrations, expressed in mEq./g. dry shell, were as follows:

Carapace	1:	13.1 mEq./g.,	2:	13.8 mEq./g.
Claw	1:	11.5 mEq./g.,	2:	12.7 mEq./g.

The total residue remaining after ashing was as follows, expressed as per cent of dry weight:

Carapace	1:	67.7%	2:	70.1%
Claw	1:	60.5%	2:	66.6%

Description of photomicrographs, microroentgenograms, and radioautographs

Figure 1. Surface view of claw shell, randomly selected from dorsal surface of claw, above point of muscle attachment.

In Figure 1a, the openings of the hair pores (h.p.) and of the tegumental gland ducts (t.d.) are visible at 30 times magnification. In Figures 1b and 1c, 250 × and 500 × magnification, one hair pore is visible in the lower left hand corner. The tegumental gland duct openings (t.d.) are visible as large, circular structures. The many fine circular structures (p.c.) probably represent the points at which the pore canals initially opened to the surface. These figures were included to demonstrate the fact that there are three main types of openings or potential openings on the surface of the claw.

Figure 2 is a microroentgenogram, enlarged 42 times, of a planar section of the claw through the principal calcified zone (lobster 1). The microroentgenogram indicates that there are two circular or oval types of structure around which the density of the mineral is increased. Carlström (personal communication) has suggested that these could be areas in which the calcium carbonate possesses a crystalline structure. The larger dark spots (h.p.) surrounded by a bright halo probably represent transverse cuts through the pores of setae. They occur with about the same frequency as do the large pores identified as hair pores in Figure 1a. The smaller dark spots (t.d.) surrounded by areas of increased mineralization are the ducts of tegumental glands. The external openings of these ducts may be seen in Figures 1a-c. The slightly elliptical appearance of some of the passages in this microroentgenogram is due to the fact that the shell is curved and some of the pores have been cut at an angle greater or less than 90°.

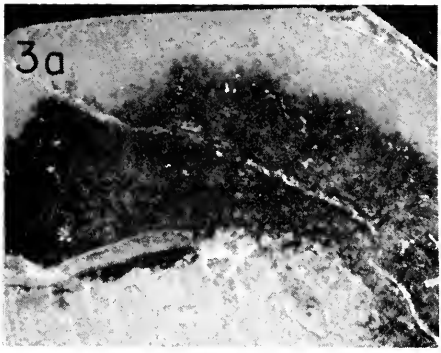
Figure 3a is a photomicrograph of a section which passes diagonally through the pigmented layer of the claw of lobster 2. The light triangular area on the right is methyl methacrylate as is the light area at the base of Figure 3a. The dark area in the center of the figure is the upper, heavily pigmented portion of the layer, and the lighter area immediately above represents the less heavily pigmented lower portion of this layer. The shell sample cracked when it was cut and polished and the break can be seen passing diagonally across the section.

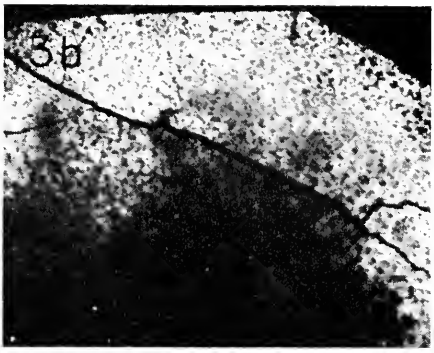
The microroentgenogram, Figure 3b, of this section of the claw indicates that there is slightly more mineralization in the outer strata than in the inner strata of this layer. Areas of lesser calcification probably represent ducts and pores or centers of cuticular prisms.

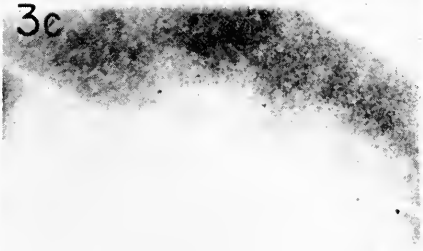
From the radioautograph of this section, Figure 3c, it is difficult to observe differential uptake of isotope in this layer. It appears that the lower and middle portions of the layer contain slightly more activity than the outermost portion.

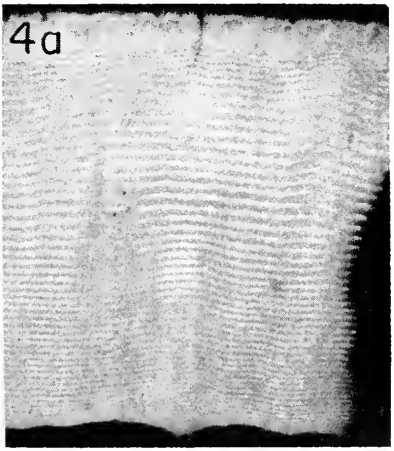
Figure 4a represents the photomicrograph of a transverse section of claw (Lobster 1). The division between the pigmented layer and the principal calcified zone can be observed. There is some indication of the possible initial location of the interprismatic septa in the pigmented layer, although in *Homarus* the septa actually fuse at an early stage after the molt is completed (Yonge, 1932; Dennell, 1960).

In the microroentgenogram of this section of claw, Figure 4b, a thin layer of greater mineralization can be observed on the outer surface of the shell. This area probably corresponds to the region of the lower epicuticle and the upper pigmented layer. Drach (1939) stated that in the Brachyura, calcite crystals









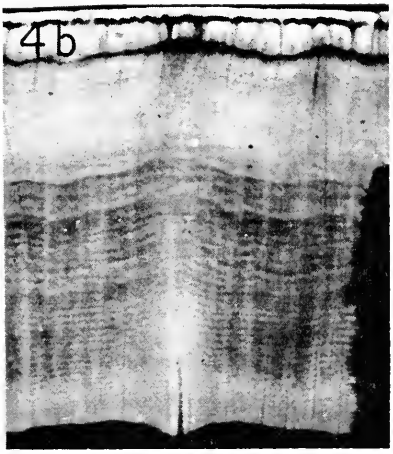


FIGURE 3. Planar section, claw 2, pigmented layer. Enlarged 48 ×.
FIGURE 3b. Microroentgenogram.
FIGURE 3c. Radioautograph.
FIGURE 4. Transverse section, claw 1. Enlarged 66 ×.
FIGURE 4a. Photomicrograph, reflected light.
FIGURE 4b. Microroentgenogram.
FIGURE 4c. Alternation of strips from microroentgenogram and radioautograph.

overlap between the two layers. The stratified nature of the pigmented layer, mentioned by Vitzou (1882), Herrick (1895), and Dennell (1960), is obscured by vertical "columns" of mineral material. The "columns" are wider at the outer surface. Dennell (1960) states that the interprismatic spaces are sites of early mineralization. From these sites, mineralization proceeds laterally and downward. It is possible that the areas which are less well mineralized represent interior portions of the cuticular prisms.

The strata of the principal calcified zone, underlying the pigmented layer, are clearly defined. The density of mineralization is actually less than that observed in the upper third of the pigmented layer. The most noticeable features of the principal calcified zone are the alternation in the density of the mineral matter in the horizontal strata and the two types of observed "ducts" which pass through the zone. The most numerous, clearly observed at the base of the principal zone, are probably the tegumental gland ducts. The large "duct" at the middle base of the principal zone is either an extremely large tegumental gland duct or the hair pore of a seta.

Figure 4c represents an attempt to correlate the strata of the principal calcified zone which may be observed in the microroentgenograms with those seen in the photomicrographs. Strips $\frac{1}{8}$ inch wide were cut, beginning at the same location, from both photomicrograph and microroentgenogram. The section was then reconstructed, using alternate strips from the two photographs. To some extent, the dark striations of the photomicrograph correspond to the dark striations of the microroentgenogram. The correlation is not perfect, and this can be regarded as suggestive only.

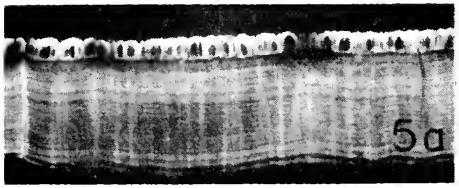
Figures 5a and 5b show the microroentgenogram and radioautograph obtained from transverse section from the claw of lobster 2. Details observed in 5a are similar to those seen in Figure 4b. There appears to be a greater concentration of the radioisotope in the outer layers of the claw.

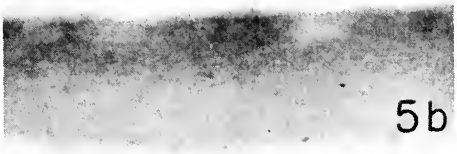
In Figure 6a, a transverse section of the carapace from lobster 2 is shown. This photomicrograph, obtained with transmitted light, merely indicates differences between outer and inner portions of the pigmented layer and the location of the principal calcified zone. The horizontal striations in the principal calcified zone, which can be easily observed at a broken edge of shell, are difficult to observe in this photograph. The very dark vertical line is an artifact.

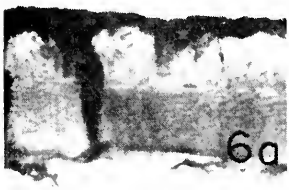
In the microroentgenogram of this section, Figure 6b, the mineral appears to be more uniformly distributed in the principal calcified zone. This may be due to greater density of the carapace shell as compared to the claw shell. In the radioautograph of the section, Figure 6c, calcium-45 activity is distributed in a much less uniform manner than in claw sections. There is little apparent relationship between uptake of isotope and variation in mineralization.

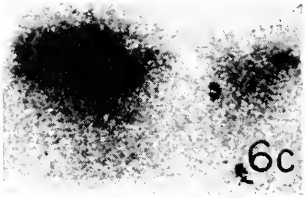
Figure 7 is a microroentgenogram of a carapace section, Lobster 1, half the thickness of that used to obtain the photographs in Figures 6a-b. There is more detail visible in the pigmented layer. However, strata in the principal calcified zone are only faintly visible, indicating that mineral is indeed more uniformly distributed in this zone in the carapace than in the claw. The duct observed is probably the hair pore of a seta.

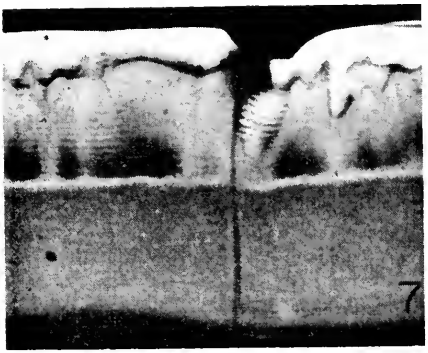
Figures 8a, b, and c were obtained using a planar section of carapace (lobster 2)

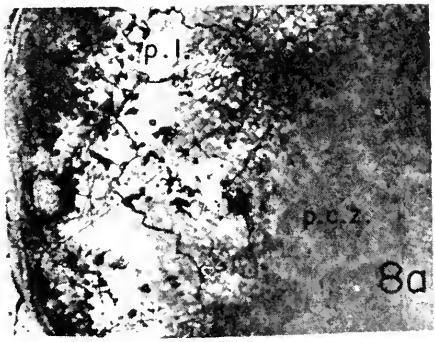












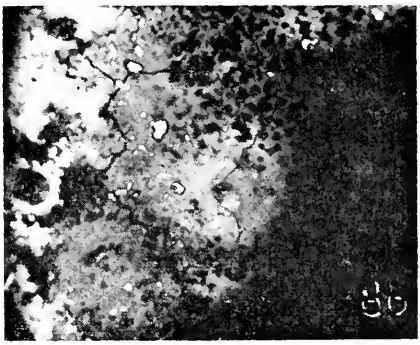


FIGURE 5. Transverse section, claw 2. Enlarged 22×.
FIGURE 5a. Microroentgenogram.
FIGURE 5b. Radioautograph.
FIGURE 6. Transverse section, carapace 2. Enlarged 66×.
FIGURE 6a. Photomicrograph, transmitted light.
FIGURE 6b. Microroentgenogram.
FIGURE 6c. Radioautograph.
FIGURE 7. Transverse section, carapace 1. Enlarged 98×. Microroentgenogram.
FIGURE 8. Planar section, carapace 2. Enlarged 60×.
FIGURE 8a. Photomicrograph, transmitted light.
FIGURE 8b. Microroentgenogram.
FIGURE 8c. Radioautograph. p.l. = pigmented layer; p.c.z. = principal calcified zone.

which passed through the pigmented layer and the principal calcified zone. In Figure 8a, the uniform gray area (p.c.z.) represents the principal calcified zone. The heavily pigmented, darker portion and the less heavily pigmented portion of the pigmented layer (p.l.) are visible.

In Figure 8b, the microroentgenogram of this section, the outermost portions of the pigmented layer appear to be heavily mineralized. The calcification suggesting interprismatic spaces is more clearly defined in the lower portion of the pigmented layer. In the principal calcified zone, the mineral is distributed in a uniform manner. The radioautograph, Figure 8c, shows again the irregular pattern of uptake of calcium-45 displayed by the carapace.

Discussion of photomicrographs, microroentgenograms, and radioautographs

Slight chemical differences have been observed among shell samples obtained from different areas of the exoskeleton of the lobster. The differences in density in these two areas apparent when Figures 4b and 5a are compared to Figure 6b could be a result of the slightly lower calcium concentration observed in the claw as compared to the carapace. The technique used to obtain the microroentgenograms is sufficiently sensitive to detect a difference of at least 15% in mineral density between two areas.

The irregular distribution of calcium-45 in the carapace, as compared to its distribution in the claw, suggests that the slightly denser mineral in the carapace contains a smaller percentage of labile calcium ions.

Drach (1939) pointed out that, in the integument of the crab, the area surrounding the hair pores may serve as a site for initial calcium deposits. The observation that some ducts and pores in the principal calcified zone are surrounded by denser mineral may bear some relation to Drach's finding.

The greater uptake of isotope by the pigmented layer, as compared to its uptake by the principal calcified zone, can be explained by the fact that the isotope was present in greater concentration at the outer surface of the shell.

General Summary and Conclusions

1. Visible light microscopy, microroentgenography, and radioautography were employed as techniques in the qualitative evaluation of the comparative distribution of mineral in the exoskeletons of lobsters and the relative exchangeability of calcium ions in the carapace and claw shells with the surrounding aqueous medium.

2. The following conclusions can be drawn from an examination of photographs obtained using these techniques: (1) In the claw and the carapace, a greater uptake of radiocalcium is observed in the outer portion of the shell. Radioactivity appears to be more uniformly distributed in the outer layer of the claw than in the same region in the carapace. (2) The outer third of the pigmented layer in the claw and the carapace contains the mineral material of greatest density. (3) In the claw, increased mineralization is observed in regions immediately surrounding the tegumental gland ducts and the hair pores; this increased mineralization is not observed in the carapace sections. The laminar nature of the principal calcified zone, observed in microroentgenograms of claw sections, is suggested only in carapace sections 50 microns in thickness. (4) The laminar nature of the pigmented layer of the carapace is obvious in microroentgenograms of cross-sections 50 microns in thickness.

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THORACIC NEUROSECRETORY STRUCTURES IN BRACHYURA. I. GROSS ANATOMY

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The pericardial plexus in decapod Crustacea was figured and described in several early investigations (Conant and Clark, 1896; Jolyet and Viallanes, 1893; Smith, 1947). Its significance, however, was not recognized until Alexandrowicz (1953) re-examined the structure, demonstrating that it contains not only nerve fibers, but also a number of arborizing terminations located just beneath the sheath surrounding the nerve trunks. This led to the suggestion, substantiated by later experiments (Alexandrowicz and Carlisle, 1953; Maynard and Welsh, 1959), that the pericardial plexus is the terminal portion of a neurosecretory system and is analogous to the sinus gland of the eyestalks. The plexus was renamed the pericardial organ (PO) on the basis of its secretory function.

Alexandrowicz's work left several aspects of the thoracic neurohaemal systems unresolved. This is the first of three papers which re-examine these systems in the brachyurans. It describes the anatomy of three neurohaemal structures in the crab thorax and suggests a correlation between PO volume and aspects of behavior. The second paper of the series will consider the histology and location of neurons contributing endings to the neurohaemal structures. The third paper will describe the histology of the terminations in the pericardial organs.

Methods

Nine species of crabs from five families were used: Cancridae, *Cancer borealis* (Stimpson); Xanthidae, *Panopeus herbstii* (Milne Edwards); Portunidae, *Callinectes ornatus* (Ordway); Grapsidae, *Grapsus grapsus* (L.), *Plagusia depressa* (Fabricious), *Goniopsis cruentata* (Latreille), *Pachygrapsus crassipes* (Randall), *Gecarcinus lateralis* (Fréminville); Ocypodidae, *Ocypode albicans* (Bosc). The *Cancer borealis* were obtained from Maine (Boothbay Harbor Crab and Lobster Co., Boothbay, Me.), the *Pachygrapsus crassipes* from California (Pacific Biomarine Supply Co., Los Angeles, Calif.), and both were examined during the winter at Ann Arbor. The other seven species were collected and examined at Bermuda during July and August.

Most of the following observations were made upon fresh, unstained material. Where necessary, certain points were confirmed with fresh material stained with methylene blue (Alexandrowicz, 1932) or with preserved specimens. In dissection the dorsal carapace was usually opened, and stomach, hepatopancreas, gonads, and bladder removed to expose the underlying ganglia and ventral thoracic structures. Complete and rapid removal of the hepatopancreas is critical—otherwise powerful digestive juices escape and destroy the finer nerve terminations—but is difficult

because of its delicacy and extensive ramifications. This problem was never completely solved, and prohibits definite statements about the absence of fine nerve branches or the ultimate destination of some nerves. Structures were considered neurosecretory if they appeared bluish-white with incident or darkfield illumination. Where there was doubt during dissection, the structure in question was removed and examined with high magnifications under darkfield illumination. Under these conditions, bluish or greenish colors can be considered a result of scattering or diffraction by small dense granules within the cytoplasm (see Maynard, 1961), and in the present series of investigations were found only in cell bodies, fibers, or terminals which could be termed neurosecretory on the basis of other criteria such as fine anatomy, staining characteristics, or physiological actions (see, also, Brown, 1940; Passano, 1953; Bliss and Welsh, 1952).

Pericardial organs were weighed individually on a fishpole balance constructed from a fine glass rod. One mm. deflection represented about 0.1 mg.; weights were estimated to the nearest 0.02 mg. In every case an attempt was made to include the entire PO but no non-PO material in the final weighing. This was generally believed successful.

Results

Three sites of storage and release of neurosecretory material are located in the crab thorax. Two, the pericardial organs and the anterior ramifications, apparently share some of the same secretory neurons, while the third, the post-commissure organ, is separate. The anatomy of the pericardial organs has been described by Alexandrowicz (1953) and others (Miyawaki, 1955; Matsumoto, 1958), but will be reviewed before proceeding to descriptions of the less known structures.

Pericardial organs

Structure. In the dozen or so brachyuran genera thus far examined, the pericardial organs show a common structural pattern. Typical organs from four species are diagrammed in Figure 1. In every case the PO appears as a plexus of nerve trunks in the lateral pericardium. Following the terminology of Alexandrowicz, this plexus may be divided into an anterior bar region and a posterior bar region, the two being joined by longitudinal trunks. Three nerve trunks pass into the anterior bar region from the ventral nerve mass. The two most anterior enter the lumen of the first branchio-pericardial (b-p) vein some distance from the pericardium; the third enters the lumen of the second b-p vein near its opening to the pericardium. These trunks, with occasional side twigs, run freely in the blood channels to the pericardium where they unite either completely to form a true anterior bar (in *Maia* and *Libinia*) or only partially to form a network of bars (most other genera, Figure 1) which lies across the combined opening of the first and second branchio-pericardial veins. Two nerves pass dorsally from the anterior bar region, one going to the dorsal thoracic muscles and one to the heart as the *dorsal nerve* (Alexandrowicz, 1932). There may be two (*Ocypode, Carcinus, Libinia*) or three longitudinal trunks joining the anterior and posterior bars. These trunks, like the bar regions, may float freely in the pericardial sinus, but often their posterior third is bound to the pericardial wall. The posterior bar lying over the opening of the third branchio-pericardial vein may be simple or complex in form.

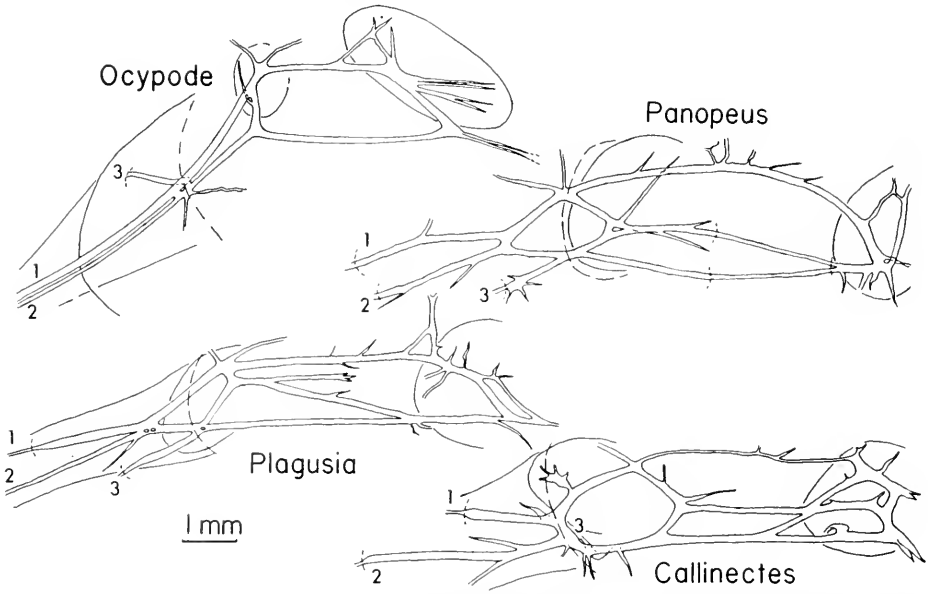

FIGURE 1. Pericardial organs of *Ocypode albicans, Panopeus herbsti, Plagusia depressa,* and *Callinectes ornatus.* Drawings from fresh preparations, organs *in situ* with openings of branchio-pericardial veins indicated. Anterior is to the left. Short dashed lines crossing nerves indicate point of egress from the pericardium or vein, or, in *Panopeus,* regions of two lower trunks which fuse with pericardial wall. The numbers 1, 2, and 3 indicate the first, second, and third segmental nerves, respectively. In the anterior bar region of *Plagusia,* three ovals mark position of anterior neuron cell bodies.

A number of trunks lead from it, some apparently going to the dorsal musculature and some to the ventral thoracic nerve mass as segmental nerves. Little attention has been given these in the present work, but at least some of the branches have been traced and described in *Pugettia* (Heath, 1941). As indicated below, the surface of all free-floating trunks may be considered a potential secretory surface. In addition, some portions of the pericardial lining and ligaments may contain secretory nerve terminations (Alexandrowicz, 1953). These latter areas, however, seem of less importance in the Brachyura than in the Astacura.

Size. Preliminary measurements on *Cancer, Carcinus,* and *Libinia* (Maynard and Welsh, 1959) suggested a correlation between relative pericardial organ weight and habitat or behavior of the species. Weights of pericardial organs were accordingly determined in a series of seven semi-tropical species from varying habitats. The results are given in Table I. The corrected pericardial organ index (weight of one PO divided by body weight, where body weight represents total weight *minus* chelipeds) is significantly higher in active swimming or semi-terrestrial forms such as *Callinectes* and *Ocypode* than in the more sluggish mud crab, *Panopeus.* Intertidal forms such as *Grapsus* and *Plagusia,* or less active terrestrial forms such as *Gecarcinus* fall between (Table II). The PO index showed no systematic variation with sex or with body weight within the samples examined. It may be of interest, however, that the uncorrected PO index (PO weight divided by total

TABLE I

Weight of pericardial organs (PO) in semi-tropical Brachyura

Animal	Habitat	n	Body wt. (gm.)	Total wt. (gm.)	PO wt. (mg.)	PO/BW (10^{-5})	PO/TW (10^{-5})
Ocypode	sandy beaches, above high tide	10	8.1–26.1	14.2–49.4	0.12–0.36	1.58±.23	0.90±.14
Callinectes	shallow bays, brackish	5	36.0–63.4	54.3–104.5	0.50–1.16	1.54±.35	0.99±.23
Gercarcinus	land, burrows	13	16.6–27.1	27.9–48.2	0.12–0.42	1.44±.42	0.87±.24
Goniopsis	mangrove swamps, intertidal	7	8.9–28.7	14.8–55.8	0.12–0.38	1.34±.11	0.76±.11
Plagusia	rocky shores, just below water	10	32.9–50.4	49.8–89.2	0.42–0.72	1.29±.11	0.80±.11
Grapsus	rocky shores, just above water	9	25.6–52.6	42.3–102.7	0.32–0.62	1.23±.17	0.68±.11
Panopeus	shallow bays, muddy bottoms	10	13.8–34.9	26.4–69.9	0.08–0.26	0.70±.21	0.39±.12

TABLE II

Significance levels of comparisons between PO indices of seven Brachyura; determined for corrected (upper) and uncorrected (lower) PO indices by Student's t test

Corrected index: PO/BW

	Ocypode	*Callinectes*	*Gecarcinus*	*Goniopsis*	*Plagusia*	*Grapsus*	*Panopeus*
Ocypode	—	ns	ns	$p = .025$	$p = .005$	$p = .005$	$p < .0005$
Callinectes	ns	—	ns	ns	$p = .05$	$p = .05$	$p < .0005$
Gecarcinus	ns	ns	—	ns	ns	ns	$p < .0005$
Goniopsis	$p = .05$	$p = .05$	ns	—	ns	ns	$p < .0005$
Plagusia	ns	$p = .05$	ns	ns	—	ns	$p < .0005$
Grapsus	$p = .005$	$p = .005$	$p = .025$	ns	$p = .025$	—	$p < .0005$
Panopeus	$p < .0005$	$p < .0005$	$p < .0005$	$p < .0005$	$p < .0005$	$p < .0005$	—

Uncorrected index: PO/TW

TABLE III

Weight of pericardial organs in temperate Brachyura (Maynard and Welsh, 1959)

Species	Habitat	Mean body wt. (gm.)	Mean PO wt. (mg.)	PO/BW (10^{-5})
Carcinus maenas	Maine coast, intertidal	102	0.54	0.53
Cancer borealis	Maine coast, subtidal	351	1.00	0.30
Cancer irroratus	Maine coast, subtidal	214	0.54	0.28
Cancer pagurus	North sea, subtidal	1810	4.0	0.22
Libinia emarginata	Mass. coast, subtidal	647	1.6	0.25
Maia squinado	North sea, subtidal	1560	2.5	0.16

weight, including claws) in the temperate or northern species examined was generally less than that of analogous semi-tropical forms (Table III).

Several factors require that interpretation of the above correlations be made with caution. First, the significant behavioral variable is not certain. Second, histological studies to be reported elsewhere (Maynard and Maynard, unpublished data) indicate that the size of the organ does not necessarily reflect the amount of secretory material contained within it. And third, the pericardial organs do not necessarily represent the only locus for storage and release of neurosecretion in the thorax, and their size alone, therefore, does not reflect the total presumptive storage volume. It seems probable that further information on the physiological function of secretions released from the PO will be necessary before a reasonable evaluation of the significance of the PO index can be made.

Anterior ramifications

Structure. The three antero-ventral trunks of the pericardial organs originate as segmental nerves in the ventral nerve mass. The most anterior of these, the first segmental nerve, corresponds to the cardio-inhibitory nerve, and the second and third to the first and second cardio-acceleratory nerves (Table IV). They leave the ventral ganglia at the level of the second, third, and fourth thoracic segments. The accelerator nerves apparently pass to the pericardium without extensive branching; they contribute fibers to the PO and one fiber each to the dorsal nerve innervating the cardiac ganglion. The inhibitor nerve, however, branches extensively in the region of the muscles supplying the maxillae. This branching forms the *anterior ramifications* (AR), and represents a second peripheral secretory structure (Figs. 2, 3 and 4). A detailed description follows.

Upon emerging from the ventral ganglion mass just behind the integumentary nerve, the first segmental nerve (inhibitor nerve) and integumentary nerve travel

TABLE IV

Terminology of anterior dorsolateral nerves in the decapod thorax

Genus	Author	Terminology			
Astacus	Keim, 1915	nervus superior primus	nervus superior secundus	nervus superior tertius	nervus superior quartus
Cambarus	Chaudonneret, 1956	nervus superior primus	nervus superior secundus	nervus superior tertius	nervus superior quartus
Cambarus	Wiersma and Novitski, 1942	—	inhibitor nerve	accelerator n.	—
Procambarus	Wiersma and Pilgrim, 1961	—	2nd root, 1st thoracic segment (t.s.)	2nd root, 2nd t.s.	2nd root, 3rd t.s.
Panulirus	Maynard, 1953	—	cardio-inhibitor nerve	first cardio-accelerator n.	second cardio-accelerator n.
Pugettia	Heath, 1941	integumentary nerve	segmental cardiac nerve x	segmental cardiac nerve m	segmental cardiac nerve I
Cancer	Smith, 1947	recurrent cutaneous nerve	inhibitory cardiac nerve	excitatory cardiac nerve	excitatory cardiac nerve
Brachyura	this paper	integumentary nerve	segmental nerve, one	segmental nerve, two	segmental nerve, three

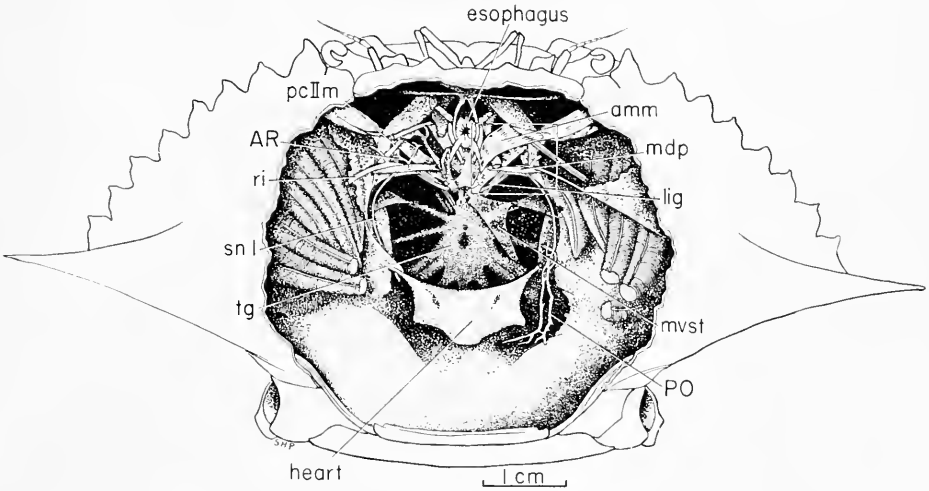

FIGURE 2. Dissection of preserved *Callinectes ornatus* showing location of pericardial organs and anterior ramifications. Digestive gland, stomach, and bladder removed. Amm, musculus abductor maior mandibulae; AR, anterior ramifications; lig, ligament; mdp, musculus dorsoventralis posterior; mvst, musculi ventrales superficiales thoracis; pcIIm, musculus proximalis coxopoditis II maxillae; PO, pericardial organ; ri, recurrent integumentary nerve; sn 1, first segmental nerve; tg, thoracic ganglia. The region bounded by a rectangle to the right of the esophagus is enlarged in Figure 3. Several overlying muscles and ligaments have been removed on the left half of the dissection.

anteriorly and laterally together to the endopleurite of the last head segment. There they pass upward through a ligament loop and angle more laterally toward the musculus dorsoventralis posterior (mdp). The integumentary nerve is often medial and anterior to the segmental nerve at this point. After passing through the ligament loop, and usually before passing beyond the mdp, the first segmental nerve gives off two branches. One angles posteriorly to the muscles attached to ligaments overlying the ventral nerve mass (musculi ventrales superficiales thoracis, Fig. 6), the other larger branch runs anteriorly beneath and beyond the mdp where it proceeds to break up into a network or plexus, the anterior ramifications. These apparently lie beneath a sinus membrane separating the ventral respiratory muscles from the dorsal thoracic cavity. Portions of the AR may dip ventrally in blood spaces between the muscles, or may spread over the membranes covering skeletal elements in the region or over the surface of a nerve (Fig. 3) apparently passing out toward the Y-organ and muscles of the mandible (Echalier, 1959). The form of the AR varies among species; for example, in *Callinectes* the ramifications give the impression of two main branches (Figs. 2 and 3), while in *Plagusia* (Fig. 4) they resemble an irregular net with no obvious main trunk. Most of the branchings and twigs of the ramifications appear to lead nowhere, as in the PO, but there are at least two branches, A and B, in which this is not so. These are particularly obvious in *Callinectes*, *Plagusia*, and *Eupanopeus*. Branch A separates under the mdp and, travelling medially, appears to enter this muscle near its medial attachment. Its final terminations have not been found. Branch B passes anterolaterally from the ramifications, paralleling the anterior branch of the integumentary nerve.

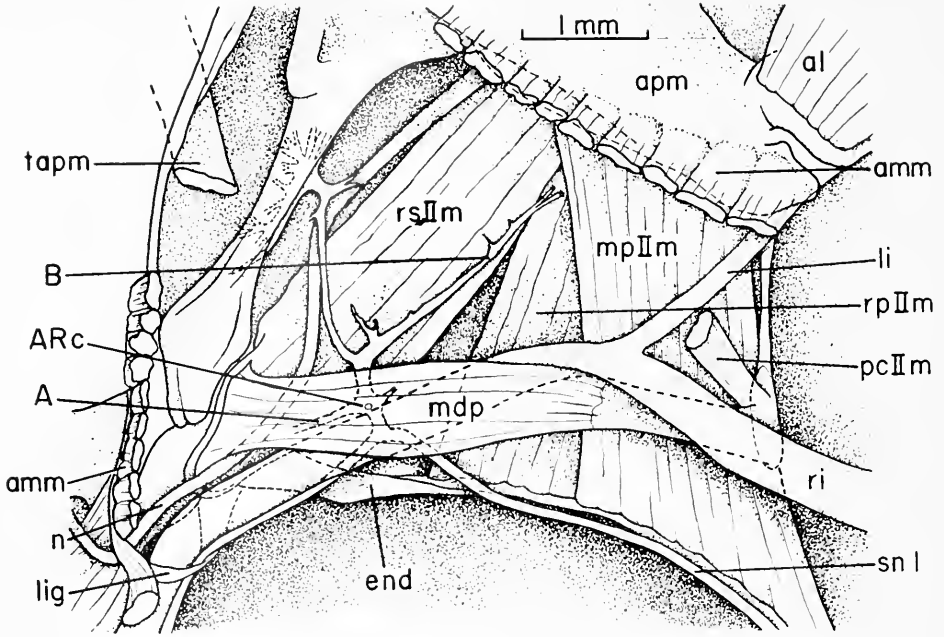

FIGURE 3. Anterior ramifications of *Callinectes ornatus;* fresh material. Dorsal exposure with digestive system removed. A, medial, posterior branch of anterior ramifications; al, musculus adductor lateralis mandibulae; amm, musculus abductor maior mandibulae, only cut segments at origin and insertion remain; the belly has been removed to expose underlying structures. Apm, apophysis of mandible; ARc, location of neuron cell body in anterior ramifications; B, anterior, lateral branch of anterior ramifications; end, endopleurite of last head segment; li, lateral integumentary nerve; lig, ligament; mdp, musculus dorsoventralis posterior; mpIIm, musculus promotor II maxillae; n, anterior dorsolateral nerve of Echalier (1959); pcIIm, musculus proximalis coxopoditis II maxillae; ri, recurrent integumentary nerve; rpIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius secundus II maxillae; sn 1, first segmental nerve; tapm, stump of tendon of musculus adductor posterior mandibulae. Note how one anterior branch of anterior ramifications spreads over anterior nerve and continues beneath the head apodeme.

In *Ocypode* this branch leads to another more lateral ramification (Fig. 5), but in other species the second ramification seems lacking and branch B disappears among muscles and membranes of the lateral, anterior carapace.

The AR are obviously located in a portion of the venous sinus system. Although the course of haemolymph flow has not been accurately determined in these regions, fluid probably passes over the ramifications as it travels from the general anterior venous sinuses of the thorax to the branchial sinuses in the gills. Perhaps of equal significance is the location of the AR in close proximity to the major respiratory muscles of the scaphognathite. Between them, therefore, the AR and PO are located just upstream from those muscles—respiratory muscles and heart, respectively—which normally exhibit continuous, rhythmic contractions and which are most concerned in the maintenance of adequate oxygen supply to the tissues of the body.

The volume of the AR is less than that of the PO, but its relative size was not measured because of difficulties in removing it without fragmentation.

Post-commissure organs

Although post-commissure organs have been identified in various shrimp and Stomatopoda, there is no published record of their presence in Brachyura. The following account describes them in *Pachygrapsus crassipes* (Fig. 6).

In *Pachygrapsus*, as in most other decapods, a pair of post-commissural nerves

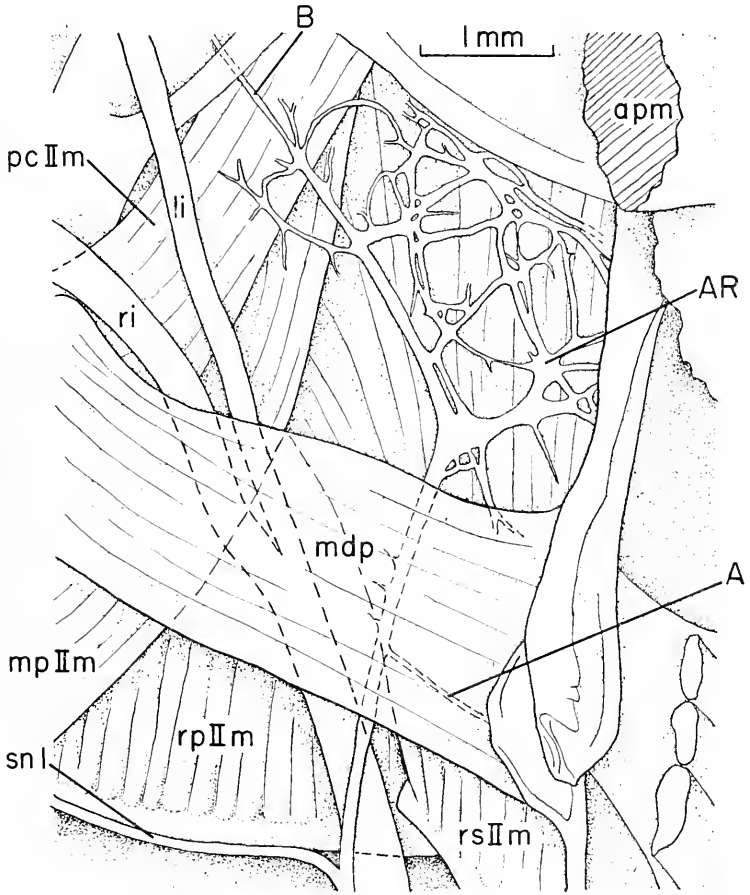

FIGURE 4. Anterior ramifications of *Plagusia depressa;* fresh material. Dorsal exposure with digestive system removed; midline to right. A, medial, posterior branch of anterior ramifications; apm, stump of apophysis of mandible; AR, anterior ramifications; B, anterior, lateral branch of anterior ramifications; li, lateral integumentary nerve; mdp, musculus dorsoventralis posterior; mpIIm, musculus promotor II maxillae; pcIIm, musculus proximalis coxopoditis II maxillae; ri, recurrent integumentary nerve; rpIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius secundus II maxillae; sn 1, first segmental nerve.

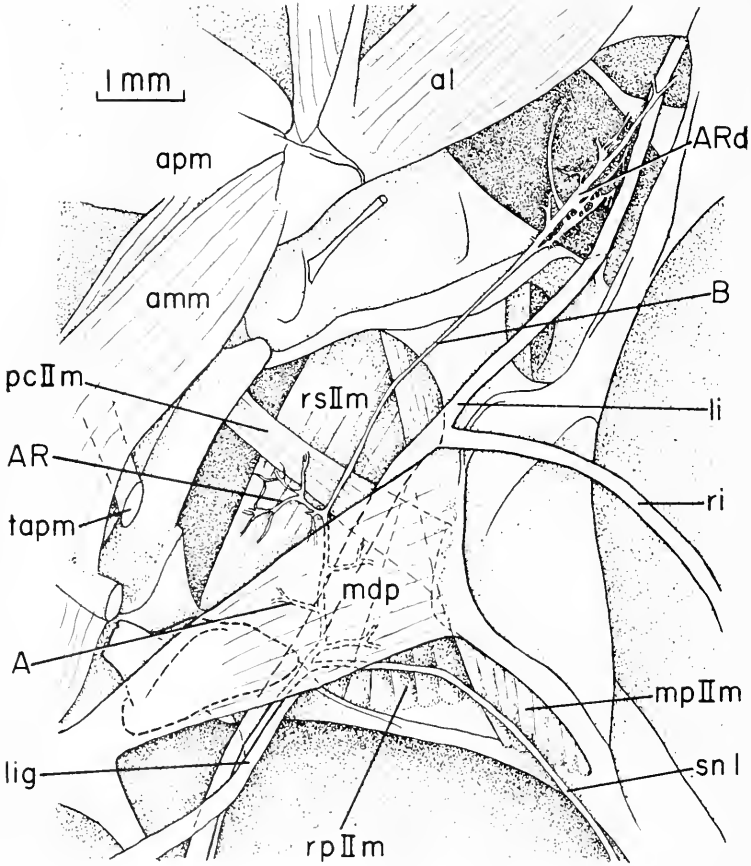

FIGURE 5. Anterior ramifications of *Ocypode albicans;* fresh material. Dorsal exposure with digestive system removed, midline to left. A, medial, posterior branch of anterior ramifications; al, musculus adductor lateralis mandibulae; amm, musculus abductor maior mandibulae (intact); apm, apophysis of mandible; AR, anterior ramifications; ARd, anterior ramifications, distal segment; B, anterior, lateral branch of anterior ramifications; li, lateral integumentary nerve; lig, ligament; mdp, musculus dorsoventralis posterior; mpIIm, musculus promotor II maxillae; pcIIm, musculus proximalis coxopoditis II maxillae; ri, recurrent integumentary nerve; rpIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius secundus II maxillae; sn 1, first segmental nerve; tapm, stump of tendon of musculus adductor posterior mandibulae.

arises from the post-esophageal commissure. Each of these passes dorsally through the posterior esophageal muscles where it is joined by a major branch of a nerve originating in the homolateral circumesophageal ganglion (see also Fig. 5; Heath, 1941). There are a number of smaller branches from these two nerves which innervate the surrounding esophageal muscles (Fig. 6). In view of the observation that in shrimp the post-commissural nerve does not send fibers to these muscles (Knowles, 1953), it seems possible that a similar situation exists in the Brachyura, and that nerve fibers innervating the esophageal muscles are derived from the nerve

originating in the circumesophageal ganglia and not the post-commissural nerve. The latter nerve passes from the post-esophageal muscles with no apparent diminution in size, and, after travelling laterally for a short distance, divides into two equal branches. One of these goes forward to terminate in a network of fibers spreading over the anterior third of the ligamentum ventrale capitis. This network is homologous with the lamellae of the post-commissure organ complex in shrimp and presumably constitutes the storage-release structure analogous to the AR and PO. It

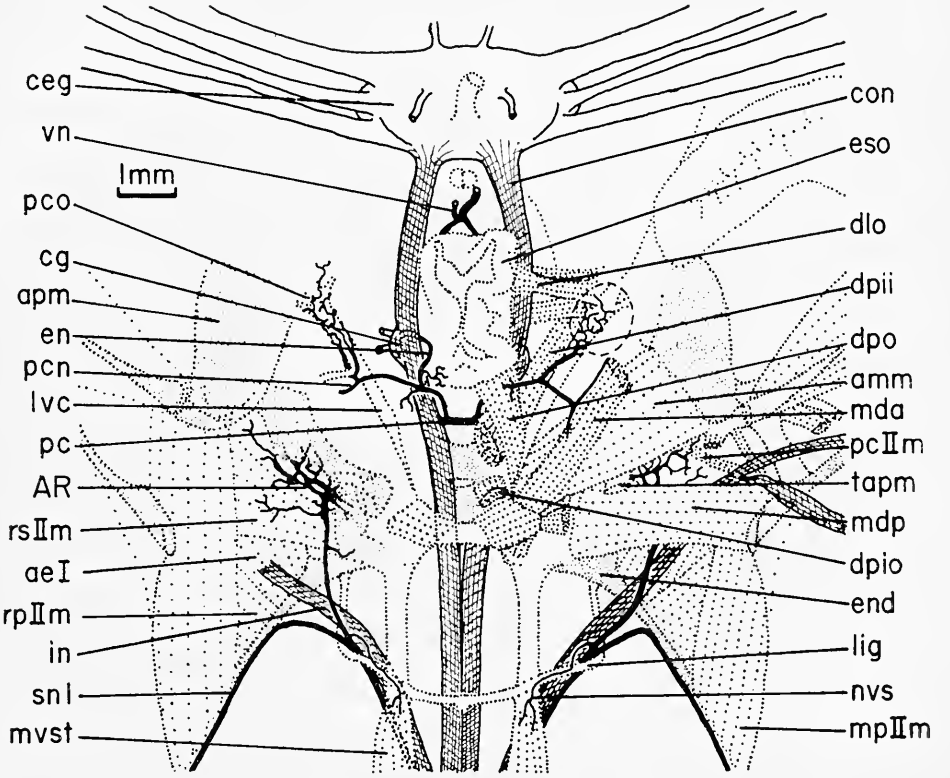

FIGURE 6. Anterior ramifications and post-commissure organs of *Pachygrapsus crassipes;* semi-diagrammatic, from fresh preparation. Dorsal exposure, digestive system removed. Ae I, musculus attractor epipoditis I pedis maxillaris; amm, musculus abductor maior mandibulae, apm, apophysis of mandible; AR, anterior ramifications; ceg, cerebral ganglia; cg, connective ganglion; con, circumesophageal connective; dlo, musculus dilatator lateralis oesophagei; dpii, musculus dilatator internus pylorici inferior; dpio, musculus dilatator externus pylorici inferior; dpo, musculus dilatator posterior oesophagei; en, nerve to esophageal muscles; end, endopleurite of last head segment; eso, esophagus; in, integumentary nerve; lig, ligament; lvc, ligamentum ventrale capitis; mda, musculus dorsoventralis anterior; mdp, musculus dorsoventralis posterior; mpIIm, musculus promotor II maxillae; mvst, musculi ventrales superficiales thoracis; nvs, nerve to ventral superficial muscles; pc, post-esophageal commissure; pcIIm, musculus proximalis coxopoditis II maxillae; pcn, post-commissure nerve; pco, post-commissure organ; rpIIm, musculus respiratorius primus II maxillae; rsIIm, musculus respiratorius secundus II maxillae; sn 1, first segmental nerve; tapm, stump of tendon of musculus adductor posterior mandibulae; vn, nerves of visceral system. The oval bounded by dashed lines above the right post-commissure organ represents the location of a dorsoventral venous channel.

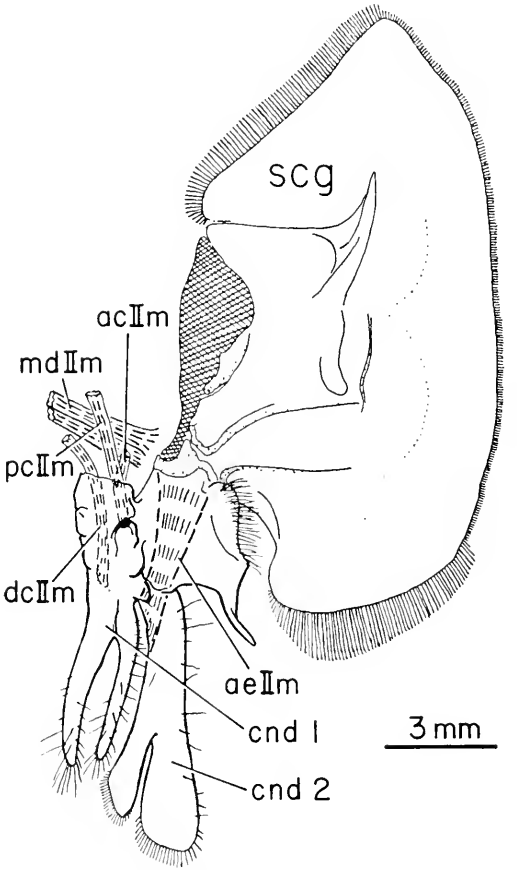

FIGURE 7. Second maxilla of *Callinectes*, showing muscles of coxopodite. AcIIm, musculus adductor coxopoditis II maxillae (no. 60, Cochran); aeIIm, musculus adductor endopoditis II maxillae (no. 61, Cochran); cnd 1, first endite of coxopodite; cnd 2, second endite of coxopodite; dcIIm, musculus distalis coxopoditis II maxillae; mdIIm, musculus depressor II maxillae (no. 59, Cochran); pcIIm, musculus proximalis coxopoditis II maxillae; scg, scaphognathite.

contains secretory granules and will be considered the post-commissure organ. The second branch of the post-commissural nerve innervates a muscle tentatively identified as the musculus dorsoventralis anterior (mda). This is possibly homologous with the "moulting muscle" in shrimp (Knowles, 1953).

In general, the entire anterior-ventral portion of the crab thorax is filled with stomach, hepatopancreas, or ramifications of the bladder. Venous haemolymph flow must therefore be largely confined to channels or spaces among the various viscera. The anterior third of the post-commissure organ is located across such a channel, outlined with dashes in Figure 6, which passes dorsoventrally between bladder protuberances beneath the stomach. Like the other neurohaemal structures of the thorax, the post-commissure organ is in a position to be continually bathed by flowing, venous haemolymph.

In *Pachygrapsus* the post-commissure organ appears the smallest of the three known thoracic neurohaemal structures.

Thoracic musculature

In the course of dissection, several muscles and ligaments were found in the anterior thorax which have not been generally recognized in the Brachyura. Since some of these are in intimate contact with the neurohaemal structures and have been used in the above descriptions or shown in figures, they are listed below with brief mention of their location. Muscles presumed homologous with those described in Macrura are named accordingly (Balss, 1941). Muscles whose homologies are uncertain are named according to their site of insertion.

Musculus dorsoventralis posterior (mdp) (Figs. 2, 6). Origin: ligaments above cephalic apodeme, runs laterally. Insertion: lateral carapace.

Musculus dorsoventralis anterior (mda) (Fig. 6). Origin: ligaments above cephalic apodeme, more medial and posterior than mdp, runs dorso-anteriorly beside stomach. Insertion: dorsal carapace just behind sockets of eyestalks (observed in *Pachygrapsus* and *Cancer*).

Ligamentum (musculus) ventrale capitis (lvc) (Fig. 6). Origin: ligaments above cephalic apodeme, runs anteriorly and slightly laterally. Insertion: carapace at origin of lateral muscles to esophagus. No muscle fibers were found in this structure in the two species examined, *Pachygrapsus* and *Cancer*.

Musculi ventrales superficiales thoracis (mvst) (Figs. 2, 6). Thin, flat muscles overlying thoracic nerve mass in thorax.

Musculus proximalis coxopoditis II maxillae (pcIIm) (Figs. 2, 3, 5, 6, 7). Origin: lateral carapace just anterior to insertion of mdp, runs anteriorly, medially, and ventrally. Insertion: inner membrane of coxopodite of second maxilla (Fig. 7). Apparently pulls coxopodite anteriorly and turns toward mouth (not figured by Cochran, 1935).

Musculus distalis coxopoditis II maxillae (dcIIm) (Fig. 7). Origin: beneath muscle 59, *musculus depressor II maxillae* (Cochran, 1935). Insertion: on medial side of coxopodite of second maxilla, distal to pcIIm. Apparently pulls coxopodite posteriorly and toward mouth (not figured by Cochran, 1935).

Discussion

The observations reported show that the peripheral neurosecretory complex in the brachyuran thorax is much more elaborate than heretofore supposed. Not only are post-commissure organs present as in the Macrura (Knowles, 1953), but the major thoracic neurohaemal structures, the pericardial organs, are but part of a still more extensive system which includes the anterior ramifications.

The total presumptive storage volume for secretion is also relatively large, as may be seen from a comparison between pericardial organs and sinus gland. The PO are the largest neurohaemal structures in the thorax, ranging from 0.25 to 1.0 mg. per 100 gm. total body weight. In *Carcinus maenas* the secretory terminations in the PO trunks form an outer layer which is about 10% of the trunk radius in thickness (Maynard and Maynard, unpublished data). The actual presumptive storage volume of the PO is therefore about one-fifth its total volume, 0.05 to 0.20

mg./100 gm. This compares favorably with the volume of the sinus gland of the fresh-water crayfish, *Cambarus virilis*, 0.2 to 0.45 mg./100 gm. total body weight (Brown, 1940).

Since functions of the thoracic structures, the PO-AR system in particular, are currently uncertain, some speculation from anatomy seems justified. Originally, the location of the PO in the pericardium led to the suggestion of a role in cardio-regulation (Alexandrowicz, 1953). Experiments showing cardio-acceleration by PO extracts supported the suggestion (Alexandrowicz and Carlisle, 1953; Maynard and Welsh, 1959). It was recognized, however, that the position of the PO is also perfectly suited for release of materials into the haemolymph for immediate distribution over the entire body, and the possibility of more general functions for PO secretion was postulated (Maynard and Welsh, 1959). The present work strengthens the latter possibility. Not only is the thoracic system remarkably extensive and voluminous for a cardio-regulatory structure, but branches of the first segmental nerve enter both the PO and AR and single neurons probably have secretory terminations in both structures (Maynard, 1961). This does not seem efficient if such neurons are concerned only with cardio-regulation, for the AR is located in a passage to the anterior branchial sinus and is separated from the heart by the full length of the afferent and efferent branchial veins. Such an arrangement is reasonable, however, if PO-AR secretion were indirectly concerned with respiratory transport. In addition to increased heart rate, one might presume that secretions from the AR portion of the complex play some role in oxygen uptake or circulation in the gills. Two possibilities may be mentioned. First, AR secretion may affect ventilation in the gill chamber by acting on scaphognathite muscle activity. A common hormone affecting both the heart and scaphognathite muscles could provide the loose coordination desirable for optimum gaseous exchange between tissue and external medium. Second, and perhaps more attractive, AR secretion may alter resistance to haemolymph flow in gill veins (Parrot, 1938). PO-AR secretions would therefore increase turn-over time by simultaneously increasing heart rate and reducing peripheral resistance. Increased turnover time would in turn tend to increase oxygen transport rates.

As mentioned earlier, the correlations found between PO volume and apparent activity of the brachyurans examined are interesting, but are very difficult to interpret. They are in line with the thesis that PO-AR secretions have some function in metabolism, water balance, or stress reactions, but hardly can be accepted as positive evidence. Appropriate comparative studies on the physiology of the brachyurans involved have not been published.

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Summary

1. Three neurohaemal structures occur in the crab thorax: the pericardial organs (PO) in the pericardium; the anterior ramifications (AR) in a ventral sinus near

the respiratory muscles; and the post-commissure organs, lateral to the esophagus and ventral to the stomach. Since the first segmental nerve supplies both PO and AR, the two are considered separate release sites of a single secretory complex.

2. The PO ranges between 0.00025 and 0.001% of total body weight in Bermuda crabs. It is largest in the most active species.

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GLUTATHIONE-CONTROLLED ANAEROBIOSIS IN CRYPTOCERCUS, AND ITS DETECTION BY POLAROGRAPHY [1, 2]

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The flagellate protozoa of *Cryptocercus punctulatus,* hind-gut symbionts of this wood-feeding roach, are generally regarded as obligate anaerobes. Cleveland *et al.* (1934) and Cleveland and Burke (1956) reported high oxygen susceptibility for *Cryptocercus* protozoa during *in vivo* studies relating oxygen toxicity with tension and temperature changes. Closely related termite protozoa are also killed by oxygen, demonstrated *in vivo* (Cleveland, 1925a, 1925b; Yamaski, 1931) and *in vitro* (Trager, 1933; Hungate, 1939). Trager's culture experiments with termite (*Zootermopsis*) protozoa, however, indicated to him that one flagellate species, *Trichonympha sphaerica,* probably requires oxygen in a very low but definite concentration.

It has been proposed that anaerobic status, *in strictu sensu,* might be assigned hind-gut-residing termite and roach protozoa if protozoa survival accompanied host survival following prolonged exposure of the host to nitrogen. Gilmour (1940) observed flagellate death in termites exposed to nitrogen and concluded that these protozoa are killed in their own metabolic end-products rather than from oxygen lack. Unpublished studies (Ritter) using *Cryptocercus* support Gilmour's conclusion. In brief, rapid cell destruction follows organic acid accumulation and pCO_2 rise with subsequent hind-gut fluid loss.

In all of these experiments it is impossible to define the exact status of oxygen (whether applied in excess of atmospheric concentration or totally omitted) and its influence upon the protozoa. Complex and critical side effects induced by secondarily altered chemical and physical constants must be assumed. Furthermore, it has not been established that a minute trace of oxygen might not be present, adequate, and quite essential for protozoan requirements. Indeed it is difficult to visualize a hind-gut content totally anaerobic considering its confinement in tissues liberally supplied with tracheal endings.

The immediate need, before further attempts to isolate the exact oxygen effect upon the protozoa of *Cryptocercus,* was development of a medium for *in vitro* culture. Actual design of the *in vitro* culture, however, was predicated upon a more certain understanding of the oxygen concentration, if any, present in roach hind-gut content.

The following study involves an apparently successful attempt to qualify anaerobiosis with respect to degree in very small samples of *Cryptocercus* hind-gut

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content with the highly oxygen-sensitive polarograph. Related phenomena are described and discussed to the extent present evidence will allow.

Materials and Methods

1. *The experimental animal*

A large collection of *Cryptocercus* was maintained at 20° C. in a metal container. Individuals dwelled within and fed upon portions of the same partially decomposed wood in which they were found. Only adult roaches were selected to supply material for the polarographic study. Hind-gut fluid was removed from the roach by holding it between thumb and forefinger, and squeezing with pressure strokes in an antero-caudad direction on the abdominal region. For a description of this insect as well as the protozoan symbionts it contains, see L. R. Cleveland *et al.*, (1934).

2. *Polarography*

Polarography, utilizing an apparatus with a sensitivity limit of approximately 10^{-6} *M* (0.001 m*M*) is one of the most efficient and accurate methods for oxygen detection (Kolthoff and Lingane, 1952). The normal hind-gut volume of an adult roach approximating only 0.1 ml., an abundance of suspended cellular and cellulose constituents in a brown and rather viscous liquid, and the likelihood of encountering a very low partial pressure of oxygen are factors which initially eliminated other techniques from consideration.

A Sargent Model XI recording polarograph was used in conjunction with a micro H-cell, possessing a solution chamber functional with slightly less than 0.1 ml. of sample. Although the micro-cell was designed and constructed with a side arm loading port suggested by Beecher, Follansbee, Murphy and Craig (1942), its use as a means of protecting the sample from air during loading proved to be unnecessary in this work. A constant flow of nitrogen was maintained while the roach hind-gut substance was squeezed into the micro-cell, during insertion of the mercury-dropping electrode, and for duration of the analysis.

Preparation of the saturated KCl-agar gel used in the salt bridge of the micro-cell and the saturated KCl solution for use in the reference electrode was performed according to Kolthoff and Lingane's method (1952). This reference and that of L. Meites (1955) offer detailed reviews of polarographic theory.

A polarogram is a plot of the current flowing through the solution in a polarographic cell against the potential of the mercury-dropping electrode. Polarograms used to document this study were traced and arranged to follow generally the order of discussion.

All polarograms were made with the same dropping electrode capillary at a constant rate of mercury drop formation. The 20° C. water bath surrounding the electrolysis vessel and all other operating conditions involved in polarographic technique were held constant.

Since the comparative method of wave height-concentration plot was used, rather than the Ilkovič equation to calculate the concentration of substance present, it is necessary to note that not all polarograms included could be recorded at the same sensitivity.

In order to record a high substance concentration, a polarograph setting was selected (*e.g.* shunt X-5 or X-10), resulting in lower apparatus sensitivity. The full wave height formed between the residual current (the lower plateau of the polarogram) and the limiting current (the upper plateau of the polarogram) wave portions, therefore, could be included within the polarographic paper margins. A low substance concentration could be recorded conveniently at a polarographic setting of shunt X-1 or X-2 while utilizing maximum apparatus sensitivity. The shunt setting, used in recording a polarogram, is included in legend data corresponding to the lettered curve figured. When a quantitative comparison is made between two polarograms, concentration reading corrections must be made for polarograms recorded at different sensitivity settings. For example, in a polarogram recorded at an apparatus shunt setting X-2, sensitivity would be one-half that utilized in a record made at a setting of X-1. Likewise, a setting X-1 indicates a recording sensitivity five times greater than a setting X-5. The wave height (corrected when necessary as directed) represents concentration, and this is read in microampere units of current. The voltage range in all polarograms extends from 0 to −0.4 volt.

3. *Glutathione assay*

The procedure used for a sodium nitroprusside assay of reduced glutathione was developed by Grunert and Phillips (1951), although in this study sodium chloride and metaphosphoric acid were not included as in Benesch, Benesch and Rogers (1953).

The alloxan (alloxan monohydrate, Eastman) "305" method of colorimetric determination, according to Patterson, Lazarow and Levey (1949), was modified only to the extent of reducing reaction volumes for adaptation to the Beckman-Spinco ultramicro analytical system. Application of the Beckman DU spectrophotometer to minute quantity measurement is described by Lowry and Bessey (1946).

The microbioassay for reduced glutathione, based on the "feeding reaction" of *Hydra* worked out by W. F. Loomis (1955a, 1955b), was performed in the Loomis Laboratory.

The polarograph, already discussed as applied, registered a decomposition voltage specific for the sulfhydryl group of a thiol compound. The comparative method of polarography identified the recorded substance to be reduced glutathione.

4. *Reagents*

Reduced glutathione as well as all other substances used in the comparative approach—polarographically to identify materials responsible for wave formation—were obtained from the Nutritional Biochemicals Corporation. Triple-distilled mercury was used in all polarographic procedures.

"Pre-purified" nitrogen (Airco) assayed 99.998% and oxygen (Airco) assayed 99.5%. Gases were passed through gas-equilibrated water and directed by capillary pipette into the micro-cell solution chamber. During routine polarographic procedure, nitrogen was passed over the surface of the gut content sample. In certain specified instances oxygen or nitrogen, as the case may have been, was bubbled through the sample *via* a capillary. The latter procedure, when applied, was carried out immediately prior to analysis.

Results

1. *Polarograms recorded immediately following sample isolation from the hind-gut*

Dissolved oxygen in solution is reduced to hydrogen peroxide at the dropping mercury electrode. Kolthoff and Lingane (1952) reported the half-wave potential of the corresponding wave, indicating this reduction is practically independent of pH, at a reading of −0.05 volt *versus* a saturated calomel electrode (S.C.E.).

More than 100 polarograms have been recorded of *Cryptocercus* hind-gut content. The samples ranged in volume from slightly less than 0.1 ml., when one individual roach was used, to approximately one ml. collected from 10–12 roaches. It was impossible to demonstrate an oxygen wave. In particular, ordinary exposure of the gut content to air, incidental to its hasty transfer from insect to the solution chamber of the polarographic cell, failed to introduce a detectable amount of oxygen (Curve A, Figs. 2, 3, 5; Curves A and B, Fig. 6).

Polarograms consistently included a prominent wave at a negative half-wave potential (−E ½), ranging between −0.19 to −0.25 v. *vs.* S.C.E. Absence of complete specificity in the −E ½ reading suggested the recorded substance within the gut content was organic and pH-dependent. According to Meites (1955), a nonspecific E ½ associated with a substance showing polarographic reversibility (as appears within gut content) is more likely attributable to a variable pH than to varying ionic strength in an unaltered medium with regard to buffer.

No direct evidence for the correlation between −E ½ and the initial gut content pH for use as a reference point was sought. It was anticipated that delay, in the event sufficient sample remained for polarographic study after pH measurement of a volume as small as 0.1 ml., would be accompanied by sample deterioration. Cell activity gradually changes the chemical nature of hind-gut content as confined metabolites accumulate. The pH of freshly obtained gut content samples ranges between 6.6 and 7.2.

Attempted sample pH control during polarography by oxygen-free buffer addition probably resulted in failure for one or more of the following reasons: (1) sudden medium alteration due to contact with lysed cell content, (2) severe sample dilution, and (3) ionic strength change leading to nonspecificity of −E ½ identical to that caused by variable pH for which control was sought ionically.

Slight variation, therefore, in −E ½ between polarograms of different freshly obtained gut content samples is attributed to pH variation between samples. Initial standardization of pH, and, for that matter, pH control during a series of recorded polarograms tracing changes produced by *aging* of any one sample as reported later, cannot be achieved successfully with buffer. Regardless, a low oxidation-reduction potential is indicated, and specificity range is sufficiently limited to allow positive identity of the reducing agent.

2. *Tests used in identification of the reducing agent*

A. Polarography

Separate polarograms for solutions of varied substances in 0.1 *M* KCl failed to produce waves even remotely resembling the shape of Curve A, Figure 2, much less a half-wave potential falling within the desired range. Among compounds studied

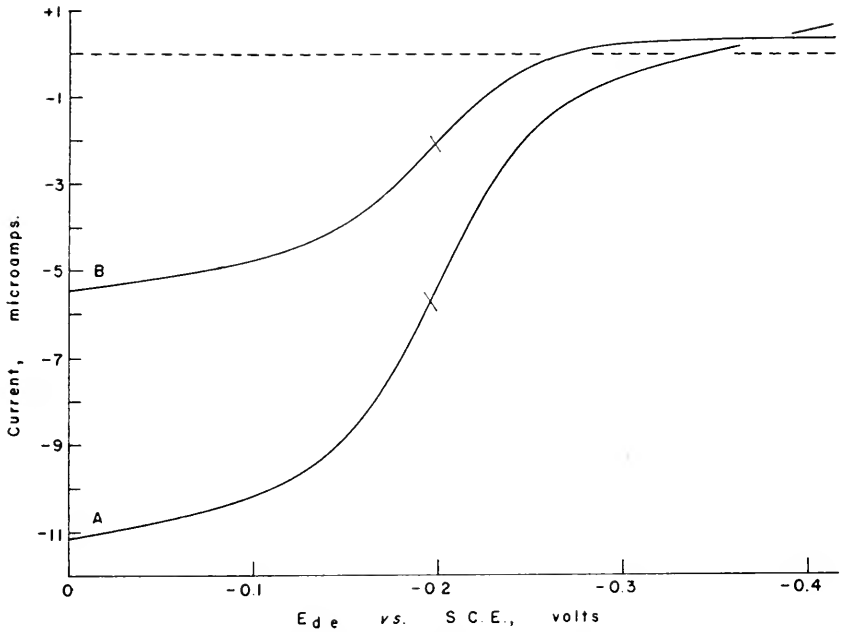

FIGURE 1. Polarograms of reduced glutathione: (A) 1.4×10^{-3} *M* GSH in 0.1 *M* KCl, (B) 7.2×10^{-4} *M* GSH in a complex salt medium also containing yeast, blood serum and methylcellulose (sensitivity X 5; pH 6.6).

were L-cysteine, adrenochrome semicarbazone, ascorbic acid, sodium anthraquinone sulfonate, acetylcholine chloride, riboflavin, and sodium thioglycollate.

A 1.4×10^{-3} *M* solution of reduced glutathione (GSH) in a supporting electrolyte of 0.1 *M* KCl at pH 6.6 produced a polarogram (Curve A, Fig. 1) closely resembling that of Curve A, Figure 2. Addition of 0.005 gm. GSH to a gut content sample increased the wave height without significant change in the previously recorded half-wave potential.

The comparative method of wave height-concentration plots indicated 7.2×10^{-4} *M* GSH in an isotonic medium of mixed salts, nutrients, and methyl cellulose results in a polarogram (Curve B, Fig. 1) almost identical to that recorded from the natural gut content (Curve A, Fig. 2). The Ilkovič equation, mentioned earlier, was not employed in the calculation of GSH concentration. Application of this equation would require a viscosity reading for hind-gut content, and this has not yet been determined.

B. Chemical

GSH produces a violet color in the presence of sodium nitroprusside. A positive reaction occurs when a thin layer of dark brown *Cryptocercus* gut content is tested on a white porcelain surface. Although ascorbic acid does not react with nitroprusside, cysteine and possibly other sulfhydryl compounds effect the positive reaction (Patterson and Lazarow, 1953).

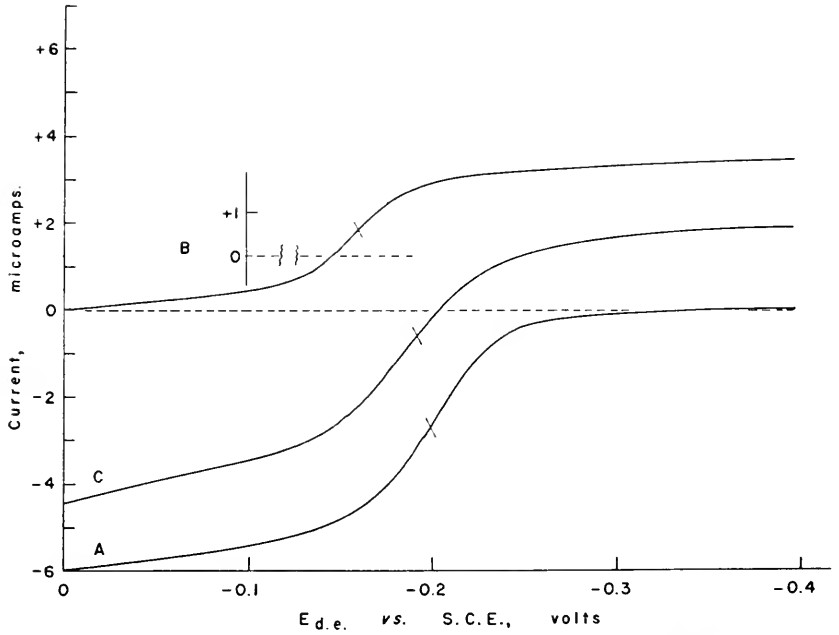

FIGURE 2. Polarograms of the same hind-gut sample from *Cryptocercus:* (A) untreated, (B) following air passage for one hour, and (C) following nitrogen gas flow, 20 minutes (sensitivity X 5; pH approximately 6.8 at start).

Patterson, Lazarow and Levey (1949) report alloxan in excess reacts with the sulhydryl of GSH forming an unknown substance with an absorption spectrum maximum at 305 mμ. By this method 3×10^{-4} *M* GSH has been identified in hind-gut content cleared of cellulose and protozoa. The authors mentioned also report equivalent molecular amounts of cysteine, cysteinylglycine, glutamylcysteine, and ascorbic acid in the presence of GSH lower the value of GSH determination.

C. Bioassay

Loomis (1955a, 1955b) showed that a concentration of 10^{-5} *M* GSH elicits a characteristic feeding response in starved *Hydra littoralis.* A 1:3000 dilution of *Cryptocercus* gut content induced a strong response, indicating a concentration of GSH somewhere in the order of 10^{-3} *M.* This bioassay, however, is no longer specific for GSH. Lenhoff and Bovaird (1960) obtained the same response with trypsin, and Cliffe and Waley (1958) obtained it with ophthalmic acid.

3. *The apparent source of GSH*

Since variables involved with polarograph operation were held constant, slight variations in GSH concentration, observed in fresh unaltered samples, must be inherent in sample fluids. The ratio between number of cells and surrounding fluid volume probably varies between hosts. If GSH is protozoan and/or bacterial in

origin, it could fluctuate with the rise and fall of these populations. If GSH is a secretion of the host tissues, its concentration could vary in direct proportion with the mass of the insect. The question of primary concern, however, was the source of GSH.

After squeezing, sufficient protozoa remain within the roach to repopulate all species back to normal level within four to six weeks. During this period, contrary to conditions in unsqueezed roaches, many cells appear in various stages of mitosis. Protozoan repopulation is a relatively slow process, but the fluid content is rapidly reestablished. An adequate volume of fluid can be removed from the same roach 24 hours after squeezing for polarographic study.

Polarograms were made at 24 hours, and at the end of the first, third, and fourth weeks during the repopulation period with two groups of squeezed roaches. Results indicate GSH concentration increases directly proportional to the increasing protozoan population.

For the present, the relatively crude method used in estimating repopulation rate during the four-six-week period appears adequate. Three stages in the process were easily recognized. (1) That time immediately following squeezing when the gut fluid contains a few random, scattered, residual cells. (2) A mid-stage follows when fluid contains a large number of cells, but also cell-free areas. (3) Maximum population is reached when a mass of cells appears, each in contact at many points with other cells.

An estimate of one week for total bacterial repopulation, following squeezing, exceeds the mean reproductive potential of such organisms. If host tissue was responsible for GSH secretion, a maximum concentration would be expected (polarographic inspection) within one week after squeezing. Usually three weeks were required before GSH concentration reached 50% of normal. This corresponds to a protozoan population judged to be at the approximate refaunation half-way mark.

Several protozoan species are intimately associated with one or more species of bacteria. The cell membrane of *Barbulanympha* shows uniform cellular arrangement for one species of bacterial rod. The association is permanent. This example of *subordinate* symbiosis, and other associations of a similar nature, prevent final decision regarding the exact source of GSH—the protozoa *per se,* intimate bacterial associates, or both. It is, however, significant to know GSH is not host elicited.

4. *The effect of oxygen and nitrogen on GSH*

The relationship between absence of a polarographic oxygen wave, following exposure of gut content to air or pure oxygen, and GSH concentration must be considered.

A sample of hind-gut content bubbled with air at a slow rate for as long as one hour in the H-cell solution chamber failed to produce an oxygen wave. GSH concentration, however, decreased approximately one-half from a reading of approximately 5.4 μa to 2.5 μa shown in a comparison of Curves A and B, Figure 2. The reduction potential also was shifted 0.04 volt more positive.

Roughly 90% of the initial GSH concentration (presumably oxidized) was reduced in the gut content during nitrogen passage for one hour. Recovery was indicated by an increase in the height of the wave (Curve C, Fig. 2) and a reduction

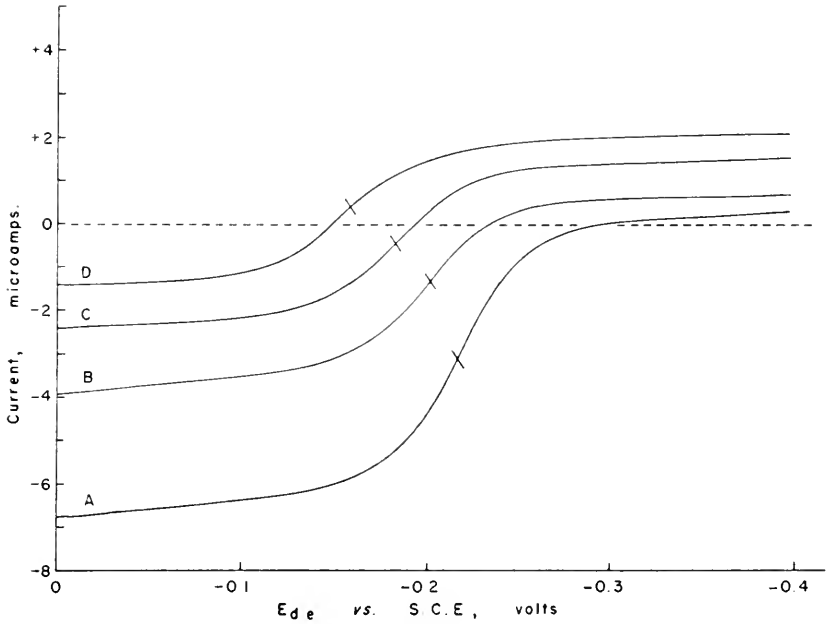

FIGURE 3. Polarograms of the same hind-gut sample from *Cryptocercus:* (A) untreated, (B) following pure oxygen passage ten minutes, (C) following a 20-minute additional period of pure oxygen passage, and (D) nitrogen gas passage, 40 minutes (sensitivity X 5; pH approximately 6.8 at start).

potential shifted back to a more negative reading. Shift of $-E\ \frac{1}{2}$ to a more negative position was first viewed as a result of alkalinity increase following a CO_2 *blow-off*. But, upon further consideration, oxygen passage would also be expected to achieve the same effect, and does not. A change in pH, nevertheless, first in one direction and then the other, appears responsible for corresponding shifts in reduction potential.

Curves B and C, Figure 3, show a progressive reduction in GSH concentration for one sample following contact with pure oxygen. Curve A represents the sample's GSH level prior to oxygen exposure. Curve A differs from the previous experiment's unexposed sample (Curve A, Fig. 2), registering approximately 0.7 μa more GSH and a slightly higher pH, judging from the more negative E $\frac{1}{2}$ position.

Curve B, Figure 3, was recorded following oxygen passage for ten minutes. From a comparison of microampere readings between Curve A (original concentration) and Curve B, estimated GSH drop was 50%.

Curve C, Figure 3, was recorded following oxygen passage lasting 20 additional minutes, in which the GSH level was further reduced. Even under these extreme conditions, in a sample less than 0.5 ml., it was not possible to demonstrate the presence of dissolved oxygen.

Continuing with the same sample, nitrogen passage—following oxygen exposure of the extreme intensity just described—failed unaccountably to demonstrate the

reversibility achieved with nitrogen after air exposure in the earlier experiment (Curve C, Fig. 2). Even forty minutes' contact with nitrogen (Curve D, Fig. 3) did not bring about a curve height increase beyond that recorded in Curve C, Figure 3. The height of Curve D, Figure 3, was depressed even further. In addition, again contrary to results obtained when the air-treated sample was bubbled with nitrogen, a comparison of half-wave potentials in Figure 3 illustrates nitrogen passage was accompanied by a reduction potential shift to a more positive reading.

The phenomena just described are based on repeated observations. Polarograms illustrated trace events related to two treated gut samples; one sample using air passage followed by nitrogen gas (results verified by fourteen experiments), and one sample using "prepurified" oxygen followed by nitrogen gas (results verified by seven experiments).

Although it has not been determined how much oxygen a small sample of gut fluid can *absorb* or *bind* without losing its potential with respect to GSH reduction during nitrogen passage, experiments suggest oxygen can overwhelm the glutathione recovery system.

A. Evidence suggesting enzymatic reduction of GSH

That partial GSH reduction can occur during nitrogen passage through a gut sample under prolonged exposure to air suggests strongly the presence of an appro-

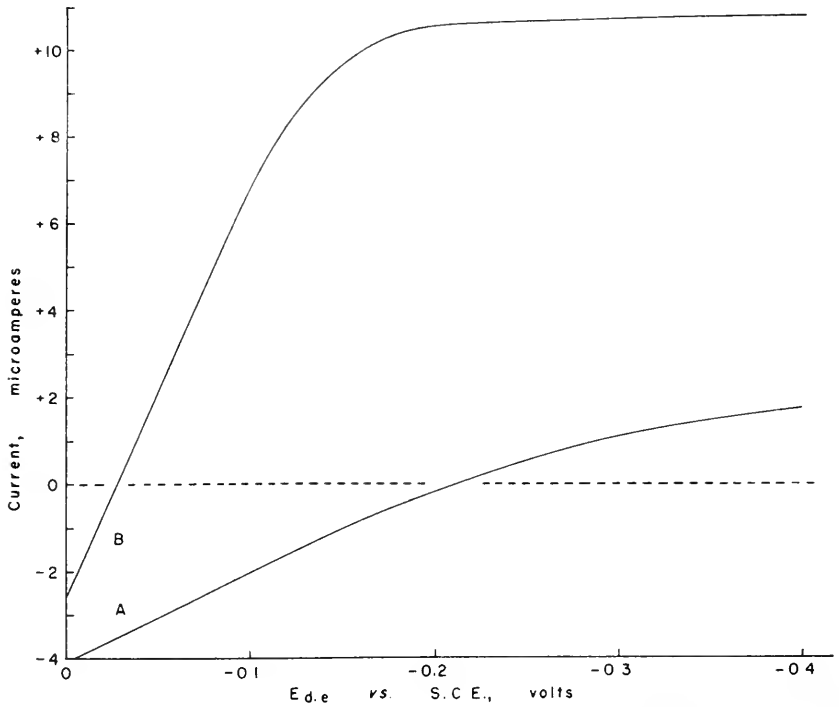

FIGURE 4. Polarograms of the same hind-gut sample from *Cryptocercus:* (A) heated ten minutes, and (B) following pure oxygen passage five minutes (sensitivity X 2).

priate enzyme system. Further evidence favoring the enzyme hypothesis appears when partial glutathione recovery, to the reduced state following air exposure, cannot be achieved in a sample altered by heating over a water bath for 10 minutes at 80° C. Even negligible atmosphere exposure encountered in transferring a heat-treated sample to the electrolysis vessel is sufficient to oxidize GSH, as indicated by disappearance of the reduction wave (Curve A, Fig. 4). Five minutes of oxygen passage then results in such marked saturation of the sample that a prominent oxygen wave (Curve B, Fig. 4) can be recorded.

Glutathione reductase and dehydrogenase assays have not yet been undertaken. Such studies will be resumed following application of present findings to protozoan culture. It is entirely possible that more than one enzyme will be found present in the hind-gut content, accounting for rapid glutathione auto-reduction demonstrated prior to heat alteration.

5. *Evidence suggesting a chemical relationship between glutathione and carbon dioxide*

Polarographic experiments and related observations repeatedly point out another phenomenon in the gut content which might support M. Calvin's (1953) hypothesis for addition of mercaptans across C = O in CO_2 in slightly alkaline media to form S-alkyl monothiocarbonate. He predicted an acid medium could reverse the reaction to release CO_2 and restore GSH:

$$GSH + \overset{O}{\overset{\|}{C}} = O + OH^- \underset{H^2}{\overset{OH^-}{\rightleftharpoons}} R - S - \overset{O}{\overset{\|}{C}} - O^- + H_2O$$

A dense resident population of microorganisms in the hind-gut of *Cryptocercus* ferments ingested cellulose. The fermentation products would be expected to be similar to those reported by Hungate (1939) for *Zootermopsis*—CO_2, hydrogen, and predominantly acetic acid. Complete acid analysis has not been made, but total CO_2 in freshly removed gut content measures 0.12 volume per cent by Scholander's (1947) method.

Fermentation probably continues briefly, following sample introduction into a polarographic vessel (due to continued cell activity), which partially accounts for increase in acidity. Twenty to 30 minutes later the pH drops from the 6.6–7.2 range to 6.4. Within five hours the pH drops to 4.8–5.1, and at 18 hours 4.4. This could explain the shift of half-wave potential in polarograms (Curves A, B, and C, Fig. 5) from −0.196 volt *vs.* S.C.E. made immediately to a more positive potential approximating −0.133 volt after 17 hours.

The protozoa are dead, most are deformed, and some are lysed when the pH reaches 6.4. Motile bacteria are no longer active. The remaining bacterial species constitute a very small number of cells in what was an overwhelmingly populated milieu. These bacterial species can be cultured in a cellulose-nutrient medium which supports growth and reproduction of five protozoan genera normally associated with the bacteria (Ritter, 1959). Bacterial metabolites produced in this culture medium in the absence of protozoa cause the pH to increase, not decrease.

Bacterial influence in this phenomenon of acidification seems to be completely ruled out following observation of toluene-treated gut content samples. Toluene addition to the extent of 20% final dilution failed to alter the pattern of pH decrease to the 4.0–5.0 range within 18 hours.

The cause of pH drop from 6.4 to 4.4 in 18 hours must be accounted for if, as seems to be the case, protozoa and bacteria are not responsible. It appears that bound CO_2 is released in sufficient quantity to cause this pH drop. CO_2 was identified as a major gas constituent, accumulating after several hours as a bubble mound at the interface of a large gut fluid sample layered with heavy mineral oil.

Correlated with this rapid accumulation of CO_2 in the absence of fermentation, polarograms (Curves A and C, Fig. 5) lend additional support to Calvin's hypothesis. As the pH registers progressively lower, GSH concentration increases. From comparative wave height measurement of polarograms A and C, GSH (which almost doubles in quantity within five hours) increases nearly four times the original amount within 17 hours.

This marked wave height increase could indicate mercaptide formation. It is generally accepted that a typical mercaptan characteristic is reaction with heavy metals such as mercury, and with organic compounds, to form mercaptides. And, apparently, there is every opportunity for this reaction to occur in a sample which rests on mercury in the electrolysis vessel.

The mercaptide phenomenon, if it occurs in this gut fluid, does not appear polarographically. The wave height increase represents GSH concentration in-

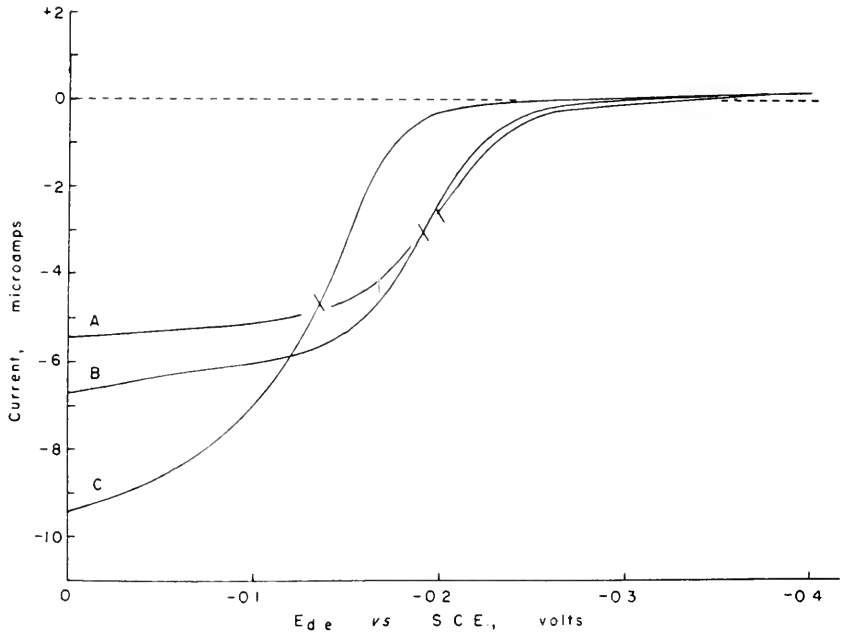

FIGURE 5. Polarograms of the same hind-gut sample from *Cryptocercus*, untreated, and recorded at intervals: (A) immediately, (B) at 20 minutes, and (C) at 17 hours (sensitivity X 5 Curves A and B; X 10 Curve C; pH approximately 6.8 at start; 4.4 at end).

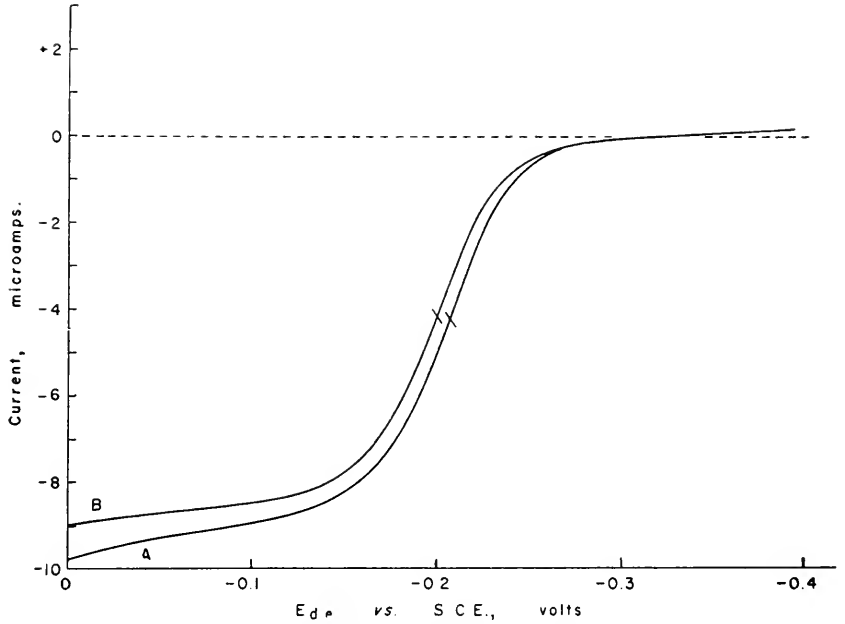

FIGURE 6. Polarograms of two different hind-gut samples from *Cryptocercus:* (A) *aged* 5 hours in the electrolysis vessel and in contact with the mercury pool, pH 5.1, and (B) *aged* 4½ hours in a vessel without mercury contact until transfer to the electrolysis vessel, pH 4.9 (sensitivity X 5).

crease, and the shift in $-E\ ½$ to a more positive reduction potential correlates with pH drop. Evidence for this conclusion rests on:

1. A fluid sample *aged* between four and five hours in a mercury-free container, after removal from the roach and deposit inside the electrolysis vessel, registers a wave (Curve B, Fig. 6) coresponding closely with a fluid sample *aged* the same period in solution chamber contact with mercury (Curve A, Fig. 6).

2. According to Benesch and Benesch (1952), more energy is required for mercaptide complex reduction than for the free compound that gave rise to the complex. Therefore, the resulting reduction wave would register at a more negative E ½ for the mercaptide—not more positive as recorded in this study—to reflect the low dissociation of the Hg-S bond.

3. Also in the event of mercaptide formation, two separate waves would be expected to appear polarographically. The difference between the $-E\ ½$ of the gradually forming mercaptide and the more negative E ½ of disappearing GSH would likely be great enough to initiate two separate reduction waves separated by a shifting plateau.

DISCUSSION

The appreciable presence of atmospheric oxygen coupled with diverse, unique, and efficient animal systems that transport oxygen suggest unspecified claims of anaerobiosis (as to degree) should be questioned. Living membranes present an

ineffectual barrier to oxygen. For that matter, full appreciation of oxygen permeability through synthetic membranes cannot be realized until experience is acquired with a *working* chamber from which all oxygen is meant to be excluded. In other words, at least a minute amount of oxygen probably reaches an organism's most remote anatomical niche. If symbionts dwelling in such a site require an absolute oxygen-free environment, either they or the surrounding host cells must chemically maintain it.

1. *Absence of molecular oxygen in* Cryptocercus *hind-gut content*

A. Direct evidence

The entire problem of determining environmental pO_2 at lower limits is hampered by inadequate indicators and mechanical devices for measurement. Polarography, in spite of extreme sensitivity for detecting oxygen in solution, is incapable of registering ultra-trace amounts. Failure to obtain typical reduction waves for oxygen in *Cryptocercus* hind-gut fluid polarograms is of great analytical significance. However, the question of degree in anaerobiosis still remains unanswered. To report that this environment is free of oxygen to the extent of 10^{-6} *M* is informative, though admittedly inadequate, for culture investigation. Technically, therefore, the term anaerobic should not be used merely on the basis of this information.

B. Indirect evidence

The polarograph also indicated concentration decrease of an unknown substance when gut content was exposed to pure oxygen. Prolonged oxygen exposure failed to leave enough molecular oxygen in solution for polarograph detection. In addition to indicating a substance is exploited in proportion to the amount of oxygen passing into gut fluid, the observation strongly suggests chemical affinity between oxygen and this unknown substance.

Oxygen most certainly diffuses into the lumen from the gut epithelium under natural conditions. Conceivably the oxygen is instantly bound molecularly. Interpretation of these phenomena, based on direct and indirect evidence obtained in polarograms, suggests *Cryptocercus* protozoa live in an anaerobic environment—anaerobic to the extent of maximum physical and chemical maintenance.

2. *Reduced glutathione in* Cryptocercus *hind-gut content*

A. Methodology in identification

Identification of the substance, which registers so prominently on polarograms and appears to function as a strong reducing agent, was difficult. Analyses offering unequivocal interpretation are rare when attempting to single out one organic constituent in the complexity of a biological medium.

Investigative methods included comparative polarography, the classic sodium nitroprusside test, the alloxan "305" method, and the Loomis bioassay technique.

Comparative polarography is a highly accurate procedure in which closely controlled physical conditions are imposed to measure known and unknown solutions. A more extensive survey of potential reducing agents appeared unnecessary because of the method's relative specificity. A known GSH solution produces

a polarogram of nearly identical structure to gut content polarograms. The supporting electrolyte of mixed salts used in the GSH medium was determined by flame photometry of the gut fluid lacking major suspended elements. Viscosity was approximated with methyl cellulose, and the pH of 6.8 required no adjustment. Variation in any one of these—especially pH—caused an expected fluctuation in the $-E\ ½$, but the polarographic curve remained essentially unchanged.

As mentioned earlier, the standard addition method of polarography confirmed the presence of glutathione. Addition of GSH to the unknown sample increased the wave height (concentration) without changing other aspects of the curve.

Following preliminary evaluation of other available methods of GSH analysis for application to this study, one-dimensional paper chromatography—of use after blocking the sulfhydryl group with N-ethylmaleimide (Hanes, Hird and Isherwood, 1950)—indicated greatest specificity. Inadequate sensitivity of the chromatographic method, using GSH concentration determined polarographically as reference, eliminated chromatographic application.

The alloxan "305" method functioned as a qualitative test only. GSH concentration determined in the supernatant portion of centrifuged gut content was less than half the amount demonstrated polarographically. Although substances already mentioned compete with GSH in alloxan reaction, it is possible only two need be considered. Cysteine and ascorbic acid, though potentially reactive at the dropping mercury electrode, do not appear polarographically. Glutamylcysteine and/or cysteinylglycine might be involved in competitive absorption. Awaiting further investigation, one might guess that γ glutamylcysteine, an intermediate in GSH synthesis probably to the exclusion of cysteinylglycine (Snoke and Bloch, 1952), is of more vital concern in evaluating potential alloxan competition with GSH.

The nitroprusside method and the bioassay technique using *Hydra* lack specificity in GSH identification. Nevertheless, nitroprusside identified the substance as a sulfhydryl compound, and *Hydra* gives a feeding response only in the presence of reduced glutathione. Trypsin, as yet unidentified in *Cryptocercus,* has not been ruled out as an alternative stimulant for *Hydra.*

This represents by no means an exhaustive methodology for assaying biologically-occurring glutathione. Each chemical test available is nonspecific or limited to some degree. General disagreement usually exists regarding the number of chemical tests necessary before identification is scientifically acceptable. In this polarographic study, however, more than mere substantial evidence accumulated to support glutathione assessment. Results derived from both nitroprusside and *Hydra* assays were conclusive yet predicated upon far less specific analysis.

On the strength of collective information afforded by these tests, along with confirming opinions of Professors K. E. Bloch, J. T. Edsall, and A. M. Pappenheimer, GSH is a constituent of *Cryptocercus* hind-gut content. GSH is responsible for maintenance of the oxidation-reduction potential observed polarographically.

B. The site of synthesis

Since more than 30 species of protozoa inhabit *Cryptocercus,* it must still be determined which of these are involved in GSH synthesis. The problem is further complicated by intimate, and, in some cases, permanent, bacterial association with the cell membrane of certain protozoan species. Evidence suggests protozoa are the site for this synthesis, but not necessarily the sole metabolic source of GSH.

C. Oxidation-reduction activity

It was impossible to assess fully results obtained in experiments in which gas was directed through freshly obtained gut content samples. It was clear that oxygen decreased the concentration of reduced glutathione. This was interpreted as an oxidative phenomenon in which GSH was converted to the disulfide form (GSSG). Although nitrogen passage was capable of partially restoring the glutathione wave height in air-exposed samples, the precise mechanism was not discernible. It has been assumed temporarily that this wave extension represents a reduction of GSSG to GSH—the nitrogen gas being most indirectly involved.

Wieland (1953) stated reduction of GSSG can be accomplished with various reducing agents, other mercaptans in excess, nascent hydrogen, Na or Li in NH_3, and by other substances. He also pointed out enzymatic reduction is frequently responsible. Whatever the cause, a point exists—polarographically demonstrated—at which nitrogen can no longer carry out its indirect role. This occurs following gut content exposure to pure oxygen in excess of ten minutes. Glutathione oxidized to this extent could not be *reactivated* as could samples exposed to lesser amounts of oxygen.

Opinions sought are totally inadequate in accounting for contradictory evidence concerning the effect of nitrogen following varied aerations of gut content—air *versus* pure oxygen. Interpretation of present phenomena obtained polarographically must await correlation with results from additional analyses now contemplated.

Since the oxidation-reduction potential of gut content can also be rendered inactive by heat, the tentative conclusion for the presence of at least one enzyme system seems justified. Glutathione reductase, according to Vennesland and Conn (1953), is the thermolabile enzyme responsible for the reduction of GSSG by tissues. GSSG is reactive with TPN-reducing systems. Accordingly: $TPNH + GSSG + H^+ \rightarrow TPN^+ + 2GSH$

D. A proposed role in pCO_2 and pH regulation

Culture studies in progress demonstrate glutathione can now be assigned an extracellular role in anaerobic maintenance. The importance of this mercaptan in growth and other physiological activities is well substantiated, but whether it can be assigned an additional function in the gut of *Cryptocercus* is still problematical.

Speculation regarding GSH capacity to combine with CO_2 to form an alkyl compound, as proposed by Calvin, is a tempting hypothesis. Culture studies of the protozoa definitely show a very narrow range of pCO_2 tolerance. Experimentally, membrane selectivity can be altered dramatically when cells are exposed to an excess of this gas, but protozoa move directly to a higher pCO_2 gradient when placed in a sub-optimal region of the culture medium.

Hind-gut protozoa, therefore, engaged in active fermentation yet highly sensitive to an altered pCO_2 threshold, would have marked survival potential if a mechanism were available for chemical regulation of this metabolite. Theoretically GSH could function as a pH-regulated pCO_2 buffer, supplementary to more direct pCO_2 control by the roach's external respiratory apparatus. Conceivably, even though pH might *trigger* this mechanism to reestablish an equilibrium between S-alkyl monothiocarbonate and GSH, pH, in turn, could be regulated, in part at least, by the mechanism. Certainly the host roach and its protozoa are confronted

by environmental factors that challenge optimal metabolism, such as conditions imposed during molting and winter.

I am very much indebted to Dr. L. R. Cleveland for invitation to work at the Biological Laboratories, for his advice, and for his criticism of the manuscript. I am grateful for the assistance of Dr. W. F. Loomis, and for advice and suggestions regarding polarography and the interpretation of polarograms from Professor J. J. Lingane and Dr. Allen J. Bard. Professors Bloch, Edsall, Pappenheimer, and Wald were consulted during various phases of this study. Their interest and counsel is greatly appreciated.

Summary

1. Polarography, employing a mercury-dropping electrode and an electrolysis vessel for analysis of sample slightly less than 0.1 ml., was applied in this study.

2. Polarograms of *Cryptocercus* hind-gut content lack an oxygen wave and indicate absence of dissolved oxygen to the extent of apparatus sensitivity (10^{-6} *M*).

3. A single polarographic wave registers a half-wave potential approximating −0.2 volt *vs.* S.C.E. within a 0- to −0.4-volt span. Specificity of this method enabled identification of reduced glutathione (7.2×10^{-4} *M*) following comparative inspection of numerous reducing agents. Supplementary assays using sodium nitroprusside, alloxan monohydrate, and *Hydra* confirmed the presence of reduced glutathione in hind-gut content.

4. Flagellate protozoa, symbionts in the hind-gut fluid of *Cryptocercus,* represent the site of glutathione synthesis.

5. Prolonged oxygen passage through hind-gut samples (0.1–0.5 ml.) fails to alter polarograms with respect to negative pO_2 registration, but reduced glutathione concentration is decreased in direct proportion to oxygen exposure intensity.

6. Partial restoration of reduced glutathione concentration following nitrogen gas passage through samples moderately exposed to oxygen cannot be explained.

7. Brief atmosphere contact with heat-altered hind-gut samples results in polarograms showing pO_2 contamination and an absence of reduced glutathione. Until enzyme analyses are completed, sample alteration by heat merely suggests presence of a mechanism for glutathione maintenance in the reduced state.

8. *Cryptocercus* hind-gut content is anaerobic to the extent of maximal physical and chemical maintenance as are the microorganisms normally living within it. Reduced glutathione is considered of major importance in this role.

9. Some evidence exists which may support Calvin's hypothesis for addition of mercaptans across carbonyls in a biological sample. The value of such a mechanism to *Cryptocercus* and its symbionts is theorized.

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COBALT AND GLUTATHIONE IN THE PRESERVATION OF FERTILITY AND LIFE OF SAND DOLLAR EGGS[1]

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The loss of fertility of the unfertilized egg of *Dendraster excentricus* within a few hours after removal from the ovary is of interest. During the breeding season the eggs may be 95–100% fertilizable for 24–48 hours when allowed to stand uncrowded in finger bowls of sea water at 18° C. After this interval, the capacity for fertilization decreases and the eggs soon disintegrate. When conditions are not optimum the eggs show poor fertility from the beginning. This is commonly the case with over-ripe eggs. The decay of fertility seems to be correlated with the onset and progression of the death processes which lead to disintegration.

Fertility may be prolonged or regained under certain conditions. In experiments involving the treatment of *Dendraster* eggs with lithium before fertilization (Rulon, 1946) it was found that a 1% ethyl alcohol solution would insure the fertility of the egg sufficiently to permit an adequate period of exposure to the test agent. In other experiments (Rulon, 1948) it was found that certain lots of *Arbacia* eggs, which were only 25–50% fertilizable, could be stimulated to 90–100% fertilization by treatment with Ca-free sea water for eight hours before the addition of a few drops of sperm suspension.

For a number of years the writer has been interested in modifying and controlling developmental patterns in echinoderm embryos with various enzyme inhibitors and other agents. In several cases interesting modifications have been caused by treatment of the unfertilized egg (Rulon, 1941, 1946). Cobaltous chloride was chosen for the present study because previous work (Rulon, 1956) had shown it to alter development in a definite manner when applied to the newly fertilized egg and the mid-blastula. In the work reported here it was found that cobalt had little effect on the pattern of the developing larva when only the unfertilized egg was treated. It was found, however, that cobalt possessed remarkable properties for maintaining fertility long beyond the normal period.

Materials and Methods

The sand dollars (*D. excentricus*) were dredged from Monterey Bay during the summer of 1959 and maintained in healthy condition in tanks in the aquarium room of the Hopkins Marine Station, Pacific Grove, California. Ova were taken in large numbers from ripe females by cutting away the oral surfaces of the tests and allowing the exposed gonads to shed. Droplets of ova were drawn into medi-

[1] This investigation was supported by the Graduate School of Northwestern University. The writer is also much indebted to Dr. L. R. Blinks, Director of the Hopkins Marine Station, Pacific Grove, California.

cine droppers and released in fresh sea water. After washing, the ova were placed in the various concentrations of cobaltous chloride and cobaltous-glutathione solutions for the duration of the experiments. At 24-hour intervals a number (50–100) of the eggs were removed to fresh sea water and washed. Fresh sperm suspension was added to test fertility. The chief criterion for fertilization was nuclear division. Throughout the work the eggs were kept uncrowded in covered fingerbowls out of direct sunlight and at 18± 1° C. Smoking was not permitted in the laboratory since previous work by Child and others has shown that in sufficient density, tobacco smoke may cause modifications in developing embryos (Child, 1941, p. 222).

Experimental

I. *The effects of cobaltous chloride on fertility and cleavage*

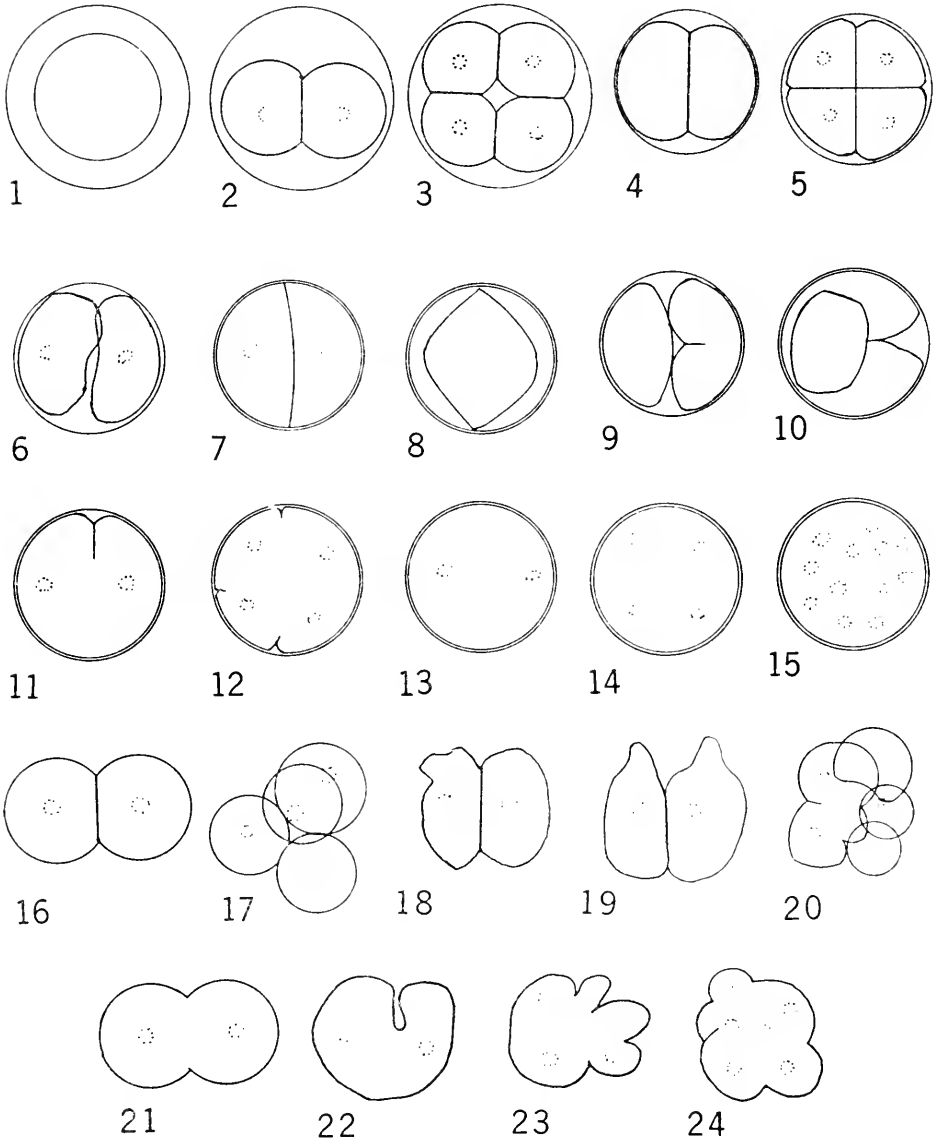
Numerous preliminary experiments indicated unfertilized eggs, in sea-water solutions of cobaltous chloride, retain their fertility long beyond those in sea-water controls. They also showed that on fertilization the cobalt-treated eggs behaved somewhat differently from the controls and that these differences were related to concentration of the cobalt and length of exposure period.

One of the most conspicuous effects of exposure to cobaltous chloride was the presence of *tight* fertilization membranes on fertilized eggs from the more concentrated solutions (compare controls, Figures 1–3, with tests, Figures 4–15). Eggs dividing in such membranes gave elongated and often incompletely separated blastomeres. If the exposure was of sufficient duration cytoplasmic division was partially or totally inhibited but nuclear division still occurred (as in Figures 11–15). If the concentration of cobalt was somewhat less but the exposure time was of long duration, the presence of a fertilization membrane could not be detected at all and cytoplasmic division graded from complete to partial (Figs. 16–24). When the blastomeres were fully cleaved they tended to become spherical and loosely attached. With long treatment many inequalities in size of cells and irregularities in cleavage patterns were to be noted.

It was soon evident that cobalt was exerting its effects largely on the cell cortex and that cytoplasmic division was more subject to inhibition than nuclear division. In general, it was found that most of the eggs that could be fertilized showed recovery in sea water and developed into plutei that were not far from normal. In some cases, however, plutei were somewhat stunted and granular but none developed in a manner comparable to those caused by post-fertilization treatment (see Rulon, 1956).

The effects of various concentrations of cobalt on the preservation of fertility are shown in Table I. Here are tabulated the results of a single series from eggs taken from one female. In this series, approximately 45% of the control eggs were fertilizable at the end of 24 hours (not in table) but none could be fertilized at 48 hours. Approximately 50% responded after treatment with *M*/400–*M*/800 for 96 hours while approximately 25% retained fertilizability for as long as 168 hours with *M*/1600–*M*/3200. Even in a weak solution of cobaltous chloride (*M*/6400) 50% of the eggs retained fertility for 96 hours.

These experiments definitely showed that certain concentrations of cobalt prolong the fertile life and even life itself of the egg of *Dendraster excentricus*.



FIGURES 1–24. Figure 1, newly fertilized egg of *Dendraster* with elevated fertilization membrane. Figures 2–3, normal early cleavage within fertilization membrane. Figures 4–10, eggs cleaving within tight fertilization membrane following exposure to strong (*M*/400–*M*/1600) solutions of cobalt. Figures 11–15, eggs with nuclear division but inhibited cytoplasmic division following long exposure to cobalt. Figures 16–24, eggs with aberrant cytoplasmic division and undetectable fertilization membranes following exposure to less concentrated solutions of cobalt.

TABLE I

The effects of different concentrations of cobaltous chloride for various intervals of time on fertilization of the eggs of Dendraster excentricus
(*Values given in percentage*)

Solution used	48 hours	96 hours	144 hours	168 hours	192 hours
Sea water (control)	—	—	—	—	—
M/200 cobaltous chloride	20	10	—	—	—
M/400 cobaltous chloride	50	60	1 —	—	—
M/800 cobaltous chloride	60	40	1 —	—	—
M/1600 cobaltous chloride	80	50	40	30	1 —
M/3200 cobaltous chloride	80	50	25	25	—
M/6400 cobaltous chloride	80	50	—	—	—

II. *The effects of cobaltous glutathione on fertility and cleavage*

It has been thought that cobalt causes much of its effect by the fixation of thiol (−SH) groups (see Marston, 1952). Zinc, which has much the same action as cobalt on differentiation but at much lower concentration, appears, also, to unite with sulfhydryl (Rulon, 1955). Since the effect of zinc may be prevented with glutathione, it was suggested that this agent may well inhibit the effect of cobalt on the preservation of fertility. Accordingly, tests were run in which eggs were placed in solutions of cobaltous-glutathione in which the concentration of cobalt varied but that of glutathione was kept constant at 0.025% (a concentration which had no detectable effect by itself on the unfertilized egg). It seemed apparent that there was actual chemical union of these two substances in that clear pink solution of cobalt becomes a clear brown when the colorless glutathione was added.

TABLE II

The effects of cobaltous chloride and glutathione for various intervals of time on fertilization of the eggs of Dendraster excentricus
(*Values given in percentage*)

Solution used	48 hours	96 hours	168 hours	216 hours	312 hours
Sea water (control)	—	—	—	—	—
0.025% glutathione	—	—	—	—	—
50 cc. *M*/100 cobaltous chloride plus 50 cc. 0.05% glutathione	95	95	95	90	—
50 cc. *M*/200 cobaltous chloride plus 50 cc. 0.05% glutathione	95	95	90	70	50*
50 cc. *M*/400 cobaltous chloride plus 50 cc. 0.05% glutathione	95	90	75	75	—
50 cc. *M*/800 cobaltous chloride plus 50 cc. 0.05% glutathione	90	—	—	—	—
50 cc. *M*/1600 cobaltous chloride plus 50 cc. 0.05% glutathione	—	—	—	—	—
50 cc. *M*/3200 cobaltous chloride plus 50 cc. 0.05% glutathione	—	—	—	—	—

* Different series of experiments.

The results of this series of experiments are tabulated in Table II where it is shown that glutathione prevented the fertility-preserving effects of cobalt only at the lower concentrations. In the stronger solutions (50 cc. $M/100$–$M/400$ cobaltous chloride plus 50 cc. of 0.05% glutathione) cobaltous-glutathione was a far more effective agent in prolonging the fertile life of the eggs of *Dendraster* than was cobalt alone. It will be noted that the eggs are still highly fertile after 216 hours in these solutions and that in another experiment there was 50% fertility after 13 days.

It was of interest to note (data not shown in table) that while with the most concentrated solution (50 cc. of $M/100$ cobalt plus 50 cc. of 0.05% glutathione) 90% of the eggs were fertile, only nuclear division resulted. In treatment with next strongest solution, where 70% of the eggs were fertile, 30% showed only nuclear division while the remaining 40% showed some cytoplasmic cleavage as well. The 75% fertile eggs from the next solution (50 cc. $M/400$ cobalt plus 50 cc. of 0.05% glutathione) showed cytoplasmic along with nuclear division.

Discussion

It does not seem likely that the effects of cobalt in the concentrations used in these experiments were due to osmotic factors. Although measurements of egg size were not made, the test eggs, before fertilization, appeared little different from the controls. In previous work by the author and many others, agents far more concentrated (*i.e.*, lithium) have been used with only slight osmotic effects. Minor changes (of the order of the concentrations used in these experiments) in salt content of the sea water produce little or no effects. It is known, however, that as echinoderm eggs age there is an increase in permeability and viscosity (see Goldforb, Landowne and Schechter, 1937, for references). It is not known that such physical changes destroy fertility in the egg but there seems to be a correlation. It should be remembered, however, that permeability to water and ions is quite different from permeability to (or penetration by) spermatozoa.

The failure of the fertilization membrane to become elevated after treatment with cobalt is difficult to explain. Tight membranes occur under a variety of conditions in these forms (*i.e.*, aging itself will cause them). It may be that long exposure to cobalt partially denatures the vitelline membrane or it may be that cobalt prevents the breakdown of the cortical granules. Certainly the subject deserves further study.

Any explanation of the mechanisms for the increase or preservation of fertility is not easy. To this worker it long has been a question as to why an ovum, containing so much in the way of nutrients, should lose its capacity to be fertilized so early. Other investigators have dealt with the problem in other eggs. The fertile life of *Arbacia* eggs may be prolonged in low-calcium sea water (Schechter, 1937). Perhaps such procedure prevents the release of calcium from its protein binding in the cortex for internal clotting (Heilbrunn, 1943, pp. 88–89) and thereby prevents or slows down processes concerned with loss of fertility and death. The fertilizable life of *Urechis* eggs may be extended with 1% alcohol or dextrose (Whitaker, 1937). It was suggested that these agents are effective by furnishing nourishment to the egg or by decreasing permeability of the egg surface. Runn-

ström (1952) reports the use of various amino acids and proteins to improve fertilization (but not to preserve fertility) in echinoderm eggs.

Of the several hypotheses given in the past it seems that the present work with cobalt may lend itself to an interpretation that is similar, in part, to one proposed by Loeb (1912, p. 155) who found that the fertilizable life of the starfish egg could be extended by cyanide or oxygen lack. He concluded that the egg becomes unfertilizable, in the normal course of events, after a damaging amount of aerobic oxidation takes place and that such agents as potassium cyanide preserved fertility by slowing down this action.

The writer has come to a tentative explanation as to the action of cobalt. This substance is well known because of its action in inducing polycythemia, possibly as the result of physiological compensation to partial anoxia, caused by the fixation of −SH groups (see Marston, 1952). It is suggested that the cortex and surface of the newly shed egg are abundant in such groupings. It is well known that essential −SH groups on an enzyme may be rapidly oxidized by atmospheric oxygen (see Singer, 1945). It seems not unreasonable to assume that under normal aerobic conditions in sea water, labile or soluble sulfhydryl compounds may be expected to unite with each other by oxidation to disulfide. In other words, simple proteins are united into large stable, insoluble protein chains in which the union of the individual members is through −S−S− bonding (see Jensen, 1959). Such a change at the egg surface may well account for the loss in fertility in aging eggs.

It is believed that cobalt prevents this chain reaction by uniting with −SH and in this manner serves in preventing the decay of fertilizability. This, indeed, seems to be the case as is shown in the experiments in which glutathione is added to the cobaltous chloride solutions. Concentrations of cobalt that were highly effective in prolonging fertility (*M*/1600–*M*/3200) were rendered partly or entirely ineffective by the addition of 0.025% glutathione. This shows the affinity of the two substances and supports the suggestion that cobalt unites with thiol groupings at the egg surface in the preservation of its integrity. But with more concentrated solutions of cobaltous-glutathione *the preservation of fertility was greatly enhanced!*

It is apparent that a different situation has now developed. The combination gives more protection to the egg surface and cortex than does the cobaltous ion alone. In the adsorption of cobaltous-glutathione, changes (oxidation of −SH) with the loss of fertility are considerably less than with cobalt alone. Such an explanation seems highly speculative until one considers the more recent work with Co^{60} Vit. B_{12} in which the combining power of this important substance with the serum proteins (alpha and beta globulins) and the protein of cerebro-spinal fluid has been so aptly demonstrated (see Meyer *et al.*, 1959). It seems quite reasonable to assume that the remarkable preserving action of cobaltous-glutathione is brought about by its union with the substances which would otherwise gradually change as fertilizability is lost.

Summary

1. A range (*M*/200–*M*/6400) of cobaltous chloride in sea water increases the fertile life of *Dendraster* eggs. The most effective concentrations (*M*/1600–

$M/3200$) prolong fertilizability for over 168 hours in 25–30% of the eggs tested. None of the control eggs is fertile at 48 hours.

2. The exposure of unfertilized eggs for long periods or to high concentrations leads to tight fertilization membranes and gradual suppression of cytoplasmic division when the eggs are finally fertilized.

3. Glutathione (0.025%) prevented the fertility-preserving action of cobaltous chloride in lower concentrations but greatly enhanced it in higher concentrations. One mixture (50 cc. of 0.05% glutathione plus 50 cc. $M/200$ cobaltous chloride) preserved fertility in 50% of the eggs for 312 hours.

4. The work suggests that cobalt and cobaltous-glutathione unite with R–SH at the egg surface (each in a somewhat different manner) preventing oxidation and a deterioration of fertilizability.

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LEARNING IN THE SPINY LOBSTER PANULIRUS ARGUS

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Although Bethe (1898) believed crustaceans in general could not learn by experience, subsequent research by many investigators has proved that cladocerans, isopods, crayfishes, hermit crabs and several kinds of brachyuran crabs can variously show habituation, discrimination learning and trial-and-error learning (reviews: Warden, Jenkins and Warner, 1940; Thorpe, 1956; Schöne, 1961; recent research paper: Datta, Milstein and Bitterman, 1960). In these different cases visual, chemoreceptor, kinesthetic or tactile elements were involved in the afferent components of the behavior patterns studied. As is typical for learning, these data (plotted as error scores) usually fall on curves resembling exponential decay functions with a large initial number of errors which, with training, are rapidly reduced at first and then later decrease more and more slowly (Hull, 1952; Hilgard, 1956; Bush and Estes, 1959).

In attempting to demonstrate polarized light vision in decapod crustaceans, training experiments were undertaken with *Panulirus* to determine whether different planes of polarization could be learned as cues for the solution of a two-choice spatial problem. While the lobsters failed to use the polarization pattern in this way, they were able to make their spatial choice on the basis of brightness discrimination or to learn always to choose the same side for attaining a goal. The present report describes these experiments and briefly considers their comparative behavioral implications.

This research was supported by NSF Grant 7387, for which Talbot H. Waterman, Dept. of Zoology, Yale University, was Principal Investigator, and by grants from the American Academy of Arts and Sciences as well as the Penrose Fund of the American Philosophical Society. Thanks are due to Dr. Waterman for his encouragement and stimulating discussions; also to Dr. William H. Sutcliffe, Jr., Director of the Bermuda Biological Station where this work was done, for his keen interest and unfailing help.

Methods

The experimental animals were juvenile specimens of *Panulirus argus* (Latreille) 8–12 cm. in body length. They were tested in an experimental discrimination box in which they were trained to walk forward (in air) to one of two compartments at the far end (Fig. 1). Each compartment had a large rectangular exit hole in its floor; one was open and led down to the aquarium below in which the lobster was normally kept; the other was closed by a wire screen which prevented

[1] Contribution number 290.

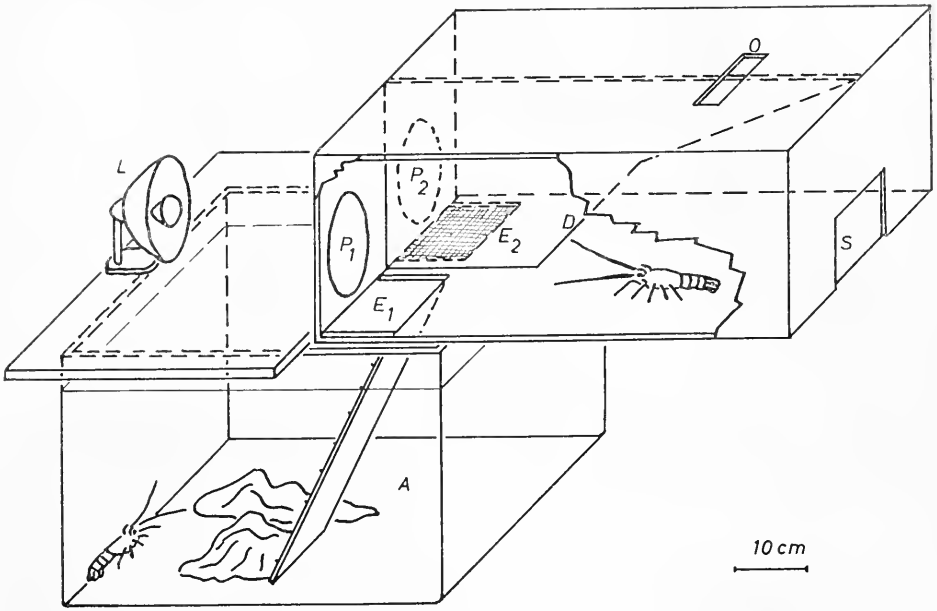

FIGURE 1. Two-choice spatial discrimination box used to train *Panulirus*. *A*, aquarium containing sea water; *D*, partition dividing front of box into two compartments; E_1, open exit from one compartment to aquarium below; E_2, blocked exit in other compartment; *L*, light source illuminating conditioning stimuli (P_1, P_2); *O*, slit for observing the lobster's behavior during tests; P_1, P_2, circular illuminated areas used in experiments training to brightness and those designed to train to plane of polarization; *S*, starting place for runs.

the animal's return home. The lobsters were observed through a peep hole and their course recorded on a floor plan of the box.

Two conditioning stimuli were presented when desired at a circular opening in the far wall of each compartment. These apertures were covered with translucent paper and illuminated from behind. The intensity of these stimuli could be altered by using additional layers of the translucent paper, and polarizers (Polaroid HN38 neutral polarizing filters) could be placed in the light paths to effect nearly 100% linear polarization of the illuminated areas.

Experiments were carried out as follows. The *Panulirus* were tested one at a time by placing them at the entrance of the experimental box facing the two goal compartments which were at the opposite end (Figs. 1 and 2). For animals which walked to the compartment with the open exit hole without entering the closed compartment a score of "correct" was kept; an error was recorded for those which walked into the closed compartment (score of "incorrect") and for those which stopped and remained anywhere in the back part of the box for five minutes ("nonspontaneous runs"), after which the lobsters were prodded into action and led in the correct direction with a small stick. When spontaneous locomotion was particularly circuitous or slow, this stick was also used to prod the lobster into action.

In addition to the error scores (in per cent per three trials) two other kinds of data were taken: (1) percentage of nonspontaneous runs and (2) percentage of runs in which tail-flexing reactions occurred.

In various experiments groups of individuals varying in number from 6 to 11 were given 2 to 6 tests per day, and the observations were repeated for 6 to 22 days. In certain cases the performances of trained and inexperienced individuals were compared.

The three main series of training experiments all followed the general procedure outlined above but differed in certain important details.

1. *Training to brightness.* Ten individual inexperienced lobsters which had never been in the experimental box before were used in these tests. The conditioning stimuli differed in intensity, and the more intense one was always on the side of the compartment with the exit open to the aquarium below (positive side). During the first 9 days I_{pos} was three times as great as I_{neg}; during the remaining 13 days I_{pos} was five times as great as I_{neg}. The first day each animal was tested three times, for the next 9 days five times per day, and for the last 12 days six times per day (about 119 runs per individual or 1188 altogether). To control against

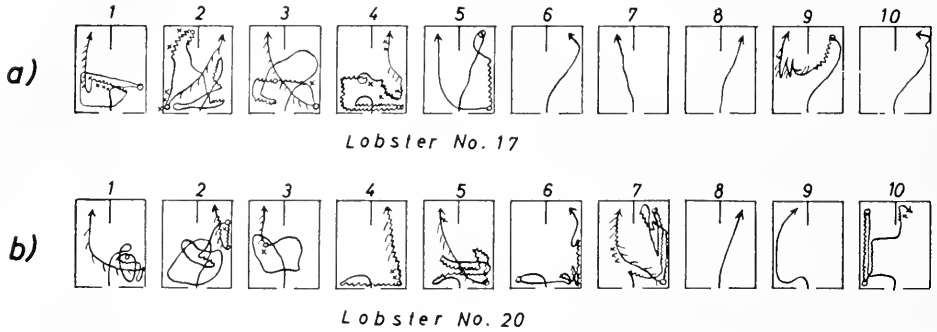

FIGURE 2. First ten runs of (a) a lobster (No. 17) which learned relatively quickly to discriminate brightness in choosing the right course to the open exit, and (b) a lobster (No. 20) which was slow in learning this task. The different parts of the animal's track are marked with the following symbol: full line = running forward; zig-zag line = running backward; small open circle = stop; cross hatching next to line = guiding by a stick; crosses = tail flexing movement.

the lobsters merely learning "side" the positive side was changed alternately from right to left for the first 12 days; then sides were shifted in random order for the next 10 days.

2. *Training to polarized light.* One series of measurements (A) was made on 11 inexperienced lobsters; a second series (B) was run with 10 experienced animals.[2] For Series A the box's wooden walls were unpainted. For Series B the walls of the experimental box were painted with aluminum paint in order to minimize reflection-refraction artifacts arising from differential effects of the walls on the total light pattern visible to the lobsters. The two conditioning stimuli had the same intensity and degree of linear polarization, but in one (the positive side) the *e*-vector was horizontal, while in the other it was vertical. Five tests per day per animal were made in each series. The horizontal polarization was regularly alternated from left (l) to right (r) in successive tests of Series B, but there were some irregularities in Series A which ran: rrllrlrlrlrrlrlrlrlrlrlrlrlr. Series A

[2] These had previously been used in the experiments on training to brightness.

lasted 6 days (about 30 tests per animal, total 327), Series B, 11 days (55 tests per animal, total 550).

3. *Training to side.* Six inexperienced lobsters were run in the experimental box for 9 days with the left compartment's exit open to the aquarium throughout. The intensities of the two light windows were equal and they were not polarized. Two tests were made per animal on the first day and three during the succeeding 8 days, making 26 per animal (total 156).

Results

A. *General behavior*

In the present experimental situation *Panulirus'* behavior was made up of activity sequences containing one or more of four locomotor components: (1) no locomotion, (2) forward walking, (3) backward walking, and (4) tail-flexing movements.[3] The actual paths followed were, of course, characteristically affected by the amount and rate of turning. Examples of the resulting trails are given as two series of 10 trials each for individual lobsters (Fig. 2).

Evidently these activity sequences differ in three major ways which are interrelated: (1) degree of spontaneity, (2) directness from starting point to end point, (3) particular locomotor components involved. Thus the most direct trails (Fig. 2a, Runs 7 and 8; Fig. 2b, Run 8) were started spontaneously, and consisted only of forward walking, at first straight ahead, then gradually turning towards the side with the open exit.

In slightly less direct paths the animal also started spontaneously and walked forward all the way but it turned more strongly towards the positive side, struck the side wall and followed this for a while until it turned in the opposite direction towards the exit (Fig. 2a, Runs 6 and 10; Fig. 2b, Run 9).

As the paths became less direct than this, more turning, stopping and backward walking appeared (Fig. 2a, Runs 5 and 9; Fig. 2b, Runs 4 and 10). Spontaneity was reduced and tail-flipping escape reflexes were evident. Finally, in the least direct runs the animals often did not leave the back part of the box spontaneously. In such cases they frequently had to be guided to the open exit with the stick. Backward walking, stopping and escape reactions occurred often (Fig. 2a, Runs 1, 2, 3 and 4; Fig. 2b, Runs 2, 5 and 7).

Some of the variation in these behavior patterns of *Panulirus* is due to individual peculiarities since the number and kind of certain loops, turns or backtracks tend to be similar in successive trials for the same lobster but to differ from one animal to another. The correlation of alterations in response with learning and the three types of training employed is considered in detail in the next section.

B. *Training experiments*

In running the two-choice maze of the present setup the short spontaneous paths over which the animals have walked directly forward to the open exit represent the best behavior if prompt return to sea water in their aquarium is considered the goal.

[3] These arise as strong flexions of the abdomen (Lochhead, 1961; Wiersma, 1961) which may result in the animal's projecting itself backward several centimeters in a response often termed an escape reflex.

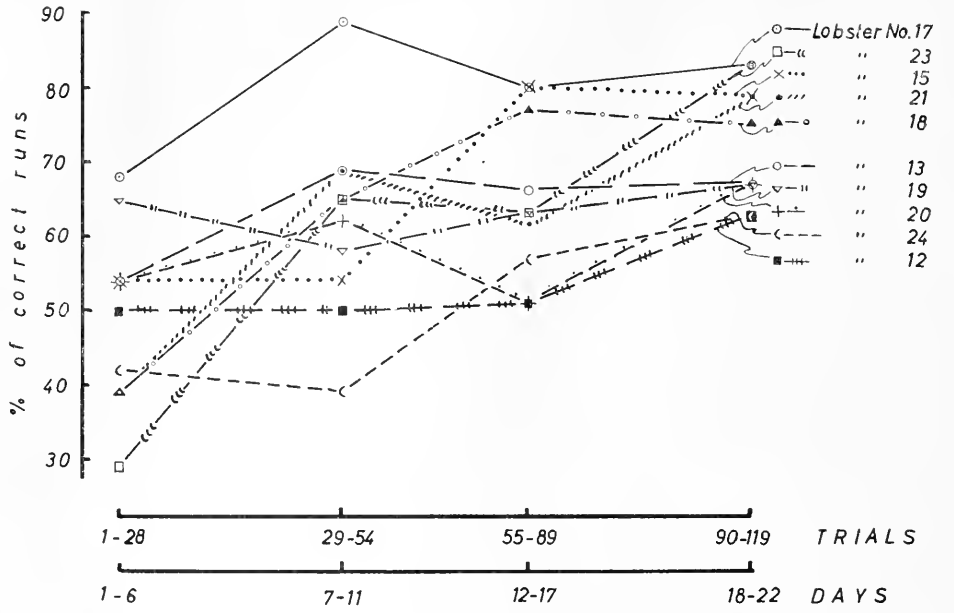

FIGURE 3. Learning curves for 10 individual lobsters training to brightness. Points indicate percentages of runs for the periods in trials (as well as days) specified on the abscissa.

Conversely, meandering runs with stops, walking backward, escape reactions and failure to reach the goal without guidance represent the worst performances. Under appropriate conditions of training and with test animals having a tendency to associate a conditioning stimulus with the goal, the poor responses shown initially by inexperienced animals improve progressively to a better performance level.

However, the amount of improvement and its rate during different parts of the training period were quite different in individual animals (Fig. 3). The factors involved in such variations remain largely unstudied. Nevertheless in certain experiments poor or insignificant improvement can be attributed to the development of certain preferences largely unaffected by the kind of associations tested. Thus in Series B of the training to polarized light, 5 of the 10 animals tested preferred one side of the box regardless of changes in the side of the open exit. This habit may be so rigid as to persist even when the positive side was never made the one preferred.

An example of this type of behavior, quite resistant to conditioning in the present setup, is shown in Figure 4. Here the alternation of positive side (a, 1; b, 1) from left to right was succeeded by the open exit being maintained only on the left (a, 2; b, 2). Despite this, Animal 12 continued to turn right, and even after a few correct responses, returned to its persistent incorrect right turn. Animal 15 showed a comparable predominance of right-hand turns for the greater part of the series but toward the end seemed to learn the left turn required to reach the positive side.

A summary of the numbers and types of runs made in each of the three kinds of training experiments is presented in Table I.

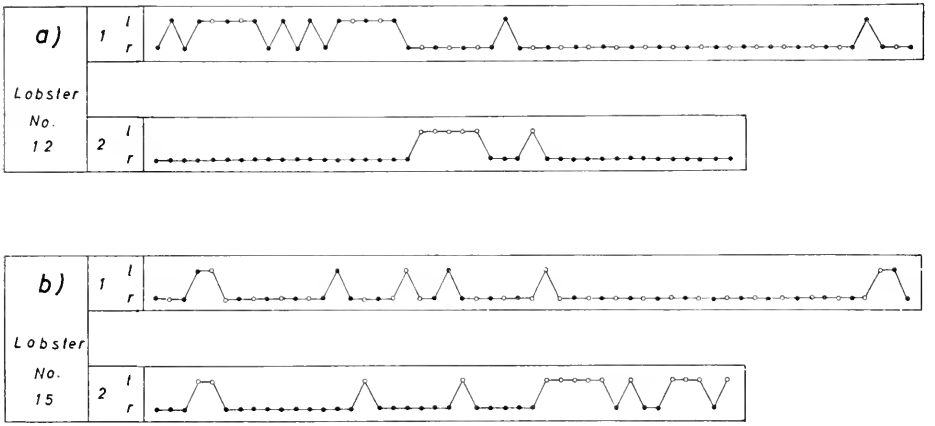

FIGURE 4. Serial representation of training runs of two experienced lobsters to polarized light (sequence from left to right). In series a1, b1 the open exit (positive side) was alternated regularly; in series a2, b2 the positive side was always to the left. Correct runs are represented by open circles, incorrect runs by filled circles. Development of a tendency to run towards the right side is shown by lobster No. 12 which kept this tendency even when the positive side remained left; lobster No. 15 did not, having finally changed to the positive side.

1. *Training to brightness.* Initially the average error score of the 10 animals tested was 93%. This had fallen to 35–40% after 30–40 trials and continued to diminish (but at a reduced rate) to about 20% by the end of the experiment (119 trials) (Fig. 5, error curve). The fraction of nonspontaneous runs was also high during the first trials (57%) but fell to about 10% after 10 trials and became zero for the last 40 trials (Fig. 5, curve of nonspontaneous runs). Similarly, the percentage of runs showing tail-flexing movement was maximum in the first trials (30%), declined irregularly during the next 30 trials and settled down to 0–7% in

TABLE I

Occurrence of tail-flexing movements during different kinds of runs in four series of training experiments

Runs	Training to brightness			Training to polarized light						Training to side		
				Series A			Series B					
	Total	With tail flexes		Total	With tail flexes		Total	With tail flexes		Total	With tail flexes	
		No.	%		No.	%		No.	%		No.	%
Correct	745	6	*1*	157	5	*3*	251	23	*9*	88	0	*0*
Incorrect*	394	82	*21*	156	66	*42*	271	86	*32*	33	5	*16*
Nonspontaneous	49	15	*31*	14	12	*86*	28	13	*46*	35	10	*29*
Total	1188	103	*9*	327	83	*26*	550	122	*22*	156	15	*10*

* Incorrect runs = error runs minus nonspontaneous runs.

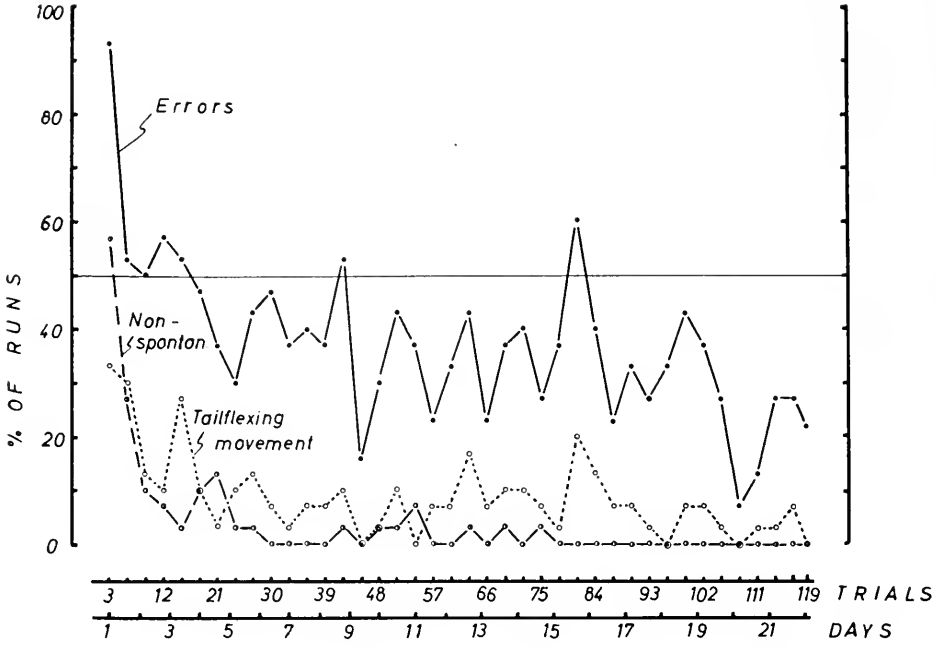

FIGURE 5. Mean learning curve for 10 lobsters training to brightness. Points represent percentages of the runs in three trials.

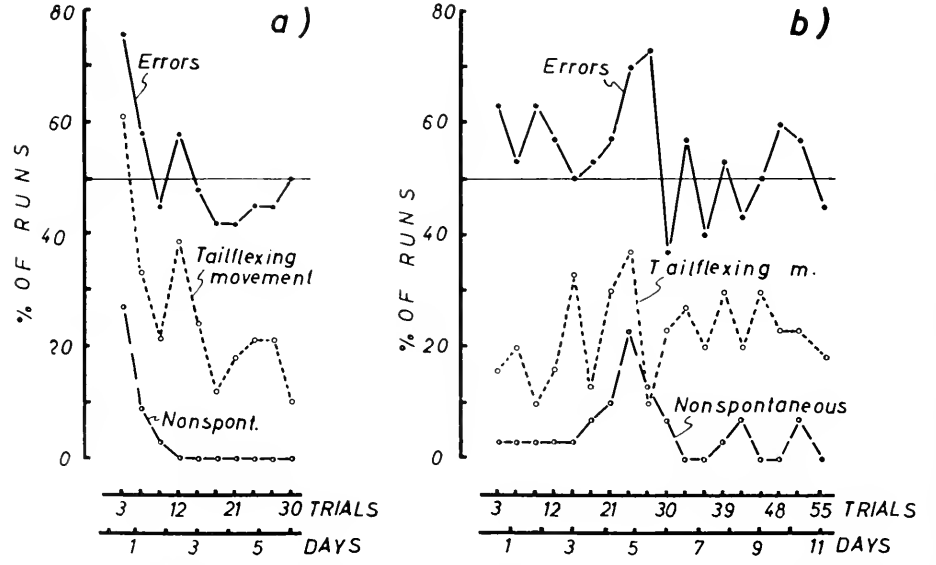

FIGURE 6. Mean learning curves for lobsters where training was attempted to plane of polarization. (a) Series A, 11 previously untrained animals, (b) Series B, 10 previously trained animals. Points represent percentages of the runs in three trials.

the last 30 trials (Fig. 5, curve of tail-flexing movement). The error curve and curve of tail-flexing movements at least show signs of flattening out, or even of a temporary upturn, starting with trial 61 when the regular right-left alternation of the positive side of the box was changed to a randomized sequence.

These results demonstrate that *Panulirus* can discriminate the light intensities used in the two compartments and utilize their position as a conditioning stimulus in goal seeking.

2. *Training to polarized light.* In both Series A (inexperienced lobsters) and Series B (experienced lobsters) the error scores were no better than random even at the end of the 6- and 11-day training periods (Fig. 6a, b). The inexperienced animals made a large number of errors (76%) in the first trials, but after 9 trials they made about 50% (Fig. 6a, error curve). Rapid decreases in the proportion

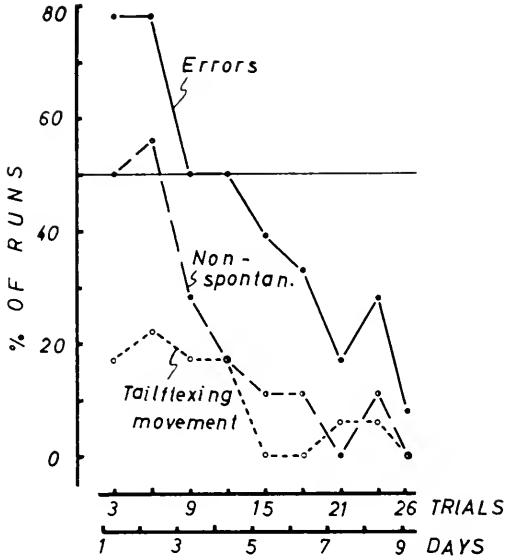

FIGURE 7. Mean learning curves for 6 lobsters training to side. Points indicate percentages of the runs in three trials.

of nonspontaneous runs and runs with tail-flexing movements also occurred in the inexperienced group (Fig. 6a, curve of nonspontaneous and tail-flexing movement runs).

In contrast, with the previously trained animals all three of these scores were about at the same level in the first as in the last trials (Fig. 6b). Although these two series showed that spontaneity and tail-flexing movements reached levels comparable to those attained in the brightness training (Fig. 5), they give no indication that the two tested orientations of the *e*-vector of polarized light could be used as a conditioning stimulus to discriminate the spatial choice required.

3. *Training to side.* The general behavior of test animals in the above experiments suggested that they readily could learn the side with a reward since they often went toward that compartment which had been the positive one in the previous test.

This was confirmed by the results of experiments carried out to study this type of training. The error score was over 78% at first but decreased precipitously and quite steadily thereafter. It was 50% after 9 trials and about 10% after 26 trials at the end (Fig. 7, error curve). A similar course was run by the proportion of nonspontaneous runs which started at 50% and decreased after 15 trials to 11%. The fraction of runs with tail-flexing movements showed a general trend to decrease from about 20% initially to zero in the last trials.

Discussion

Experience indicates that rate and extent of learning in various animals depend strongly on the particular problem tested and the sensory modalities involved in the afferent components of the process. Also the strength and type of drive and reinforcement involved obviously will markedly affect learning (Hull, 1952). Nevertheless, it seems clear in the present experimental situation that *Panulirus* did not learn even at best to make two-choice spatial discrimination either very quickly or very accurately. Mean error scores for a day never were substantially less than 20% and even these took more than 20 trials to achieve. Thus in the training-to-side experiment the error score dropped markedly below 20% only after 26 trials (Fig. 7) and in the training-to-brightness series an error score markedly below 20% first appeared after 108 trials (Fig. 5).

The differences in learning shown in these two sets of training experiments may reflect the relative importance of different sensory modalities for the type of conditioning involved. The more rapid achievement of $< 20\%$ errors in choosing one side than in discriminating between the two circular areas of different brightness suggests that some tactile or kinesthetic cues are more important than visual ones in finding the open exit hole. Such an interpretation is supported by the cases cited above (Fig. 4) where persistent preference for one side was manifest in certain individual *Panulirus* and by previous work on the fiddler crab *Uca* (Schwartz and Safir, 1915) and the crayfish *Pacifastacus* (Gilhousen, 1927) which demonstrated turning predominantly in one direction or following of one wall. Such a preponderant influence of mechanoreceptor afferent information for *Panulirus* is consonant with the animal's normal rocky surroundings in submarine holes and crevices, coupled with its essentially nocturnal habit.

The lack of evidence for the lobsters learning to discriminate between vertically and horizontally polarized light sources is consonant with the conclusion that visual cues are not as significant as certain others in the present spatial choice problem. However, it does not seem to reflect the finding in the crab, *Ocypode*, that vertically polarized light has a significantly greater effect on eyestalk displacement than horizontal polarized light of the same photometric intensity (Schöne and Schöne, 1961).

Previous attempts with other animals to demonstrate discrimination of different planes of polarization by training techniques have yielded both positive and negative results. In the pigeon, food-training discrimination tests gave no evidence for polarized light perception but indicated good light intensity discrimination (Montgomery and Heinemann, 1952), thus resembling some of the present results with *Panulirus*. On the other hand, in the honeybee a significant ability to discriminate vertically from horizontally polarized light was reported on the basis of food-seeking

training (Stockhammer, 1956).[4] Reward and punishment training in *Octopus* has similarly demonstrated that animal's ability to discriminate vertical from horizontal polarized light as well as between oblique planes of polarization at 45° and 135° to the vertical (Moody and Parriss, 1960, 1961).

However, the present failure to get discrimination learning to polarized light cannot be taken as evidence that the animal does not perceive the plane of polarization, as a great variety of arthropods, including decapod crustaceans, must do (Stockhammer, 1959; von Frisch, Lindauer and Daumer, 1960; Jander and Waterman, 1960; Waterman, Jander and Daumer, unpublished; Schöne, unpublished data). Negative evidence of the sort obtained here merely demonstrates an absence of training effect in the current experimental situation without decisive prejudice to the more general question.

In the learning curves for the error scores of inexperienced lobsters (Figs. 5, 6a, and 7), the occurrence of initial levels significantly above the random choice value of 50% results directly from the way errors were counted. The inclusion of nonspontaneous runs as errors contributed substantially to the initial excess over what would be expected, since inexperienced animals frequently required prodding to initiate and maintain walking.

On the other hand with experienced animals the initial error scores were near 50% (Fig. 6b) and the nonspontaneous runs were much less frequent during the first trials. This raises two points of interest. (1) Retention of training must occur for the period between experiments (3 days). (2) Learning, as evidenced by the reduction in nonspontaneous runs, took place even when the *Panulirus* were not learning to find the open exit. Such data suggest that the test situation consists of at least two stages: (1) learning to walk forward (probably related to the general factor of drive or appetitive behavior (Tinbergen, 1951), and (2) learning to discriminate the positive compartment.

Another point of interest is the correlation of tail-flexing movements with inexperience and with progress in learning. The tail-flexing movements appeared to be characteristic of inexperienced individuals (Figure 2 and the first trials of the appropriate curves of Figures 5 and 7) and of those showing little tendency to learn (Fig. 6). In both of these cases tail-flexing is correlated with error runs. The detailed correlations of tail-flexing movements with correct, incorrect and nonspontaneous runs are shown in Table I which indicates that tail-flexing movements were most prominent in nonspontaneous runs, less so in incorrect runs, and at a minimum in positive runs where the open exit was reached spontaneously.

The protocols show that tail-flexing movements were particularly associated with "indecisive" behavior. Thus, if the lobster (1) was inactive unless prodded with the stick, (2) chose the negative compartment and found the exit blocked, (3) made turns in more than one direction, stopped or walked backwards, then tail-flexing movements were likely to appear suddenly. In cases (1) or (2) above the tail-flexion response could be interpreted as an escape reaction from a threatening situation as it often appears to be for lobsters in the sea when strong external stimulation occurs (such as the nearby appearance of a large fish). However, when the same reaction appeared as a component of "indecisive" behavior patterns lacking

[4] In these experiments no control is mentioned against the possibility that intensity artifacts due to differential reflection of the polarized light were involved in the training process.

any obvious external threat, the tail-flexing movement would seem to be a displacement activity in the ethological sense (Eibl-Eibesfeldt and Kramer, 1958; Schöne, 1961). Thus, its diminution with experience could be considered as habituation to initially startling or conflicting situations.

Summary

1. Juvenile spiny lobsters (*Panulirus argus*) could be trained in air in a two-choice discrimination box to use two stimulating lights which differed in intensity by a factor of 3–5 times in learning to reach an open exit hole leading to an aquarium containing sea water.

2. Comparable training procedures yielded no better than chance selection of the two compartments (one with the open exit, the other with its exit blocked) when the conditioning lights were of equal intensity but were both linearly polarized, one horizontally, the other vertically. Demonstration that lobsters did not use polarization pattern in this spatial discrimination situation does not prove that the plane of polarization was not perceived by *Panulirus*.

3. Learning was more rapid than it was to intensity differences when the animals were trained always to go to one side of the discrimination box to reach the open exit. For intensity discrimination initial high percentage errors were reduced to less than 20% only on the twentieth day after 104 trials per animal, whereas for training to side less than 20% errors were achieved after 20 trials per animal.

4. Learning indicated by the curve of mean error scores per day resembles an exponential decay function and is accompanied by decreases in percentage of nonspontaneous runs and runs showing tail-flexing movements. Tail-flexing movements were correlated positively with error runs and occurred as a result of "indecisive" behavior.

5. Learning indicated by reduction in percentages of nonspontaneous runs showed retention of training between experiments and continued in cases where exit discrimination was not being learned. Hence over-all training involved: (a) learning to walk directly forward, and (b) learning to discriminate the side with the open exit.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 1961

ABSTRACTS OF SEMINAR PAPERS

June 26, 1961

Varieties of ephaptic transmission in crayfish. Harry Grundfest and Akira Watanabe.

The septal membranes of the abutting segments of the lateral giant axons of the crayfish nerve cord form bidirectional ephaptic junctions. The properties of the junctional membranes are described accurately in terms of a simple (non-reactive) resistance which links the reactive, electrically excitable and spike generating membranes of the two segments. Thus, the septal membranes show no rectification, at least for a range of polarization of ±25 mv., nor do they respond with electrogenesis to depolarization by 70 mv. The electrical inexcitability of these ephaptic membranes accounts for the bidirectional transmission of the junctions. In contrast, the membranes of the unidirectional ephaptic junctions between the cord and root giant axons have an electrically excitable component which is responsible for the high degree of rectification of these membranes. The available data are insufficient to indicate whether the conductance change is due to K-activation, as in frog slow muscle fibers (Belton and Grundfest, this issue) or to Cl-activation, as in *Raia* electroplaques. Bidirectional ephaptic junctions are also formed by the commissural connections between the branches of corresponding segments of the two lateral giant axons. Since these connections form a ladder of cross-linkages at the different segmental levels, circus propagation of impulses from one axon to the other is possible. These ephaptic pathways can give rise to reverberating activity of both lateral giant axons at rates as high as 400 impulses per second. This activity presumably can initiate corresponding activity in other neurons of the nerve cord.

July 3, 1961

The advance of electrical models for cells and axons. Kenneth S. Cole.

The electrical models of cells and axons have advanced rather steadily from the model for the red blood cell given by Fricke in 1925, which demonstrated the molecular dimensions of living membranes, to the detailed model for the squid giant axon given by Hodgkin and Huxley in 1952 in which much of classical electrophysiology is explained in terms of membrane ionic conductances obtained from voltage clamp experiments.

July 10, 1961

The components of the resting potential in crayfish and lobster muscle fibers. Lucien Girardier, John P. Reuben and Harry Grundfest.

In short range experiments involving changes of external potassium, lobster muscle fibers depolarize with a slope of 58 mv./decade increase of K, according to the thermodynamic (Nernst) relation. Under similar experimental conditions, crayfish muscle fibers do not manifest the theoretical $E_m - \log K$ relation. The latter is attained, however, when thermodynamic conditions are established, *e.g.* by keeping the product $K \times Cl$ constant. Furthermore,

the crayfish fibers exhibit a long-lasting electrochemical disequilibrium after modification of the bathing solution. Thus, remarkably slow transient potential changes are induced by altering the Cl-content of the medium. These properties are consonant with the high membrane resistance of crayfish muscle fibers and with their high volume-to-surface ratio, and the preparation is thus particularly suitable for the study of the pharmacological effects on the ionic fluxes which can be deduced from the kinetics of the membrane potential. The synaptic agents, GABA and picrotoxin, which respectively initiate and block activity of the inhibitory synaptic membrane in these fibers, respectively increase and decrease the membrane resistance and the net Cl-flux in the resting membrane. Although the inhibitory synapses of the fibers operate through increased Cl-conductance, the effects of the drugs on net Cl-flux are due to complementary actions on the non-synaptic membrane component. They are produced only by high concentrations of the agents, well after the maximal effects on the synaptic membrane had been attained. Barium, which converts the normally graded responses of the fibers to spikes and also increases the membrane resistance, decreases the transport number for K. Procaine, which also converts the graded responses to spikes, but has little or no effect on the resting resistance, exerts no clear-cut action on any one ion flux. In lobster muscle fibers, by comparison, the synaptic drugs and Ba have similar actions on the membrane resistance. However, only Ba modifies the $E_m - \log$ K relation of lobster muscle fibers, as may be expected, since the relation in these muscle fibers is determined primarily by the K.

July 11, 1961

Temperature adaptation in Drosophila melanogaster pupae. Roger Milkman.

Day-old (at 23° C.) *D. melanogaster* pupae respond to treatment at 39.5°–41.5° by showing quantitative defects in the posterior crossvein. Longer treatments lead to death. Both responses have a Q_1 of 2.3, and both depend upon the temperature at which the first day of pupal life is spent.

The higher the temperature, the greater the resistance, both to crossvein defect production and to death. Temperatures at 1° intervals between 20° and 30° have been tested. The *rate of increase* in resistance per degree rises greatly with higher temperatures in the range.

The temperature coefficient measurements, previously published in detail, imply that these crossvein defects result from thermal protein denaturation. Moreover, duration-response curves for crossvein defects at 40.5°, using pupae aged at 23° and at 28°, are now shown to be parallel, the duration threshold being greater for the 28° pupae. This implies similar protein denaturation rates, and that the 28° pupae had more of the relevant protein to begin with.

Since resistance to morphological effects and death is similarly temperature-dependent at various pupal stages in *D. melanogaster* and other *Drosophila* species, it is suggested that one mechanism of temperature adaptation may be the change in concentration of certain proteins, or perhaps proteins in general.

Short prior exposures (5–60 minutes) to 32°–38° obliterate the crossvein response and defer death. Production of crossvein defects and both forms of temperature adaptation have been shown in *D. virilis, D. willistoni, D. pseudoobscura,* and *D. busckii.*

This work supported by grants from NSF and NIH.

Endoplasmic reticulum in the asters of invertebrate eggs. Lionel I. Rebhun.

Electron microscopic evidence was presented showing an orientation of the endoplasmic reticulum in the asters of the egg of *Spisula solidissima.* Such an orientation can be seen during formation of asters of the first polar body spindle after germinal vesicle breakdown and during aster formation, just prior to first cleavage. In eggs which have been centrifugally stratified during periods in which asters are present, the endoplasmic reticulum can be seen as a layer 4 to 5 microns wide, tightly pressed beneath the lipid layer. This corresponds to a layer distinguishable in the stratified egg with the light microscope and in the same sublipid position. During redistribution after stratification, asters reappear usually in the form of "one-sided" radiate bodies at first, and later, as full asters. In the electron microscope the endoplasmic reticulum shows a similar one-sided radial orientation, later changing to a

fully radiate body, again suggesting that an important component of the aster is the endoplasmic reticulum.

The orientation of centrioles in dividing cells and its significance. DONALD P. COSTELLO.

The centrioles of the resting first cleavage metaphase of the eggs of the acoel, *Polychoerus carmelensis,* are curved rods about 5 microns long, oriented at right angles to each other and to the spindle axis (Costello, 1961). By light microscopy a smaller rod, projecting at right angles from the middle of each centriole, but at an oblique angle to the spindle axis, can be discerned. At the end of this accessory rod is a small, clear vacuole, containing a stainable (with iron haematoxylin) granule. Extending into the centrosomal area, away from this vacuole, are other similar vacuoles, each with a granule. As Heidenhain (1894) pointed out, and Schreiner and Schreiner (1905) demonstrated, centrioles in many species reproduce by budding, with the daughter centriole produced at right angles to the mother centriole. The small rods are therefore centriolar buds (daughter centrioles) which will grow and separate, presumably at the angle indicated by their mode of origin, from the mother centriole.

Comparing this centriolar orientation and behavior (including daughter centriole origin) in *Polychoerus* with that in the spermatocyte divisions of Gryllidae (Johnson, 1931) and Hemiptera (Payne, 1927) in relation to the arrangement of daughter cells, leads to a new hypothesis, which may account, in part, for cleavage patterns:

(a) The orientation of the centrioles and their mode of reproduction determines the path of separation of daughter centrioles from mother centrioles (or of daughter centrioles from each other).

(b) This, in turn, determines the position of the main axis of the spindle for the next division, and hence, the relative position of the daughter cells with respect to each other.

(c) This arrangement of daughter cells is maintained, for a time, by the primary cell connective, of which the spindle remnant is the significant portion.

(d) These relations obtain in the absence of secondary intervening factors.

Aided by a grant from the National Institutes of Health, RG-5328.

JULY 18, 1961

The nature of lamprey eel fibrinopeptide material. RUSSELL F. DOOLITTLE.

When various components of the mammalian blood coagulation scheme are reacted with components from lower vertebrates, the ensuing clotting times are invariably longer than when all the components come from the same or closely related animals. An investigation of the chemical nature of this "species" specificity was undertaken, using purified preparations of lamprey eel thrombin and fibrinogen and their mammalian counterparts. Human thrombin will completely clot lamprey eel fibrinogen, but the reaction proceeds at only 50% of the rate of human thrombin clotting bovine fibrinogen. Lamprey eel thrombin will clot bovine fibrinogen at less than 10% the rate at which it clots lamprey fibrinogen.

The clotting of mammalian fibrinogen by mammalian thrombin is effected by the enzymatic release of two different negatively charged peptides. It has been postulated that the negative charge clusters keep the fibrinogen molecules mutually repelled and that their loss permits aggregation of the monomeric units. There has been some dispute as to whether or not it is necessary to cleave both peptides in order for the fibrinogen to completely clot. When lamprey eel thrombin clots bovine fibrinogen, only peptide A is released, in spite of the fact that over 90% of the original protein is incorporated into the clot. Human thrombin and lamprey thrombin yielded identical products when exposed to lamprey fibrinogen. One electronegative peptide and one peptide bearing a positive charge at neutral pH were detected. The amino acid composition of the two lamprey peptides was determined and compared with bovine fibrinopeptides. The nature of restricted and permissive mutations in the evolution of the thrombin-fibrinogen interaction was discussed. The details and methods of this work will be published elsewhere.

Equal and unequal effects of unit dosages of ultraviolet and x-ray irradiation of Arbacia gametes as recorded by cinephotomicrography. Ralph Holt Cheney and Carl Caskey Speidel.

Eggs and sperm were subjected separately to either 2537 Å ultraviolet or x-ray irradiation. UV-rayed gametes were exposed for periods ranging from one-half second to 4 minutes, the rate of delivery being 103 ergs per sq. mm. per second. Gametes exposed to x-rays received from 4 to 120 kr. Fertilization combinations were then made and ensuing developmental stages recorded by cinephotomicrography as follows: (1) elevation of fertilization membrane, (2) first cleavage, (3) 5-hour cleavage, (4) initiation of blastular motion at about 8 hours, (5) 24-hour development. Motion picture scenes of stages 3, 4, and 5 were especially useful in demonstrating that there was a striking difference in the relative effects induced by UV treatments as compared with x-ray treatments.

A unit UV dose to eggs caused *far less* developmental delay and injury than an equal UV dose to sperm. Even a 4-unit dose to eggs caused less damage than a 1-unit dose to sperm. An 8-unit dose to eggs, however, induced delay and damage approximately equivalent to a 1-unit dose to sperm. With x-ray irradiation, on the other hand, although a unit dose to eggs was followed usually by less damage than an equal dose to sperm, the difference was much less marked. A 2-unit dose to eggs, or even less, was sufficient to induce delay and damage equivalent to a 1-unit dose to sperm. *Thus, there was a sharp contrast between the two types of irradiation.* A ratio of 8:1 describes the relation between the dosages of UV irradiation to eggs and sperm, respectively, to induce equivalent effects; a ratio of not more than 2:1 describes this relationship for x-ray.

Time-lapse motion pictures of stages 1 and 2 illustrate varieties of fertilization membrane elevation (concentric, eccentric, and partial), also movements of intracellular structures.

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July 25, 1961

Fine structure of an unusual sperm in the flatworm Plagiostomum. A. Kent Christensen. (*See Lalor Fellowship Reports*).

Observations on the Golgi apparatus and related lysosomes. Alex B. Novikoff.

Nucleosidediphosphatase has been localized in Golgi lamellae and a staining method developed that permits delineation of the Golgi apparatus from sites of high acid phosphatase activity. In epididymis and many other cells the acid phosphatase-rich lysosomes show a close topographical relation to the Golgi lamellae. Essner's observations in our laboratory on differentiated hepatoma cells suggest that there may be a "membrane flow" to and from the Golgi apparatus: elements of the endoplasmic reticulum (ER) transform into Golgi cisternae (in one case, this apparently involves flattening of individual smooth-surfaced sacs derived from the rough ER; in the other, Golgi cisternae appear to arise *in situ* by transformation and reorientation of the smooth ER); the cisternae enlarge and separate into vesicles (lysosomes) containing condensed secretory products. Light microscopy and electron microscopy show that the ER (and nuclear membrane) of these cells, and another interesting group, possess nucleosidediphosphatase activity, and that the acid phosphatase reaction product is localized in the lysosomes of these tumor cells. The lysosomal hydrolases may be involved in the biochemistry of secretory granule condensation. How the enlarged Golgi vesicles acquire hydrolytic enzymes is unkown, but there is some suggestion that this may occur via small lysosomes (pinocytosis vacuoles?). In the zymogenic cells of parotid, pancreas and jejunum, acid phosphatase-rich granules are restricted to the Golgi area, and thus only the newly formed secretory granules appear to be lysosomes. Of many cells tested, only the spermatogonia and their derivatives always show high acid phosphatase activity in the Golgi apparatus. Acid phosphatase is present in the acrosome (frog sperm) which may be considered a specialized lysosome with a decisive role in fertilization.

Functional analysis of the corpus allatum of the insect, Leucophaea maderae, with the electron microscope. BERTA SCHARRER.

In adult females of *Leucophaea,* periodic changes in corpus allatum activity are paralleled by structural variations whose details become particularly evident in electron micrographs. In the inactive gland, which due to inhibitory action of the brain does not furnish gonadotropin, the nuclei are densely packed and surrounded by scarce cytoplasm; their chromatin appears clumped. Neighboring cells interdigitate by elaborate foldings of the cell boundaries. Furthermore, the cells interlock by means of long, gradually thinning processes. Following cessation of inhibition, intermediate stages lead to the fully activated corpus allatum and the concomitant maturation of eggs. A considerable increase in cytoplasmic content of the corpus allatum cells is accompanied by gradual straightening of their cell membranes. The number of mitochondria rises, and endoplasmic reticulum appears in the form of juxtanuclear spherical units which may be paired. In addition, small clusters of unattached ribosomes become more numerous, while Golgi elements remain inconspicuous. Electron-dense cytoplasmic granules are especially noticeable at the surface of the gland. With increasing nuclear diameter the chromatin becomes more evenly distributed. One, or not rarely two large nucleoli show a characteristic morphology. The folding and straightening of the cell membrane, as its content shrinks and expands with each reproductive cycle, demonstrates its structural identity. The stellate shape of the corpus allatum cells, which is observed also in fresh tissue dissociated by means of trypsin, seems to be maintained throughout the changes of volume. Branches of the basement membrane which ensheaths the corpus allatum and the corpus cardiacum penetrate between the corpus allatum cells. Some of these processes enclose neurosecretory elements. This morphological intimacy between the two organs reflects their close functional relationship.

JULY 31, 1961

Biological transducer mechanism. WERNER R. LOEWENSTEIN.

The data available presently on charge transfer through the receptor membrane of the nerve ending of Pacinian corpuscles can be fitted to the following excitation scheme: the receptor membrane separates two media of different ionic concentration. Mechanical stimulation causes the conductance of the membrane to increase in a relatively non-selective manner, and ions to flow through along their electro-chemical gradients. An activation energy barrier of about 16,000 cal./mole limits this process.

The membrane reaction that leads to the conductance change is highly localized. It is localized at and confined to the membrane spot that has been stimulated mechanically, and does not spread to adjacent spots within the same receptor membrane by local circuit excitation. This stands in striking contrast to the well-known propagated nature of excitation of the adjacent axon membrane that gives rise to the action potential. The peculiarity of the receptor membrane is that excitation is produced by the mechanical stimulus alone, but not by the resulting local flow of current. As a result, the amount of charge transferred through the membrane, namely the generator current, increases with area of membrane excited. The area-generator current relationship is non-linear; the current increases progressively at a diminishing rate with excited area. The area of membrane excited (that is, the lumped membrane conductance) is the main factor, if not the only one, in determining the intensity of the generator current.

Repeated excitation leads to a transient inactivation of the receptor membrane. The effect manifests itself as a reversible depression in generator current which has low energy requirements and is independent of resting membrane potential. Like excitation, inactivation is a localized process.

AUGUST 7, 1961

Current voltage relations in Nitella. UICHIRO KISHIMOTO.

The measuring cell and the voltage clamp setup (Cole and Moore, 1960) for squid giant axon was used for the *Nitella* experiment, because the size and shape of *Nitella* internode are just

about same as those of squid axon. Two microtips were inserted into vacuole of *Nitella* internode instead of the axial wire-microtip system used in the squid giant axon, since penetration of the 50- to 100-μ axial wire caused a sudden rapid loss of vacuolar sap due to high osmotic concentration gradient between inside and outside of *Nitella*. One microtip is used for monitoring the potential between vacuole and the reference electrode just outside of *Nitella* membrane. Another is used for current supplying through the membrane. Space clamp condition is achieved with this arrangement, since the output of the feedback amplifier appears to be a perfect square wave, which is probably due to a large space constant of *Nitella* (2–3 cm.). The membrane current during step potential change is very similar to that of squid giant axon. The membrane current consists of three components. The first one is the sudden rise or fall of current which corresponds to leakage current, the second is the transient inward current and the third is the steady outward current which increases slowly with time and finally approaches to a constant level. Ions which contribute to the leakage current are not identified as yet. However, there is a good reason to suppose that the second one corresponds to the marked increase of chloride efflux and the third one to that of potassium efflux.

Increase of outside chloride concentration to 10 m*M* causes a reduction of the transient inward current to about half. Increase of outside calcium concentration to 10 m*M*, maintaining the outside chloride concentration constant, brings about a longer duration for action potential without changing the height of the latter. The transient inward current is not influenced by high calcium. Threshold for excitation was increased and the leakage current decreased markedly. Decreasing the temperature to 7° C. promptly increased the leakage of the *Nitella* membrane and the threshold for excitation was increased greatly. The recovery from the effect of lowered temperature takes about an hour. Application of prepulse (subthreshold intensity) of 1–3 seconds in duration has no influence on the inward and outward currents, but the leakage current tends to decrease for hyperpolarization prepulse and to increase for depolarization prepulse.

August 8, 1961

The physiological basis of acclimation of Tetrahymena to high NaCl medium. Philip B. Dunham.

It has been known for some time that fresh-water ciliates can acclimate to concentrated media. However, little was known of the physiological basis of acclimation. *Tetrahymena pyriformis* strain W was acclimated to a 1%NaCl–2% proteose-peptone medium, and compared to a normal (unacclimated) culture of the same animals. An investigation of ion regulation in normal *Tetrahymena* has been reported in this journal, in which it was shown that these animals have a sodium extrusion mechanism with a saturation level at 20 m*M*. medium Na and an apparent free sodium space in the cells of 48%. The acclimated culture was started without intermediate steps. Only 5% of the normal animals survive to divide in the high NaCl medium. The acclimated animals have a much lower sodium content (39 m*M*./l. of cells) than do normal animals equilibrated in high NaCl medium (103 m*M*./l. of cells). The potassium and chloride contents of the two cultures of animals are not significantly different. A comparison of the relationships between cell sodium and medium sodium in acclimated and normal animals suggested two kinds of mechanisms operative in the acclimation: (1) a decrease in apparent free sodium space in the cells from 48% to 20%; (2) an increased saturation level of the sodium extrusion mechanism from 20 m*M*. Na to 45 m*M*. The acclimated animals have a slower growth rate, are smaller, and are differently shaped.

Excretion rate of radio-isotopes as indices of metabolic rates in nature: biological half-life of zinc-65 in relation to temperature, food consumption, growth and reproduction in arthropods. Eugene P. Odum.

Laboratory experiments and a preliminary field experiment with two species of terrestrial insects (*Tenebrio molitor* and *Oncopeltus fasciatus*) and a marine isopod (*Idothea baltica*) indicate that the excretion rate of tracer amounts of Zn^{65} is proportional to certain activity rates, especially food consumption and egg production rates. Individuals were allowed to

ingest contaminated food or water in a "tagging chamber," then transferred to a clean environment where the loss of radioactivity was followed by making periodic whole body counts in a well scintillation detector. Semi-log plots of radioactivity against time strongly indicated two pools of isotope: (1) a non-assimilated pool which is lost rapidly at a rate dependent on the level of the original "tag" and (in aquatic environments) on the physio-chemical nature of the environment, and (2) an assimilated pool which is distributed throughout the tissues of the body with some concentration in digestive organs and gonads and which is excreted much more slowly at a rate more directly related to metabolic processes. Biological half-life ($Tb_{1/2}$) of the assimilated pool varied from as long as 250 days in inactive adults kept at low temperatures to 20 days in active egg-laying *Oncopeltus* females or rapidly growing *Tenebrio* larvae. Young adult *Tenebrio* kept for 30 days at 10°, 20°, and 30° C. averaged 163, 40, 23 days $Tb_{1/2}$, respectively, which is roughly inversely proportional to expected metabolic rate at these temperatures. $Tb_{1/2}$ of 5 adult *Oncopeltus* which had been confined to small bottles in the laboratory decreased 5-fold (125-25 days) when released for 10 days in an outdoor habitat, indicating much greater activity in the field. It is concluded that the rate of loss from the assimilated pool provides a "metabolic clock" for comparing laboratory and field rates of energy flow in arthropod populations.

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August 22, 1961

In vitro culture of human tumors on explants of chick embryo organs. Emilienne Wolff and Etienne Wolff.

Previous researches of our laboratory have shown that it is possible to culture malignant cells from mammals on chick embryonic organs explanted *in vitro*. Mesonephros is one of the most favorable organs for tumor proliferation. Up to now, cells originated from fresh mouse tumors and from standard human strains have been cultured by this method.

Recent improvements of our technique made it possible to obtain new developments of this investigation.

1. A thin membrane, the vitelline membrane of the hen's egg yolk, is placed between mesonephric explants and cancer cells, originated from the human strains HeLa, KB, Fogh, J III, Detroit 6 and Detroit 116. Malignant cells multiply intensely at the expense of substances which pass through this dialysing membrane. They form aggregates, the histological structure of which is similar to the primary tumor, from which they were originated. The same results were obtained with crude extracts of mesonephros or with "conditioned" media, *i.e.* media in which mesonephros was cultured during several days and then carefully removed.

2. Pieces of fresh human surgical tumors were wrapped up in a vitelline membrane together with explants of chick mesonephros. In many cases, such as epithelial carcinomas of stomach and uterus, an abundant proliferation of the cancerous cells takes place at the expense of the mesonephros which becomes more or less completely invaded. The growing tumorous masses retain the same histological structures as in the *in situ* tumors.

The question arises whether the substances provided by the mesonephros are banal nutrients or specific growth promoting substances.

GENERAL SCIENTIFIC MEETINGS

AUGUST 28–31, 1961

Abstracts in this section (including those of Lalor Fellowship reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read by Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

Molybdenum uptake of marine plankton algae. Robert J. Barsdate and Robert R. L. Guillard.

Molybdenum uptake by three species of marine planktonic algae was investigated using Mo-99 as a tracer. *Coccolithus huxleyi* (BT-6) and *Cyclotella nana* (clone 13-1) were isolated from the Sargasso Sea; *Synechococcus* sp. and *Cyclotella nana* (clone 3-H) are estuarine forms. All were grown in pure culture at 22° C. and 2000 lux in enriched sea water containing both nitrate and ammonia, and buffered to pH 8 with "Tris." The total molybdenum content of the medium was varied from 15 to 40 μg./l. (1.5 to 4 times the Mo concentration of Woods Hole Harbor water).

C. nana was grown in Mo-99-tagged media for about 7 cell divisions. Cell counts and molybdenum assay were then made, from which a concentration factor of *ca.* 6.5 was established. Labelled algae in darkness maintained their molybdenum concentration without change for 24 hours. There was no significant difference between the oceanic and estuarine clones.

When *C. nana* cells were exposed in darkness to an increase in molybdenum concentration of the medium, they equilibrated completely (to a concentration factor of 6.5) in four hours, after which there was no change. However, in illuminated flasks the addition of molybdenum resulted in an initial uptake exceeding that corresponding to a concentration factor of 6.5. After 12 hours the concentration factor was again 6.5. The molybdenum uptake by chloroform-killed *C. nana* cells was 70% that of living organisms (at 40 μg./l. of molybdenum).

The coccolithophoride, *C. huxleyi*, with a cell volume 2.5 times that of the diatoms, had a concentration factor of 4.8. *Synechococcus* sp., with a cell volume *ca.* 10^{-3} that of the diatoms described, had a concentration factor of about 70.

This study constituted one of of the staff-student research projects of the Marine Ecology Course (NIH Training Grant 2G–535) and was supported in part by a NSF grant.

Formation and possible function of the "secretory packets" of the starfish tube foot. Alfred B. Chaet and Delbert E. Philpott.

Further studies were carried out on the secretory structures found near the walking surface of the tube foot of *Asterias forbesi*. The "secretory packets" were seen with the electron microscope to be formed intracellularly in an area proximal to the bottom surface of the tube foot where the "packets" were released to the outside. In most cases the cytoplasm of the cell consisted mainly of endoplasmic reticulum, a Golgi apparatus, and associated mitochondria. Within these cells, several "secretory packets" generally were being formed. It appeared that a clear area first formed in the cytoplasm, which then contained a "secretory packet." This newly formed elliptical "packet" had darkly staining (osmophilic) ends between which were attached 50 or so fibers (200 Å diameter). During formation the osmophilic ends of the "packet" were attached to the rest of the cell by cytoplasmic strands, thus holding the "packet" in the center of the small clear area.

Attempts to follow the individual paths of the "secretory packets" from their intracellular origin to the secretory surface at the base of the tube foot have been unsuccessful, due to the tortuous nature of the long narrow channels.

In an effort to further the hypothesis that these "packets" functioned as a "glueing" mechanism, sections of the tube foot were stained by the P.A.S. technique. Observation with the phase contrast microscope demonstrated the "secretory packet" to be P.A.S.-positive, further suggesting their function as an adhering mechanism. These structures were not observable in the light or phase microscope until stained by the P.A.S. method.

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Blood coagulation and fibrinolysis in the smooth dogfish. Russell F. Doolittle.

The hemorrhagic nature of elasmobranch blood can be remedied *in vitro* by the presence of unphysiologically large concentrations of calcium ions. In a milieu containing 50–75 m*M*./L.

calcium, dogfish blood plasma generates thrombin activity equivalent to mammalian systems maintained at physiological calcium levels. Under such conditions, other phases of the event become greatly exaggerated. Fibrinolysis occurs at a rate proportional to thrombin generation, and complete clot dissolution can be effected within 60 minutes of clot formation. Concomitantly, an intense serum inhibition develops which will prevent clotting when added to similar dogfish plasma systems. The inhibition is thermo-labile, partially dialyzable, and not reversed by toluidine blue. The site of inhibition is the thrombin-fibrinogen interaction or the subsequent polymerization of fibrin monomer units. If serum is separated from the fibrin clot before fibrinolysis occurs (*e.g.,* 30 minutes after clot formation), the inhibition does not develop, indicating that the fibrinolytic breakdown products are instrumental in the serum inhibition. Thrombin activity in such serum is neutralized as usual, however, suggesting two separate control devices. Comparisons of the ability of the generated thrombin to clot dogfish fibrinogen with its power to split the artificial substrates, tosyl-L-arginine methyl ester (TAMe) and L-lysine ethyl ester (LEE), showed that fibrinogen clotting power is neutralized much more rapidly than is the esterase activity. Other studies with TAMe and LEE suggest other esteratic or proteolytic events before and after clot formation which are distinct from the ability to clot fibrinogen. These include an initial sharp peak for TAMe esterase activity before the generation of thrombin and a slight increase in LEE esterase activity after clot formation.

This study was aided in part by a grant from the ONR Research Contract No. 1497.

In vivo and in vitro recovery of irradiated gametes of Arbacia punctulata. PATRICIA MCCLEMENT FAILLA.

It is known that exposure to ionizing radiation of either sperm or eggs of *Arbacia punctulata* delays the time of first cleavage of the fertilized egg. The magnitude of cleavage delay decreases exponentially with the time the irradiated eggs remain in sea water before fertilization (*"in vitro* recovery"). No such recovery occurs in irradiated sperm. Use of the electrical stimulation method to procure gametes has permitted investigation of the recovery process in gametes remaining inside irradiated urchins for periods of time up to two weeks (*"in vivo* recovery"). It was found that some recovery occurs in these eggs but at a rate about 100 times less than in the *in vitro* case. After about eight days no further recovery is detectable, but a slight cleavage delay persists. As in the *in vitro* case, no significant recovery was found for sperm *in vivo*. Also the ability of irradiated eggs to recover *in vitro* is no longer present when the eggs have remained in the animal more than four hours after irradiation.

It was thought that the slower recovery rate *in vivo* might be due to a lower metabolic activity of the eggs while in the ovary. The *in vitro* recovery process was followed, therefore, with the metabolic activity of the eggs reduced by keeping them in sea water in a nitrogen atmosphere for an hour after irradiation. Preliminary tests indicate that this recovery process is not affected by marked decreases in the oxygen tension. On the other hand, irradiation of the eggs in a nitrogen atmosphere caused a considerable reduction in the cleavage delay. The rate of subsequent *in vitro* recovery in air was reduced as well.

Absorption, accumulation and loss of radioactive scandium by marine macroalgae. JOHN GUTKNECHT.

Uniform discs of freshly collected *Ulva lactuca* and *Porphyra umbilicalis* were exposed to 20 uc./L. concentrations of high specific activity Sc^{46} in initially sterile and strongly buffered pH 8.0 sea water solutions in the light and the dark. Radioactivity of the algae was measured directly in a well scintillation counter.

Uptake-time curves were characterized by a roughly hyperbolic rapid absorption phase, followed by a more linear accumulation phase. In 48 hours the light *Ulva* absorbed 35% more Sc^{46} per unit weight than the dark, while the light *Porphyra* took up 15% more than the dark. High wet weight concentration factors were observed for both species, 2600 and 1500 times the external concentration in *Ulva* and *Porphyra*, respectively. Adsorption accounted for 40–60% of the total uptake as estimated by the intercept of the line of regression of the linear

part of the time curve. Thalli transferred after 48 hours from light to dark radioactive media showed a definite retardation of the Sc^{46} uptake rate. Algae killed with ethylamine absorbed more scandium than live material.

Loss of Sc^{46} was measured in buffered sea water in the light and the dark. Both species displayed a slow linear loss of scandium, less than 1% per hour. The light and dark algae showed no difference in loss rate.

Research supported by NIH Grant 2G–535 (MBL Ecology Training Program).

Properties of a newly isolated bacteriophage of luminescent bacteria. J. W. Hastings, A. Keynan and K. McCloskey.

Phage was isolated from a squid incubated with inoculations of marine mud and four strains of luminous bacteria. Phage containing samples were obtained by filtration and by treating with chloroform. Typical bacteriophage plaques were produced when tested with either *A. fischeri* or strain 60L (isolated from the Pacific Ocean by C. B. Van Niel) as host. The phage showed clear host specificity; with five locally isolated bacteria strains and one from Jamaica no plaques were observed. However, various "dark" mutants of the sensitive strain were susceptible to infection.

Plaques developed well in soft agar at temperatures between 20° and 30° C. The plaques always showed a turbid center, suggesting that the phage is a temperate one. In older plaques the phage-resistant bacteria completely overgrew the cleared areas, to an extent such that it was difficult to visualize the plaques. Observed in the dark, however, the formerly phage-cleared areas could be seen as non-luminescent spots on the background of the luminescent host strain. From this it was concluded that the phage-resistant cells growing in the center of the plaques were non-luminescent. "Dark" bacteria isolated from the center of the plaques exhibited the following properties which remained stable through several single colony isolations: (1) Ten of the fifteen isolates emitted light not visible to the eye but photometrically detectable; the others were intermediate in brightness. (2) All isolates were resistant to phage infection. (3) After being re-isolated four times from single colonies, all produced phage.

These observations support the view that these are lysogenic bacteria produced by phage infection, and that lysogenicity is in some way inversely related to light production.

The isolation and characterization of dark mutants of luminescent bacteria. A. Keynan and J. W. Hastings.

Numerous authors (Beijerinck, Van Niel, McElroy) have noted that dark mutants of luminescent bacteria accumulate in cultures. Biochemical studies of different dark mutants could yield information concerning the genetic and physiological control of bioluminescence. The present study is concerned with conditions under which dark mutants are found, their frequency and selection, and their physiological properties. Three strains were used in this work; (1) *Achromobacter fischeri,* (2) a strain isolated by Van Niel (L-60), and (3) a strain with a low temperature optimum isolated locally (W-14). From cultures started from a single bright colony, maintained on solid media, no dark colonies (out of several thousand) were seen; from similar cultures maintained in either minimal or complete liquid culture for extended periods (three days to three weeks), many dark colonies were observed upon plating out. From cultures maintained at high temperatures (up to 37°) a high proportion (30%) of dark colonies were found. Fifty-one dark mutants have been isolated and studied. Although several isolates may be derived from the same parental mutant, many are clearly different in origin and in physiological characteristics. All of the mutants which are dark to the eye emit some small amount of light which may be detected photometrically; the different mutants vary enormously with respect to their ability to emit, the intensity of the dimmest mutant being 10^{-5} of the wild type. The quantity of dark cells found in cultures suggested that the conditions gave selective advantage to dark mutants. This possibility was tested and supported in experiments where different proportions of wild type and dark mutant were mixed. It will be of interest to see if conditions where the wild type has a selective advantage can be found. This might be an experimental approach for studying the possible competitive advantage of bioluminescence.

Recent observations on Botryllus schlosseri. ROGER MILKMAN AND SYLVIA BYRNE.

We have confirmed Bancroft's observation of tremendous morphological diversity in the offspring of individual colonies of *Botryllus schlosseri* and his finding that the more closely related two colonies are, the more likely they are to fuse vascular systems when contiguous. Oka and Watanabe's demonstration of vascular budding in *B. primigenus* has been extended to *B. schlosseri*. Sabbadin's demonstration of selfing has been repeated.

Outcrossing, shown experimentally by Sabbadin, must be common in nature, too, for the great genetic heterogeneity of individual colonies would not otherwise be present. Fertilization by sperms arriving over a period of time probably accounts for the continuous variation in developmental stages we find in individual colonies of this viviparous organism, in contrast to the well-known synchrony of budding. Embryos may be raised outside the colony from before gastrulation. Attempts at external fertilization have failed.

Numerous traits, stable in asexual reproduction, have been characterized. Most of these depend upon the nature, quantity, and distribution of pigments; they are components of the colonies' over-all color patterns, of which there must be thousands. Inbreeding should permit development of homozygous strains to extend the recent observations of others on the genetic control of colony fusion and of pigmentation. *B. schlosseri* is notable for the unusual degree to which its genetic diversity is visibly obvious.

This work supported by a grant from NIH (Milkman) and an NSF Cooperative Summer Fellowship (Byrne).

Cortical granule extrusion during maturation of the egg of Asterias forbesi. ARTHUR K. PARPART.

The cortical granules of immature and mature eggs of *Asterias forbesi* were observed by television microscopy under conditions where individual cells were exposed to 1 *M* solutions of glycerol, erythritol, d-xylose, glucose or sucrose. In no case did these egg cells alter in volume, therefore these compounds do not enter the egg in the time period of observation, one hour.

The cortical granules of these eggs, however, reacted quite differently, dependent on the stage of maturation. The cortical granules of immature eggs showed no change, when exposed to the above solutions. The mature eggs exhibited a definite and violent explosion of their cortical granules in 1 *M* glycerol, erythritol or d-xylose; while no such explosion occurred in either 1 *M* glucose or sucrose. Prior to first polar body formation no cortical granules exploded under these conditions. Within a half hour after polar body formation all of the mature eggs had cortical granules which exploded, and showed membrane elevation.

These experimental observations suggest that the cortical granules of immature eggs are in the egg cytoplasm and that during the maturation process these cortical granules are extruded through the egg's plasma membrane and come to lie between it and the vitelline membrane. This is indicated by the fact that the polyhydric alcohols and sugars used cannot penetrate the plasma membrane. On the other hand a penetrating alcohol, ethylene glycol, caused the explosion of the cortical granules in both immature and mature eggs, and subsequently cytolysis. The fact that the above 1 *M* solutions caused an explosion of the cortical granules indicates that the granule's membrane must be permeable to the polyhydric alcohols and d-xylose but not to glucose and sucrose.

The α, β-unsaturated ether (plasmalogen) content of the tissues of several mollusks. MAURICE M. RAPPORT.

It was recently shown that the lipids of a number of marine invertebrates contain high concentrations (10 to 15%) of plasmalogens having the α, β-unsaturated ether structure (Rapport and Alonzo, J. Biol. Chem. **235**: 1953, 1960). Comparative studies have now been made of the lipids of individual tissues from 8 species of mollusk including 6 pelecypods (*Mya, Mytilus, Ostrea, Pecten, Spisula, Venus*), one gastropod (*Busycon*), and one cephalopod (*Loligo*). The α, β-unsaturated ether content of 5 or more tissues from each of these species was determined with respect to two reference bases: tissue dry weight and total lipid weight.

Among the pelecypods it was found that gill tissue contains the highest plasmalogen concentration, attaining values as high as 50 μmoles per gram dry weight and over 400 μmoles per gram of lipid (about 30% by weight). These values compare with those found in mammalian (rat) brain, the mammalian organ in which plasmalogens are most concentrated, namely, 60 μmoles per gram dry weight and 140 μmoles per gram of lipid. High plasmalogen concentrations are found in most pelecypod tissues, the lower values usually being associated with tissues of low lipid content (adductor muscle). Only crystalline style (*Venus, Pecten*) was found to be devoid of plasmalogen. In *Loligo*, a species in which plasmalogen concentrations are rather low, highest values were also observed in gills. In *Eriocheir,* the posterior gills, a salt-concentrating organ, were found by Dumont (Arch. Internat. Physiol. **66**: 373, 1958) to contain a high concentration of plasmalogen phosphatide, and this led him to suggest the possibility of a role for this lipid in ion transport. The findings in this study of mollusk tissues indicate that elevated plasmalogen concentrations may be a general characteristic of gills of all species, and are consistent with the suggestion that plasmalogens are involved in some type of transmembrane transport process.

Supported by N.I.N.D.B. research grant B–1570, U. S. Public Health Service.

Studies on the binding site of pinocytosis inducers. Ronald C. Rustad and Lynne C. Rustad.

Pinocytosis is a method of transport in which the formation of membrane-limited vacuoles is dependent on temperature and aerobic respiration. The first step in this process involves the attachment of inducers to the cell surface. In *Amoeba proteus* basic dyes act as inducers. These dyes compete with proteins for the same surface sites. The molecular orientation of the binding sites can be demonstrated by the birefringence colors of the attached dye. The dye colors obscure the natural surface birefringence; hence, the attachment is in immediate proximity to the major oriented component in the surface. Since proteins, which would not cross the permeability barrier, act as inducers, the binding region must be located outside the cell membrane.

The Alcian blue staining of sections of amoebae indicates that the extracellular coat is an acid mucopolysaccharide. This conclusion is further substantiated by the demonstration *in vivo* of alpha metachromasia when toluidine blue is used to induce pinocytosis.

This work suggests that pinocytosis inducers become attached to highly oriented sites in the extracellular acid mucopolysaccharide coat of the amoeba.

Culture of gonads and neural gland of Molgula manhattensis on various natural and synthetic media. Philippe Sengel and Madeleine Kieny.

The gonads and the neural gland were obtained from immature and mature animals. The gonads, including testes and ovaries, were explanted, together with the lateral wall of the body to which they are attached. The neural gland was cultured together with the brain and the vibratile organ.

Several media were tested as to their ability to provide adequate conditions for the survival of the organs *in vitro*. Some media contained 7 volumes of a 1% agar saline solution, 5 volumes of sea water and one volume of either sea water (medium M), chicken plasma, horse serum (medium HS), chick embryo extract, or Parker's solution no. 199. Other media were made of 7 volumes of the agar saline solution and either 6 volumes of horse-shoe crab serum or three volumes of sea water and three volumes of horse-shoe crab serum.

Immature gonads were associated on media M and HS with neural glands, including brain and vibratile organ, obtained from both juvenile and adult animals. In some cultures, however, the gonad was associated with the neural gland alone.

The explants were cultured for seven days and survived throughout this period. In most cases, the vibratile activity of the ovarian cavity, the spermiducts and the vibratile organ continued *in vitro* until the end of the cultivation period. The explants mostly maintained their organotypic organization, and very little outgrowth of cells occurred.

The histology of the cultured gonads and the possible action of the neural gland on their differentiation will be studied and described ulteriorly.

Effect of temperature, salinity, and food intake on the excretion of Zn^{65} in small marine fish. JUDITH SHULMAN, I. LEHR BRISBIN AND WALTER KNOX.

Fish of three species were tagged by feeding *Venus* meat soaked in $Zn^{65}Cl_2$ solution of approximately 1 μc./ml. concentration, and biological half-life ($Tb_{\frac{1}{2}}$) of assimilated isotope determined by making periodic whole-body counts in a well counter over a 15–20-day period. Fish were kept in clean tanks with running water or frequent changes of water.

Oxygen consumption measurements were also made on two species, using the Winkler method.

At 20° C. and 35‰ salinity, $Tb_{\frac{1}{2}}$ was 13 days for *Menidia menidia* as compared with 58 days for *Fundulus heteroclitus;* the metabolic rate of the former is about two times that of the latter. For *Fundulus,* $Tb_{\frac{1}{2}}$ was 75 days when kept at 10° C. and 35 days at 30° C., suggesting an inverse relationship of environmental temperature and excretion rate.

A preliminary experiment showed that *Fundulus's* $Tb_{\frac{1}{2}}$ was not different at 8‰ and at 35‰ salinity, indicating that gill salt excretion at high salinity does not materially increase the exchange of zinc.

No effect of varying food intake could be demonstrated. Zn^{65}-tagged specimens of *Tautogolabrus adspersus* at 20° C. and 35‰ salinity averaged a $Tb_{\frac{1}{2}}$ of 44.5 days, at food levels varying from 153.7 to 58.1 cal./g. fish/day, over a 17-day period. $Tb_{\frac{1}{2}}$ of one specimen of *T. adspersus,* which was starved for the 17-day period, was 59 days.

Research supported by N.I.H. grant 2G–535 (M.B.L. Ecology training program).

Aminopterin effects on Arbacia punctulata development. RICHARD G. SKALKO.

Aminopterin, a potent inhibitor of folic acid metabolism, produces a variety of developmental abnormalities in mammalian embryonic systems. This study was undertaken to determine whether this compound also interfered with the normal development of the sea urchin, *Arbacia.*

Eggs and sperm were obtained from mature *Arbacia* by mild electrical shock. Aliquots (100–300) of eggs were added to stender dishes containing the anti-metabolite dissolved in filtered sea water, either 30 minutes after fertilization or at the blastula stage. They were allowed to develop in a constant-temperature incubator (19° C.) and observations were made during cleavage, at hatching, at pre-pluteus and mature pluteus stages.

Exposure at fertilization over a range of doses (200-50 μg./10 ml. filtered sea water) produced no visible effect during cleavage. Beyond this stage, however, the effects were dosage-dependent. At 200 μg./10 ml., the eggs rarely became free-swimming blastulae and were killed at that stage. Eggs treated with 100 μg./10 ml. hatched normally but never developed beyond the blastula stage. They exhibited a characteristic circular swimming pattern and survived 10 to 14 hours beyond the 200 μg.-treated eggs. Those treated with 50 μg./10 ml. were completely inhibited as pre-plutei.

When the eggs were exposed to the same doses of aminopterin at the blastula stage, a different range of effects was observed. Those exposed to 200 μg./10 ml. developed into pre-plutei and rarely into immature plutei. Those treated with 100 μg./10 ml. developed into pre-plutei and immature plutei, while those treated with 50 μg./10 ml. developed into mature, immature and pre-plutei.

The results indicate that the effects produced by aminopterin are both dosage-dependent and time-dependent, the embryos being most sensitive during cleavage stages. It appears that, during cleavage, the anti-metabolite is interfering with a metabolic process that is essential for further development beyond the blastula stage.

Difference in chlorophyll a content of Cyclotella caspia cells grown on nitrate and uric acid. MARGARET J. WALDREP AND R. R. L. GUILLARD.

We determined the chlorophyll *a* content per cell and per unit of cell volume of *Cyclotella caspia* (clone 10–5) grown in pure culture in enriched sea water having either nitrate (100–400 μM) or uric acid (25–100 μM) as nitrogen source.

At the higher concentrations of either nitrate or uric acid the chlorophyll remained relatively high, varying from $5.1–8.0 \times 10^{-7}$ μg./cell, or from $3.8–6.9 \times 10^{-9}$ $\mu g./\mu^3$ of cell volume.

At the lower concentration of nitrate the chlorophyll *a* was lower, varying from 1.1–3.3 $\times 10^{-7}$ μg./cell or from 1.1–2.8 $\times 10^{-9}$ μg./μ^3 of cell volume. At the lower concentration of uric acid the chlorophyll remained high (4.5×10^{-7} μg./cell, or 4.2×10^{-9} μg./μ^3) during early growth of the culture but then fell in one day to 2.6×10^{-7} μg./cell or 2.2×10^{-9} μg./μ^3 of cell volume.

All observations are consistent with the hypothesis that availability of nitrogen rather than the specific nature of the nitrogen source is responsible for the variation in chlorophyll *a* content. However, the availability of nitrogen is influenced both by the nature of the source and its concentration.

C. caspia can utilize all the nitrogen atoms in the uric acid molecule (at 25 μM). Photosynthesis per cell, determined by C^{14} uptake, was about 30% greater when cells were supplied uric acid (100 μM) than nitrate (400 μM).

Active transport of oxygen into the eye of fish. Jonathan B. Wittenberg and Beatrice A. Wittenberg.

The transport of oxygen against a concentration gradient has heretofore been described in only a single organ, the swimbladder of teleost fishes. M. Biot, in 1806, established that oxygen could be brought into the swimbladder against a pressure gradient of over 100 atmospheres. A century later W. N. F. Woodland, and J. S. Haldane showed that gas secretion was accomplished by a complex of two organs—a gas gland and a rete mirabile. The rete consists of a regular and intimate intercalation of venous and arterial capillaries, which follow parallel courses and carry blood to and from the gas gland. An entirely similar rete mirabile supplies blood to the choriocapillaris underlying the retina of teleost fishes. The pigment cell layer of the retina may be regarded as analogous to the gas gland. It occurred to us that the rete mirabile-pigment epithelium complex might build up a large tension of oxygen behind the retina, which would provide a pressure head for diffusion to supply the vigorous oxygen demand of the relatively avascular retina.

We have used a polyethylene-covered polarographic oxygen electrode to measure the oxygen tension in the vitreous humor immediately in front of the retina. Eight species of teleost with well developed retia exhibited oxygen tensions greatly in excess of that in air (160 mm. Hg); tensions ranging from 400 to over 1000 mm. were commonly encountered. Two species of teleosts with poorly developed retia exhibited tensions not too different from air; three elasmobranchs and two species of teleosts, which have no rete, displayed oxygen tensions at the retina substantially less than that of air.

Our finding establishes a second instance of oxygen secretion against a large concentration difference. The parallel between the degree of development of the rete and the oxygen tensions measured at the retina constitutes strong evidence that the choroid rete is an important member of the oxygen-secreting complex.

PAPERS READ BY TITLE

Cytochemical observations of oxidative activity in the developmental stages of Chaetopterus pergamentaceus, using succinate and DPNH as substrates. M. Jean Allen.

Activity of DPN diaphorase and succinic dehydrogenase was demonstrated in whole mounts and serial sections following incubation with DPNH-Nitro-BT and succinate-Nitro-BT. At the germinal vesicle stage, using DPNH as substrate, purple to blue granules of diformazan (indicating DPN diaphorase activity) are concentrated in a perinuclear ring. From the nuclear membrane a decreasing gradient of activity extends into the cytoplasm surrounding the endoplasmic spherules. As the germinal vesicle breaks down, activity becomes concentrated in a broad crescent-shaped area (side view) of non-spherular endoplasm and residual substance around the spindle. At interphase of 2- and 4-cell stages, diformazan granules are present as a perinuclear ring and as a centro-peripheral gradient in the endoplasm. Perinuclear rings of activity were observed in later cleavage stages. In early blastulae, activity was clearly demonstrated only in the surface blastomeres. Oxidative activity in swimming larvae of 8 to 13 hours is primarily in the ectodermal cells (yolky endoderm showed no activity), as is

true in 24-hour trochophores except for the large mesotrochal cells which show no activity. For 1½- to 7-day trochophores, results are similar except that two small surface mucous glands at the level of the intestine show comparatively high activity.

Activity of succinic dehydrogenase was much less marked. Incubation time in succinate was 2½ to 3 hours instead of 7 to 10 minutes (DPNH incubation time) and even then, evident particularly in later stages, a pink rather than a purple to blue coloration may be noted in stages observed *en masse* as well as microscopically. The faint pink coloration was difficult to localize. The mucous cells of 1½- to 7-day larvae were not demonstrated following succinate incubation; this contrasts with the high concentration of oxidative demonstrated in these cells following incubation in DPNH.

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Techniques for obtaining, dissociating, and culturing cells and organs of Loligo pealii embryos. JOHN M. ARNOLD.

Some technical problems of obtaining and handling eggs and embryos of *Loligo pealii* were solved in the following manner.

It was possible to stimulate captive adults to lay eggs by suddenly exposing them to a naturally laid egg mass. This immediately resulted in both the males and females "investigating" the egg mass, mating, and egg-laying by the females. This is apparently a completely visual stimulus since an artificial egg mass made of water-filled polyethylene tubing would elicit the same response. Squid, in nature, have deposited fresh eggs on an egg mass hung from a piling after dark. In this way, the exact time of laying could be observed and very young stages of naturally laid eggs could be obtained.

It was found that dechorionated embryos, isolated organs, and single cells could be cultured in whole adult blood. This corroborates similar results by H. Hibbard and H. Hamilton (personal communication). Sterile culture techniques were used and antibiotics were added to prevent contamination. These embryos and organs appeared to develop normally.

It was possible to dissociate the cells of these embryos and to obtain solid aggregates within 15 minutes. These cells were dissociated in two different ways. In the chorion they were dissociated by ten minutes of violent shaking in sea water at pH 5. This treatment removes the jelly coating as well. In culture the cells were dissociated by crushing the embryo and mechanically selecting the single cells. Aggregates from both treatments survived up to 72 hours. As of yet, histological examination has not been made.

A longer paper giving further details is planned.

Observations on the mechanism of cellulation of the egg of Loligo pealii. JOHN M. ARNOLD.

Cleavage of the zygote of *Loligo pealii* leads to the formation of a blastoderm in about 12 hours. The blastoderm expands over the surface of the yolk so that the whole embryo is eventually composed of a surface layer of cells and a central yolk mass. The mechanism by which the yolk mass becomes covered by cells was the subject of this investigation.

Adult squid were kept in a deep sea-table and stimulated to mate and lay eggs. Eggs that were about one-half covered with cells were dechorionated and placed in depressions in a wax-bottomed watch glass containing sterile sea water. The embryos will live for about 24 hours under these conditions. The surfaces of the cells were marked with carmine powder, carbon particles, and/or Nile blue sulfate. Marks were made in the region above, at, and below the margin of the cellulated area. The margin of the cellulated area progressed further down the surface of the yolk and passed under the marks, but in no case did any major movement of the marks take place, although minor rearrangements did occur. This indicates that cellulation of the zygote takes place by peripheral cell division rather than by epiboly.

To negate the possibility of a non-moving surface coat, Nile blue sulfate marks were made on the eggs in addition to the particulate marks. These marks did not move in relation to each other. When the embryos at this stage were placed in 1:10,000 colchicine, cleavage was stopped and the cellulation of the zygote also stopped. Direct observations support this hypothesis also.

Evidence of a marginal zone of proliferation was found in aceto-orcein squashes which show a higher rate of mitosis at the edge than in the center of the blastoderm.

Responses of the mud snail, Nassarius, to experimental reversals in direction of very weak magnetic fields. FRANKLIN H. BARNWELL AND H. MARGUERITE WEBB.

Snails emerged from a magnetic-south-directed aluminum funnel-shaped corral. The mean amount of turning was expressed in terms of angular deviation from south (designated 0°) as the snails reached a point 3 cm. from the exit. The field into which the snails emerged was asymmetrical, black to left and white to right; the mean path of the snails preferred the white. To avoid the known daily fluctuation in mean path, all series were run between 8 AM and 12 M. Each daily experiment comprised assaying mean snail paths when the H component of magnetism was experimentally reversed to produce north-directed fields of 0.04, .1, .2, .4, .8, 2, 5, and 10 gausses. Control runs in the earth's field alone preceded and followed each pair of experimental fields. Each experiment comprised moving up the series with five paths in each, and then moving back down the series. Differences between experimental and adjacent control paths were determined. A significant difference ($P < .02$) in mean path from the controls was found only for the reversed 0.2 gauss experimental field, with only slightly weaker response in the 0.1 gauss field. The 0.1 and 0.2 gauss field effects exhibited essentially a unimodal distribution of values while the remainder were more or less bimodal, suggesting the fields to be inducing right *or* left turning. A study of the structure of the regressional relationship between mean path of all snails in a given series and magnet response revealed that when the path was more weakly (+2.5° to +12.5°) or more strongly (+15.5° to +24.5°) positive than near average (+ 12.5° to + 15.5°) the 0.2 gauss field was turning them highly significantly ($P < .001$) to the right; the .04 gauss field induced left-turning. The controls displayed a substantial lunar tidal fluctuation ($P < .001$) in mean path.

This study was aided by a contract (1228–03) with the Office of Naval Research and by grants from the National Science Foundation (G15008) and National Institutes of Health (RG–7405).

In vitro incorporation of C^{14}-labeled amino acids into goosefish islet tissue proteins. G. ERIC BAUER AND A. LAZAROW.

Islet tissue (2–6 mg.) was incubated for two hours in a modified Krebs-Ringer bicarbonate buffer containing leucine-C^{14} ($2.1 \times 10^{-3}M$) plus valine-C^{14} ($3 \times 10^{-3}M$). Specific activities were 6–10 mC./mM. Proteins were precipitated and washed with 5% trichloroacetic acid, extracted with 95% ethanol, ethanol-ether, and ether. Aliquots of both the alcohol-soluble fraction (ASF) and the final insoluble protein were removed and the radioactivity determined. When I^{131}-labeled insulin was added to islet tissue as a marker, 80–90% of the radioactivity was recovered in the ASF.

The average two-hour C^{14} incorporation into the ASF in 33 samples was 5720 cpm/10 mg. of tissue. Incorporation of C^{14}-amino acids into the ASF increased progressively during 9 hours of incubation (3900 cpm at one hour, 15,000 at four hours, and 23,500 at 9 hours). The incorporation was oxygen-dependent (up to 90% inhibition by anaerobiosis). When the amino acid concentration in the incubation medium was decreased ten-fold the counts incorporated into the ASF were decreased by only 50%. The addition of glucose (at concentrations of 100 and 200 mg. per 100 ml.) likewise decreased the counts in the ASF by 50%.

Further studies on purified ASF (dried, ether-extracted, dissolved in acid alcohol, and reprecipitated by acetone) using I^{131}-insulin as a marker, indicated that the amount of insulin precipitated by acetone was dependent upon the amount of unlabeled insulin present. Purification of the ASF obtained from the amino acid incorporation experiments indicated that 65–89% of the counts were precipitated by acetone. The average specific activity of the purified ASF was 129,000 cpm/mg. protein (*i.e.,* three times greater than that of the insoluble protein residue). These studies suggest that amino acids, added to islet tissue *in vitro,* are incorporated into insulin.

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The ionic factors in the electrogenesis of the electrically inexcitable and electrically excitable membrane components of frog slow muscle fibers. PETER BELTON AND HARRY GRUNDFEST.

The depolarizing electrogenesis of frog slow muscle fibers is due to postsynaptic potentials (psp's) generated in electrically inexcitable synaptic membrane by neural volleys or by cholinomimetic agents. However, the fibers also possess a membrane component which responds to sufficiently large depolarizing stimuli, which may be the psp's themselves, or depolarizations from intracellularly applied currents of sufficient magnitude to cause depolarization above the threshold for this electrically excitable response. The response is a hyperpolarizing (or repolarizing) electrogenesis, with which is also associated increased conductance of the membrane. The electrogenesis of the synaptic membrane appears to be due to increased conductance for sodium and potassium, as appears to be also the case in frog twitch muscle fibers and *Raia* electroplaques. Removal of chloride from the bathing medium, which inverts the normal hyperpolarizing inhibitory psp's that operate through increased chloride-conductance and which causes prolonged "spikes" by the chloride-conductance operated electrically excitable rectification of *Raia* electroplaques, does not affect the depolarizing psp's of the frog slow muscle fibers. Removal of chloride also does not affect the electrically excitable responses of the muscle fibers, while removal of potassium increases the hyperpolarization of the fibers. Thus, the electrically excitable electrogenic response of the frog slow muscle fibers appears to be due to potassium-activation.

Electron microscopic observations on the effects of homologous egg water on echinoderm spermatoza.. MAURICE H. BERNSTEIN.

Egg substances in contact with spermatozoa of the same species are credited with causing the formation of a filament from the substance of the acrosome. This acrosome reaction is sometimes accompanied by an agglutination of the spermatozoa. An electron microscopic examination of three species of echinoderms has provided a morphological basis for these egg water reactions and some further insight into the nature of acrosome filament formation and the mechanism of sperm agglutination.

The spermatozoa of *Arbacia punctulata, Echinarachnius parma,* and *Asterias forbesii* share a relatively simple morphology, being made up of an acrosome, head, middle piece, and flagellum. Sea urchin and sand dollar spermatozoa have a simple acrosome with a single, membrane-limited apical granule set in an anterior depression of the head. The starfish acrosome has a number of components and is of considerably greater complexity. In all three species the middle piece is dominated by a single ring-shaped mitochondrion.

The response of spermatozoa to treatment with egg water can be considered as a two-step reaction. In the first phase, the substance of the acrosome loses its structured appearance, apparently undergoing a solubilization or hydration. In the second phase, contact with any one of a variety of relatively non-specific surfaces will elicit the formation of an acrosome filament. These non-specific surfaces may include other eggs, glass, plastic films or particles.

The change in the appearance of the acrosome on egg water treatment is accompanied by an expansion or swelling of the middle piece. The cristae of the mitochondrion are disarrayed and partially disintegrated and the sperm membrane is distended to accommodate the increased volume of the middle piece. In agglutination, adhesion is mediated by contact with the surface of the swollen middle piece.

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The corneal endothelium of the scup (Stenotomus chrysops). LASZLO Z. BITO AND CLIFFORD V. HARDING.

The mammalian eye is known to have a sheet of endothelial cells completely covering the posterior surface of the cornea. This endothelium is known to have a very important

function in the metabolism of the cornea. The present studies demonstrate that there is a similar layer of cells in the scup eye and that this endothelium is a suitable tissue for *in vitro* studies of DNA synthesis. Whole-mount preparations of the posterior surface of the cornea were stained with Harris hematoxylin. Faintly stained nuclei were apparent in some areas, but for the most part, the more heavily-stained underlying stromal elements obscured the picture. This difficulty was overcome by making extremely thin preparations from corneas which had been stained according to the Feulgen procedure. Such preparations showed distinctly stained nuclei, quite evenly distributed over the entire posterior surface of the cornea. These nuclei showed a greater diversity in morphology than nuclei of similar preparations of mammalian (rabbit) corneal endothelium, and the number of cells per unit area is smaller than in the rabbit.

DNA synthesis was studied by autoradiography of endothelial preparations after the corneas had been incubated in teleost Ringer containing H^3-thymidine. Corneas incubated for 2–6 hours showed incorporation by a varying number of cells around the periphery of the endothelium. Similar incorporation can also be obtained after 24–48 hours of non-radioactive pre-incubation, indicating that these cells retain their DNA synthesizing ability *in vitro* for at least 48 hours. Corneas isolated and incubated 24–48 hours after a central penetrating needle injury showed marked DNA synthesis in the central area of the endothelium. An *in vitro* injury of the endothelial surface of freshly isolated corneas did not stimulate thymidine incorporation 24-48 hours after injury. Mitotic figures were not evident in any of the endothelial preparations.

Preliminary measurements of filter-feeding activity of the soft-shell clam, Mya arenaria, by use of radioactive algae. JOHN W. BLAKE.

Filtration rates (volume of water from which all particles are removed in unit time) of the soft-shell clam, *Mya arenaria*, were studied with single-species cultures of unicellular marine algae labelled with P^{32} (methods developed by Drs. T. R. Rice and W. A. Chipman, present work initiated while visiting the Radiobiological Laboratory, Bureau of Commercial Fisheries, Beaufort, North Carolina).

Data were obtained relating filtration rates to combinations of the following parameters: clam size (5 sizes, ½ to 2 inches shell length, 0.1 to 11.0 gms. wet meat weight, respectively), species and size of algal cells, algal population density, and salinity.

Preliminary findings indicate similarities between *Mya* and the hard-shell clam, *Mercenaria mercenaria* (data from Rice and Smith 1958, Smith 1958). Filtration rate of *Mya* appears independent of salinity (32 vs. 16‰), and algal population densities from 3×10^6 to 1.5×10^9 cells per liter (filtration is inhibited severely at 3×10^9 cells per liter). Filtration rate does vary with size or species of algal cells (2-inch clam filters *Nitzschia closterium*, $5 \times 30\,\mu$, at an average of 2.0 liters per hour, *Carteria* sp., $16 \times 19\,\mu$, at 1.6 L./hr., *Nannochloris atomus*, $2 \times 2\,\mu$, at 0.9 L./hr.), and also varies with clam size (2-inch clam filters *Carteria* at 0.15 L./hr./gm. clam meat, ½-inch clam at 3.0 L./hr./gm.).

While trends are similar, soft-shell clams, especially the smaller sizes, maintain an advantage over the hard-shell clams in the efficiency with which they are able to remove small particles, *i.e., Nannochloris*, from the water. This may relate to the greater abundance of smaller sized phytoplankton species and cells in the more estuarine habitats of the soft-shell clam.

Supported by a grant from the Aquacultural Research Corporation, Chatham, Massachusetts.

Comparison of the osmotic activity of ocular fluids with that of arterial plasma in the dogfish. M. BLOETE, D. C. NAUMANN, H. S. FRAZIER, A. LEAF AND WILLIAM STONE, JR.

It has been previously demonstrated that the aqueous humor is slightly, but significantly, hypotonic to the venous plasma in the smooth dogfish (*Mustelus canis*). The aqueous is currently assumed to be secreted into the posterior chamber, circulated anteriorly through the pupil, and reabsorbed finally at the base of the iris. The observed hypotonicity, therefore, might result from secretion of an isotonic fluid into the posterior chamber with subsequent dilution by reabsorption of solute. To test this hypothesis, tonicity of fluid from anterior and posterior chambers, as well as of the vitreous body, was determined.

Minute droplets of fluid were obtained under oil from the chambers of the eye of lightly curarized dogfish. Arterial blood was collected from the caudal aorta. Osmolality of these fluids was determined with a Ramsey-Brown Osmometer. Duplicate measurements, agreeing within 1%, were made with a Fiske Osmometer when sample volumes were sufficient.

In eleven dogfish, the osmolality of aqueous humor averaged 963.7 ± 3.8 mOsm./kg. water (S.E. mean) while arterial plasma averaged 978.3 ± 2.8; the mean difference is 14.5 ± 3.8 and $p < 0.01$. This result substantiates the difference previously reported between aqueous humor and venous plasma. Additional measurements showed: arterial plasma ($n = 15$) averaged 981.3 ± 2.2 (S.E. Mean), anterior aqueous ($n = 15$), 960.7 ± 2.8, posterior aqueous ($n = 12$), 952.8 ± 3.7; and vitreous ($n = 6$), 948 ± 1.3. The mean difference between plasma and anterior aqueous was 19.9 ± 4.1 ($p < 0.01$) and between anterior and posterior aqueous 7.4 ± 1.9 ($p < 0.01$).

Thus, there is a concentration gradient: arterial plasma > anterior aqueous > posterior aqueous > vitreous. Hence, (1) this sequence is incompatible with the gradients expected from the original hypothesis; (2) this sequence could not result from entry of water through the cornea from the more hypotonic sea water: (3) no metabolite is present in an amount sufficient that its utilization by the retina could account for the osmotic differences.

In the teleost (*Stenotomus chrysops*) whose plasma is hypotonic to sea water, limited measurements indicate that ocular fluids are also hypotonic to plasma.

Organismic orientation relative to magnetic axes, in responses to weak magnetic fields. Frank A. Brown, Jr. and Franklin H. Barnwell.

The path of mud snails, *Nassarius,* initially directed in each of eight equally spaced angular relationships to the horizontal component of geomagnetism from 0° to 270° was determined between 1 and 4 PM every two to three days for the period, June 21 through August 18, 1961. The average paths of four groups of ten snails were obtained on each day, with five snails emerging at each orientation starting at 0° and five snails passing in the reverse order. The snails emerged into an unchanging asymmetrical field with black to left and white to right. A total of 1000 snail paths in each of the eight relationships was obtained. The snails, favoring the side of brighter illumination, displayed a maximum right-turning when magnetic north-directed and minimum when northeast (45°) directed. ($P_{diff} < .001$.)

Although the snails turned progressively more to the right as the angle increased from 45° to 270°, they turned significantly ($P < .005$) further left when the magnetic axis was at 45°, 135°, 225°, and 315°, than at adjacent parallel or right-angle positions. The effect of magnetic N, E, S, and W relationships was confirmed (north and east difference, $P < .001$) by experiments on alternating afternoons for the same period. That magnetism itself was in some manner involved was demonstrated by a parallel series of experiments in which the orientation of the snail chamber itself remained unaltered but a horizontal *5 gauss* field was rotated and control observations interpolated. A significant difference ($P < .02$) in response to the magnet as a function of magnetic orientation was observed, of the same general amplitude, but closely mirror-imaging the corresponding relationship in the earth's 0.2 gauss field. Other experiments established ($P < .005$) that the snails showed left-turning in response to 2- and 5-gauss vertical fields.

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Lysergic acid and growth in Fundulus. Joseph A. Burke and Joseph C. Orlando.

Previous immersion experiments have shown that lysergic acid diethylamide (LSD-25) inhibits growth of eggs of *Fundulus heteroclitus.* To ascertain more precisely the developmental stage at which LSD exerts its greatest effect, eggs were placed in 100 microgram/ml. solutions of LSD-25, (1) during fertilization; (2) from the 2-cell stage (Op. 3) to blastula (Op. 8); (3) from Op. 8 to Op. 15 (closure of the blastopore); the eggs were then placed in pure sea water. LSD does not inhibit fertilization nor are there any apparent effects from exposure (1) or (2). Eggs treated as in (3) were noticeably retarded about Op. 19–20 when the

nervous system is being laid down. Mean heart rates for all days up to three days after hatching were: controls, 144 beats/minute; test embryos, 99/minute. However, unlike eggs grown from fertilization to hatching in LSD, these embryos gradually recover. Three to four days after hatching, test embryos look like controls and their pulse is the same. In another series of experiments, to determine the lasting effects of LSD, eggs at Op. 11 were placed in a 1 mg./ml. solution of LSD-25 for periods of 1, 4, 10, 24 and 72 hours, respectively, and then returned to sea water. Depending on time of exposure to LSD, all embryos showed some inhibition. First measurable heart rates averaged: controls, 120 beats/minute; 1-hour embryos, 108/minute; 4-hour embryos, 95/minute; 10-hour embryos, 87/minute; 24-hour embryos, 72/minute; 72-hour embryos, 69/minute. By two days after hatching, all embryos had recovered, except the 72-hour embryos which died before hatching due to severe abnormalities in the nervous and circulatory systems. In the 1-, 4-, 10- and 24-hour embryos, the melanophores were more dispersed and stellate in form than in the controls, indicating a still persistent effect of the LSD substance. Histological studies of preserved test embryos will be made to ascertain whether there is any damage at the cellular level.

Supported by grant MY-3235 from National Institute of Mental Health.

Organization of the cockroach respiratory center. James Case.

The isolated ventral nerve cord of the roach, *Blaberus craniifer,* continues to generate rhythmic volleys, judged to be respiratory motor since they appear only in the innervation of the respiratory musculature and are highly sensitive to pCO_2. In such preparations localized application of CO_2 to any ganglion intensifies and accelerates the rhythm. Contrasting with this widespread sensory capacity, the rhythm does not appear unless certain combinations of ganglia are present. Specifically required are the first abdominal ganglion and a minimum of either adjacent ganglion. Dissociated from ganglia anterior to the second abdominal, the cord remains highly sensitive to CO_2 but responds to it only with increased random spike activity. The respiratory control center would thus seem to function by rhythmically applied inhibition of tonic motor neuron activity in the remainder of the cord. Because single electrical stimuli applied at any point in the cord or to the roots of the segmental nerves interrupt a respiratory volley in progress and induce a series of ten or so brief volleys which gradually return to pre-excitation duration and rate, it appears that the normal respiratory rhythm also involves excitation of the inhibitory center by respiratory motor activity throughout the cord.

Effects of acids on an isolated insect respiratory center. James Case.

Under relatively normal conditions CO_2 has been considered to act as a respiratory stimulus in insects by means of some specific property other than the acidity it generates in solution. Experiments with the isolated ventral nerve cord of *Blaberus craniifer* show that this is not correct. Under the proper conditions, any of the thirteen weakly dissociated acids tested were able to induce respiratory rhythms in a completely reversible manner. These acids were not effective at the same pH so it may be concluded that hydrogen ion alone is not the governing factor. The more highly dissociated the acid the lower the pH had to be before respiratory volleys were generated. Thus, while unbuffered butyric acid saline was effective at pH 4.1, maleic acid solutions had to be carried to pH 3.6 before becoming effective. Further, the effective pH of carbonic acid-bicarbonate solutions varied uniformly with the bicarbonate concentration, from pH 5.0 for .001 *M* to pH 6.7 for .04 *M* bicarbonate. These observations are taken to imply a respiratory control mechanism sensitive to hydrogen ion concentration exists in the roach central nervous system, and that it is isolated by a barrier with preferential permeability to undissociated molecules.

Further studies on the gamete "shedding substance" from radial nerves. Alfred B. Chaet and Robert A. Rose.

The presence of a gamete "shedding substance" extracted from starfish (*Asterias forbesi*) radial nerves by lysis in distilled water has been reported in a previous communication. Nerve

extracts, when first autoclaved and then dialyzed against large volumes of sea water, induced shedding of gametes (within one hour) in starfish receiving an intracoelomic injection (0.15 cc./gm.). These results suggested that the "shedding substance" was non-dialyzable. Further experiments, however, indicated that the "shedding substance" did indeed dialyze through Visking tubing prior to autoclaving, but did not dialyze if the extract was autoclaved prior to dialysis. Such results suggested that autoclaving allowed the "shedding substance" to complex either with itself, or with other material in the extract, thus changing its dialyzable properties. The active component ("shedding substance") in a distilled water extract diffused across a membrane if dialysis was carried out against sea water, but did not dialyze if the dialysis was carried out in the absence of salts. The "shedding substance" found in a dialysate of sea water was ether-insoluble and was destroyed by autoclaving, but was only partially inactivated by boiling for 20 minutes. The "shedding substance" can be released not only by lysing radial nerves in distilled water (1 cc./nerve), but was also released when nerves were exposed to ethanol for 30 minutes. The fact that the active component in starfish nerves was not follicle-stimulating hormone (FSH), luteinizing hormone (PLH), 4-aminobutyric acid, or DL 2-aminobutyric acid, was seen when injections of the above solutions failed to induce the release of gametes from ripe starfish.

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Mutarotase inhibition by urea and guanidine hydrochloride. Aurin M. Chase and Hildegard C. von Meier.

The activity of kidney mutarotase preparations was measured polarimetrically by determining the first order reaction rate constant from the change in optical rotation when alpha-D-glucose was converted to the equilibrium mixture of the alpha and beta forms in the presence or absence of the enzyme, and in the presence of the inhibitors. All experiments were done at 26° C. in pH 5.3, 0.05 *M* acetate buffer, phosphate buffer having been found to have itself a greater catalytic effect on mutarotation than acetate and to inhibit mutarotase activity.

Guanidine hydrochloride was about ten times as effective as urea, but an analysis of the data representing relative activity as a function of inhibitor concentration indicated that about four molecules of either urea or guanidine combined with one mutarotase molecule (or active site) to produce the inactive complex.

An analysis by the Lineweaver and Burk method showed that the inhibition by guanidine hydrochloride is of the competitive type. Mutarotase would therefore fit in the general category of enzymes considered by Rajagopalan, Fridovich, and Handler (J. Biol. Chem., 1961), who found that those with organic substrates were competitively inhibited by such substances as urea or guanidine.

Exploratory experiments indicate that the percentages of inactivation of mutarotase produced by various concentrations of guanidine are essentially the same whether the enzyme is catalyzing the mutarotation of alpha-glucose or of beta-glucose.

The time required for half-completion of the enzyme-catalyzed mutarotation of alpha-glucose increases as the initial concentration is increased, over the range from 2½ to 10%.

Supported in part by a grant from the National Science Foundation.

Zygote versus gamete irradiation in Arbacia. Ralph Holt Cheney and Carl Caskey Speidel.

The damaging effects of x-ray irradiation of zygotes and gametes in *Arbacia* contrast sharply with those following 2537 Å ultraviolet irradiation. Extensive series involving graded doses of both types of radiation were studied with special reference to developmental delay and injury in the progeny during the first two days. X-ray dosages ranged from 1 to 128 kr. Ultraviolet exposures ranged from 30 seconds to 12 minutes.

X-ray effects. (1) A 1-unit dose to zygotes (irradiation begun one minute after mixing normal eggs and sperm) caused damage *equal* to or *greater* than a 1-unit dose to both gametes subsequently intermixed for fertilization. (2) A 1-unit dose to zygotes also induced damage

equal to or greater than an 8-unit dose to eggs alone which were then fertilized with normal sperm, or than an 8-unit dose to sperm alone which were then combined with normal eggs.

Ultraviolet effects. (1) In contrast with no. 1 above, a 1-unit ultraviolet dose to zygotes (irradiation begun one minute after intermixing normal gametes) caused *much less* damage than a 1-unit dose to both gametes. Furthermore, a 4-unit or even 8-unit dose to zygotes also caused less damage. An essentially similar result, though the contrast was not so great, was obtained from zygotes *shaken* throughout the irradiation period. (2) In contrast with no. 2 above, a 1-unit dose to *unshaken* zygotes caused damage about equal to a 2-unit dose to *unshaken* eggs alone; also a 1-unit dose to *shaken* zygotes caused damage about equal to a 2-unit dose tc activated sperm alone.

Pigment distribution in degeneration (recorded by time-lapse motion pictures). · Strong x-ray or ultraviolet irradiation of zygotes, both gametes, or eggs alone often induced violent internal upheaval followed by localized massive pigment concentration. Strong irradiation of sperm alone, however, usually induced abnormally scattered, spotty pigment distribution.

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Further experiments on the role of the polar lobe region in embryonic determination in Ilyanassa. Anthony C. Clement.

Through removal of the D macromere (and hence of the polar lobe region) from the egg of Ilyanassa at successive cleavage stages, the following types of partial eggs were produced: (1) ABC, (2) ABC + 1d, (3) ABC + 1d + 2d, (4) ABC + 1d + 2d + 3d, (5) ABC + 1d + 2d + 3d + 4d. All of the types produced partial larvae except the last, which gave rise to a complete veliger of reduced size.

Types 1 and 2 gave partial larvae of the sort obtained after removing the polar lobe at first cleavage. Larvae of type 3 were similar to the preceding except that occasional individuals bore a small external shell. Larvae of type 4 were much superior to the preceding, often showing an organized velum, paired eyes, foot, and well developed shell. Heart and intestine appeared only in larvae of type 5.

The difference in development of larvae of types 3 and 4 cannot be attributed solely to the presence or absence of the third micromere. In a different series of experiments the third micromere was removed from otherwise whole eggs; the resulting larvae showed foot deficiencies, but good development of eyes, velum and shell. The polar lobe region appears to play a role of induction or activation in the determination of certain larval organs.

Fertilizability and developmental capacity of germinal vesicle and non-germinal vesicle eggs of Hydroides. Laura Hunter Colwin and Arthur L. Colwin.

Batches of eggs shed by the female upon removal from the dwelling tube were examined. The germinal vesicle, though usually intact in the full sized egg, was often found to have broken down in a few eggs, and in some batches it had broken down in as many as 50% of the full sized eggs. When the germinal vesicle has broken down, an ill-defined clear area lies in the cytoplasm nearest the animal pole.

Eggs of both types were isolated, inseminated, and compared as to fertilizability and capacity to develop. Uninseminated controls of both types were isolated from the same batches. It was found that the egg can be fertilized in either the germinal vesicle or non-germinal vesicle condition. Inseminated eggs of both types can develop into normal trochophores. From some females the "non-germinal vesicle eggs" gave higher percentages of normal development than did the "germinal vesicle eggs." In other batches, both types of egg gave about the same percentages of normal development. None of the uninseminated control eggs underwent any real development whatsoever. However, the uninseminated non-germinal vesicle eggs eventually undergo a type of fragmentation which bears some superficial resemblance to cleavage.

It is suggested that the eggs in which the germinal vesicle normally breaks down soon after shedding are those which have most nearly reached the peak of their physiological maturity.

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Studies on the site of action of alloxan: lack of penetration into cells. S. J. Cooperstein and James A. Jackson.

As part of a project designed to determine the mechanism by which alloxan selectively kills the insulin-producing cells of the islets of Langerhans, we have previously compared the distribution of injected C^{14}-alloxan with that of C^{14}-mannitol, C^{14}-thiocyanate, C^{14}-inulin, and C^{14}-urea in islet and other tissues of the toadfish. Since the distribution of injected alloxan paralleled that of mannitol, inulin, and thiocyanate (compounds whose distribution is limited to the extracellular fluid), it was suggested that alloxan does not enter the cell but exerts its diabetogenic effect by acting on the beta cell membrane.

In the present study the distributions of C^{14}-alloxan and H^3-mannitol were measured simultaneously in the same fish; this procedure permits a more precise comparison between these two compounds than was possible when separate animals were used. A mixture of tracer doses of these two radioactive compounds was injected intravenously into toadfish; 7 fish were killed at 2 minutes after injection, 5 at 5 minutes, and 6 at 15 minutes. Samples of islet and other tissues were removed and the amounts of C^{14} and H^3 present were determined using the Packard Tri-Carb liquid scintillation counter.

The results showed that at all times following injection the relative amounts of C^{14}-alloxan and H^3-mannitol in islet, heart, muscle, gill, kidney, brain, and blood were the same as that in the injected mixture. Liver differed from the other tissues; it contained a relatively greater amount of mannitol, due to the penetration of this compound into liver cells. These findings support the thesis that alloxan does not enter the cell during the period when it is known to exert its diabetogenic effect, and that the beta cell membrane is a primary site of alloxan action.

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Comparison of serum-proteins from young and old Limulus. Armando A. de la Cruz and Alfred B. Chaet.

It has previously been reported that young horseshoe crabs (*Limulus*) exhibited a resistance to burn toxin which proved fatal to older animals. These findings suggested a comparative study of the serum-proteins between young and old horseshoe crabs (*Limulus polyphemus*). The blood from small-young (2.0–3.0 grams) animals was obtained by heart puncture, pooled, and the supernatant serum removed for future study. Aliquots of sera of large-old crabs (3.2–4.5 kg.) were obtained in the same manner. The sera were dialyzed against buffer (pH 8.6) and analyzed by starch-gel electrophoresis. Such dialyzed samples gave considerably better resolution than comparable non-dialyzed samples. Since spectrophotometric measurements (490 λ) demonstrated more haemocyanin in the serum of the older animals, the serum from large crabs was diluted (with buffer) until it approximated that of small crabs.

Electrophoresis of the sera from both age groups was carried out by running the two samples in the same gel. The small Limulus serum revealed 7 distinct bands, all migrating towards the anode area, whereas the serum from large crabs revealed only 4 distinct bands and a fifth slower migrating diffused band. The 4 fastest migrating bands in the large Limulus serum were comparable in mobility and stainability with that of the 4 fast bands of the small Limulus serum. The remaining 3 fractions present in small Limulus serum migrated considerably faster than the fifth diffused band of large Limulus serum and thus were not comparable.

Preliminary evidence indicated that a blood protein, migrating like a gamma globulin towards the cathode area, was present in whole blood aliquots and sometimes in sera of young crabs. To date, this gamma globulin-like fraction has not been found in the sera of older animals. Since gamma globulins have not previously been found in the blood of invertebrates, this observation is under further study.

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The effect of volume on the termination of pregnancy. A. I. Csapo and M. A. Lloyd-Jacob.

Earlier experiments indicated that an increase in uterine volume facilitates parturition and that this effect of volume on delivery can be balanced by progesterone therapy. In the present experiments we kept the progesterone treatment constant and altered uterine volume so as to demonstrate conclusively that an increase in uterine volume hastens delivery.

Twenty-five days pregnant rabbits were ovariectomized and their placentae dislocated. Endogenous progesterone was substituted by a 6 days' decremental progesterone treatment ranging from 2.0 mgm. to 0.5 mgm./day. Such animals with a total uterine volume of about 200 ml. began to deliver 24–48 hours after surgery at the 1.5–1.25 mgm./day progesterone level. Delivery was prolonged for several days and was often only partial. However, if the content of one uterine horn was transferred into the other, delivery usually began within twelve hours after operation and the excess volume was quickly delivered.

When the uterine volume was controlled by dummy babies made of a mixture of paraffin and wax it was the horn carrying a larger volume which delivered first. Delivery from a horn with exceedingly small volume often failed altogether. All these observations point to the same conclusion; namely, that volume and progesterone have opposing effects on the parturient activity of the uterus. When the ovaries were removed in a group of animals subjected to decremental progesterone therapy and the content of only one horn was replaced by dummy babies, the dummies were first delivered in spite of the excess volume of the intact horn due to fetal growth. This finding is in good agreement with earlier observations that functional placentae exert a local effect on the myometrium, and also with the conclusion that excess progesterone can balance the effect of increasing uterine volume on facilitating delivery.

Ionic relations in the Fundulus egg. Philip B. Dunham and David C. Shephard.

Jacques Loeb's experiments on heart beat cessation in intact *Fundulus* embryos immersed in KCl solutions have been repeated. His observation that the heart beat fails to recover as long as the embryo is left in distilled water, but resumes after being placed in sea water, was confirmed. His suggestion that the excess K cannot leave the egg unless there are positive ions outside for exchange was checked by following the net changes in the Na and K contents of 8–12-day *Fundulus heteroclitus* embryos in different solutions. When eggs are placed in 0.5 *M* KCl, the K content increases and the Na content decreases. Net changes are effectively complete in 30 minutes. When returned to sea water, the K and Na contents return to their original levels in a similar period of time. Fluxes of Na^{24} showed rapid equilibration of most of the Na content of the eggs. However, the K content of eggs in sea water is very slowly exchangeable with K^{42} (25% in 75 hours). When KCl-poisoned embryos were placed in distilled water, the K content decreased rapidly to its normal level, but the heart beat did not recover unless the eggs were subsequently returned to sea water. K^{42} efflux from KCl-poisoned eggs is rapid and of the same rate in sea water and distilled water. The Na content of embryos moved from sea water to distilled water was significantly higher than that of the KCl-poisoned embryos, although lower than in embryos in sea water. Thus, the failure of the heart beat to resume in distilled water is clearly not due to the retention of the initial high K content as suggested by Loeb, but is probably due to an imbalance in the Na:K ratio in the blood of the embryo. This hypothesis was tested by experiments on exposed hearts of dechorionated embryos. The heart beat stopped in 5 seconds when exposed to solutions with Na:K ratios of 5:1 or lower, but recovered quickly if the ratio was increased to 10:1. These results were independent of total concentration over at least a two-fold range of concentrations.

Studies of melanin biosynthesis in the ink sac of the squid (Loligo pealii), Thomas B. Fitzpatrick, Makoto Seiji, Robert Simpson and George Szabo.

Melanin particles contained in the cells lining the ink sac and in the ink "milked" from the ink sac have been isolated by differential centrifugation method, followed by density-

gradient centrifugation. Tyrosinase activity was studied in the isolated particles. In mammals, the *melanosome* is a varying-sized cell organelle with a distinctive internal structure and tyrosinase activity. The *melanin granule* represents a later stage of development of melanosome; the melanosome becomes progressively melanized and the tyrosinase activity disappears, presumably because it is masked by the deposition of the melanin polymer on the particle. The melanin granule is a heavily pigmented particle without detectable tyrosinase activity. This sequence of events from an enzymatically active particle to an inert dense particle also appears to occur in the squid ink sac. Melanin particles isolated from homogenates of the ink sac have marked tyrosinase activity, while those in the ink have no tyrosinase activity. It was possible to solubilize the tyrosinase from the melanin particles in the ink; thus the ink particles contained a masked tyrosinase. In contrast to mammalian pigmented tissues, however, the ink sac has been found to contain most of the tyrosinase activity in the soluble fraction. Characterization of the ink sac tyrosinase showed it to be similar to mammalian tyrosinase: tyrosine and DOPA were the most active substrates, copper-binding agents inhibited enzyme activity, and the same type of lag in the oxidation of tyrosine occurred.

Although the pigment in the eye and in the "chromatophores" of the skin of the squid occurs in granular form, similar in size and density to melanin granules, these do not contain measurable tyrosinase. The pigment in the granules has the solubility and other properties of ommochromes which have previously been demonstrated in squid skin and eye.

Influence of tail skin, epidermis, and dermis on forelimb regeneration in the axolotl. Richard W. Glade and Nancy J. Scott.

Tail tissues were transplanted between black and white axolotls (body lengths 7 to 11 cm.) to 43 fore- or upper arm stumps. Whole mock grafts and portions of actual grafts were analyzed for percentages of epidermal, glandular, connective tissue, and muscle nuclei. Tail skin, epidermis, and dermis were transplanted singly or in combination with spinal cord from the tail. Contralateral limbs, amputated at the same level as the experimentals, served as controls. Epidermis and dermis were isolated by allowing pieces of skin to remain in 100% Holtfreter's solution 1–2 hours at room temperature. The tissues could then be peeled apart, and proved to be viable. Dermis-free epidermis was also obtained by removing the wound epithelium from denuded sections of tails.

The cartilage structure of 22 useful experimental regenerates has been studied to date and correlated with percentages of component cells in the grafts. When epidermal cells constituted 90–95% of the graft (isolated epidermis), the regenerates were either normal, in one out of five cases (1/5 cases), or slightly inhibited (3 digits, 4/5 cases). When connective tissue cells formed 22–28% (whole skin) or 88–92% (isolated dermis) of the graft, more severely inhibited regenerates appeared. Moreover, in 10/17 of these cases, cartilages developed which did not appear to be homologous to those of the limb. In 5/10 cases, these were of the axial type.

The frequency of various degrees of limb inhibition and the development of non-limb or axial cartilages could not be correlated with the implantation of spinal cord. The same lack of correlation applied to the presence of tail muscle fibers which adhered as a contamination (maximum 0.2% of the nuclei of the graft) to some of the grafts.

It is concluded that tail skin may express its own regionality in the cartilage pattern of the forelimb regenerate. The factors responsible probably reside in the dermis.

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Strontium utilization by Arbacia punctulata. I. P. Goldring and H. I. Hirshfield.

An artificial sea water containing strontium as a substitute for calcium was used to test its effect upon the activation, fertilization, and subsequent development of *Arbacia*. The medium was free of phosphate and sulfate, due to their low solubility with strontium in water. The bulk of the anions were in the form of chlorides with a small quantity of bicarbonate present.

A control solution (an artificial sea water containing equivalent amounts of calcium and the same components as the strontium medium) was used. Parallel studies using calcium-free artificial sea water and filtered sea water were carried out.

The visible effects of the artificial media, whether it was calcium or strontium, indicated that anion deficiencies existed, probably sulfate and phosphate. The purity of the salts used was critical. The results with the calcium and strontium artificial media were surprisingly similar. Insemination of strontium-exposed eggs with sperm in strontium sea water resulted in no activation or fertilization. Normal sperm, however, were capable of activating a high percentage of eggs in strontium medium. Actual cleavage numbers were lower than in normal eggs and many abnormal forms were present. A higher degree of mortality and asynchrony in development were noted. Neither medium could support development past the prism stage. Aliquots containing different stages of development, up to early gastrulae, from either medium transferred into normal filtered sea water produced normal plutei. Mixtures of the artificial strontium and calcium media could not produce normal long-armed larvae. Developmental arrest at the prism stage in the artificial media or their combinations could be maintained for at least 5 days.

Skeletal spicules were formed in both types of artificial media, but their composition is unknown.

Comparisons of the above studies with similar studies of *Chaetopterus* indicate that there is a greater need for skeletal metabolites in *Arbacia* than in *Chaetopterus*. Although the mortality rate was higher, trochophores were produced which were mostly abnormal.

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An accessory small nerve fiber in a mechano-receptor. K. Goto and W. R. Loewenstein.

Besides the well-known myelinated axon (large fiber), a fine non-myelinated axon (small fiber) enters the Pacinian corpuscle. The large fiber is clearly the afferent axon of the sense organ: it transmits the impulses elicited by mechanical stimulation of the receptor. It conducts at 25 m./sec. (32° C.). The small fiber is not excited by mechanical, thermal, or chemical stimulation of the corpuscle. It conducts at 0.65 m./sec. Under certain conditions, we have found it to have a facilitatory action on the receptor process. Single mesenteric Pacinian corpuscles were isolated together with a length of its extra-corpusclar nerve supply containing the large and the small fiber. When the small fiber was stimulated selectively outside the corpuscle so that only the small fiber impulses (but not the antidromic fast fiber impulses) run into the corpuscle, the generator potential of the nerve ending of the fast fiber in response to a given mechanical stimulus was reversibly enhanced by about 10%, and the mechanical threshold for firing of impulses in the fast fiber lowered by about the same amount. However, the effect could be shown only in 12 out of 91 preparations; and even in the rare successful cases, it lasted only a few minutes. The physiological role of the small fiber is thus as yet unclear. Because of its insensitivity to various modalities of sensory stimuli, it is likely to be an efferent axon. But whether receptor facilitation is its function, as in the case of the sympathetic small fiber system of certain cutaneous touch receptors (Loewenstein, J. Physiol., **132**: 40, 1956), or whether its role is merely to control the tonus of the small blood vessel inside the corpuscle, is quite uncertain.

A densitometric technique for measuring the rate of reaggregation of dissociated sponge cells. Bernice Grafstein.

Classical studies of reaggregation of sponge cells, based on the number and size of aggregates formed after a finite period of time, provide only an indirect and qualitative estimate of the rate at which aggregation proceeds. With the present method, the aggregation rate was determined quantitatively by measuring the rate of disappearance of unaggregated material.

Dissociated cells were prepared by squeezing 10 g. of sponge through #14 standard bolting cloth into 50 ml. calcium- and magnesium-free sea water at 0° C. Suspensions made from either *Microciona prolifera* or *Haliclona permollis* contained between 12×10^4 and 20×10^4 cells per mm.^{3} Aggregation was initiated by adding 1 ml. cell suspension to 4 ml. filtered sea water in a petri dish. After an interval of between 2 and 60 minutes, the aggregated material was removed and aggregation arrested by pouring the 5-ml. sample through three layers of

bolting cloth into a cold test tube containing 25 mg. crystalline sodium citrate. The "unaggregated" material remaining in suspension consisted of dissociated cells and aggregates no larger than 15 μ in diameter. The optical density of this material was read on a Coleman Junior Spectrophotometer at 660 or 570 mμ. Readings were converted to cell numbers by means of appropriate calibration curves.

There was found to be a marked difference between the rates of reaggregation of *Microciona* and *Haliclona* at 14.5° C. With *Haliclona,* there was a logarithmic decrease in the amount of unaggregated material, with a half-time of 45 minutes. With *Microciona,* there were two phases of logarithmic decrease. In the initial 15 minutes, the half-time was 10 minutes; subsequently it was about 220 minutes. This difference may be important in the process of separation of cell types that occurs in the aggregation of a mixture of cells from different sponges.

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Effect of aconitine on the giant axon of the squid (Loligo pealii). WALTER H. HERZOG.

Aconitine is known to cause repetitive responses in nerve after a number of short shocks. The following observations were made with either internal double wire stimulating and recording electrodes or internal KCl-filled glass electrodes and external stimulation. In axons with action potentials greater than 100 mV. or resting potentials greater than 53 mV. there was no oscillation following action potentials produced by short square wave shocks. Repetitive firing (maximum of five impulses) occurred in most axons with suprathreshold shocks of 15 to 20 milliseconds duration.

Without stimulation aconitine (1×10^{-6} g./cc.) had no effect on resting potential or on size and shape of action potential. However, if the axon was stimulated at a regular rate, resting potential decreased gradually by five millivolts and, concomitantly, damped oscillations appeared which increased in size until repetitive firing occurred. The repetitive firing usually continued for 20 to 70 seconds. Higher stimulation rates and/or longer time was necessary to elicit repetitive responses with lower concentrations (1×10^{-7} and 3×10^{-7} g./cc.). At the end of the repetitive burst the axon was depolarized by ten to fifteen millivolts. Repolarization occurred in 10 to 15 minutes. Repolarization occurred in less than three minutes if the axon was placed in sodium-free solution (sodium replaced by choline). Aconitine (1×10^{-4} g./cc.) produced a depolarizing block without stimulation.

Measurements were made of current-voltage relation in these axons. Without stimulation aconitine produced no change in current-voltage curves. With stimulation at a rate producing oscillations the current-voltage curve indicated a decreased membrane resistance. Decreasing external sodium from 423 to 168 mM./L. decreased the height of the action potential and oscillations, and increased the membrane resistance to control levels.

This evidence indicates that the action of aconitine in squid axons is due at least in part to increased sodium conductance.

Nucleotide metabolism in developing Arbacia. HENRY I. HIRSHFIELD, SELMA B. ZIMMERMAN AND ARTHUR M. ZIMMERMAN.

Eggs of *Arbacia punctulata* were incubated in 0.5 μC./ml. thymidine-H^3 sea water for one hour, or five minutes prior to insemination. They were maintained in this medium until the metaphase of the first mitotic cleavage and then placed in Bouin's fixative. Subsequently they were embedded and sectioned at 5 μ. The prepared slides, stripped with AR. 10 autoradiographic film, were incubated 25 days prior to development of the film. Similar studies were made using 1.12 μC./ml. uridine-H^3. Uptake of the thymidine and its incorporation into chromosomal DNA was confirmed by the use of DNA-ase. No specific localization or incorporation of the uridine was found.

The duration of incubation with thymidine was not significant. Incorporation of thymidine into the chromosomes in those eggs kept one hour prior to fertilization and those eggs kept five minutes prior to fertilization was about the same. This suggests that active uptake and utilization probably occurred during or after fertilization.

The analogous utilization of cytidine in DNA synthesis was investigated by immersion in cytidine-H^3 1.6 μC./ml. for one hour prior to fertilization and five minutes after fertilization. Surprisingly, no comparable localization of cytidine to thymidine was found within the nucleus. The autoradiograms of cytidine were similar to those of uridine-H^3.

The lack of incorporation of cytidine and uridine cannot be easily explained. One possible interpretation is that uridine and cytidine or precursors are readily available in early cleavage stages. Thymidine may be present in extremely low concentrations and produced as needed from other nucleotides or precursors. Its rapid uptake and incorporation in the chromosomes indicate that, when available, thymidine will be incorporated into DNA, possibly sparing conversion of other nucleotides or thymidine precursors.

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Effect of electrical currents on the initiation of nerve impulses in a Lorenzinian ampulla. NOBUSADA ISHIKO AND WERNER R. LOEWENSTEIN.

When the internal pressure of Lorenzinian ampullae (*Mustelus canis*) is raised above the external pressure, a generator potential is produced in the receptor elements. The generator potential and the frequency of the resulting nerve impulses increase with the pressure differential. The impulse frequency at any given pressure differential is markedly altered by the flow of electrical currents through the ampulla. Current flow in the direction of ampulla swelling to pore enhances, and, in the reverse direction, depresses the frequency. The minimal effective current is 5×10^{-9} amps. (Current was passed with a micro-electrode placed on the swelling and a silver-silver chloride wire placed at the pore opening.) The effect is not due to a direct excitation of the nerve ending or fiber of the receptor, but requires the presence of a generator potential produced by pressure: when the pressure-generator mechanism is turned off by setting the pressure differential near zero, currents as high as 10^{-5} amps. are ineffective in eliciting generator or action potentials (the nerve fiber was normally excitable by directly applied electrical stimuli). The minimal current required to flow through the ampulla in order to produce a given enhancement of background impulse frequency (minimal current) is inversely related to pressure or generator potential. For example, the minimal current at 60 cm. (Hg) pressure is about one hundredth of that at 10 cm. (Hg). (This pressure change corresponds to an approximately four-fold change in generator potential and a five-fold change in background frequency.) At constant pressure, the enhancement or depression of frequency increases roughly linearly with the logarithm of current.

Effect of x- and gamma radiation on the growth rate and conidia formation in Neurospora crassa. JOHN KEOSIAN.

Unless otherwise specified, radiation was from a 182 Kv. machine, inherent filtration of 0.15 mm. Cu, dose rate 4860 r per minute, total dose 9000 r.

1. A small but consistent post-irradiation increase in growth rate was previously reported in *Neurospora* cultured in growth tubes. Present studies using longer tubes, showed the increased growth rate to persist to the end—a period of more than four days.

2. Irradiation of the growing frontier of the mycelium causes the early appearance of a heavy band of conidia at the frontier. The effect of radiation was tried on an albino, aconidial, slow surface-growing strain of *Neurospora*. No conidia appeared, but the irradiated cultures, after a short lag, changed to a faster, heavier, aerial growth. The same change occurred in the controls two days later.

3. In a deficient medium the wild type shows a slow, sparse, surface growth which does not develop conidia. Irradiation of the frontier of these cultures caused the appearance of a sparse band of albino conidia. Four successive irradiations of these tubes, permitting about two inches of growth after each exposure, produced four successive bands of albino conidia in each tube.

4. The R.B.E. (relative biological effectiveness) of gamma radiation (Cs-137 irradiator) was tested by exposing *Neurospora* cultures in Falcon plastic petri dishes to doses ranging

from 1000 to 100,000 r. No frontier conidia formed. X-radiation using plastic dishes also gave negative results. X-radiation produces frontier conidiation in *Neurospora* grown in Pyrex dishes. The role of Pyrex vs. plastic on this effect is under investigation.

Krebs and pentose cycle dehydrogenase systems in eggs of Spisula as measured with a tetrazolium salt. Evelyn Kivy-Rosenberg and Frances Ray.

The quantitative study of substrate-dependent dehydrogenase activity was continued with the investigation of uninseminated and inseminated eggs of the bivalve *Spisula*. The series of substrates utilized included those which require no cofactor as well as those with DPN or TPN as cofactors. Among the substrates were four involved in the Krebs cycle (isocitrate, using TPN as cofactor; alpha-ketoglutarate, and malate using DPN; succinate with no cofactor) and two in the pentose cycle (glucose-6-phosphate and 6-phosphogluconate, both requiring TPN). As has previously been reported, the tetrazolium salt utilized as hydrogen acceptor was 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT).

Since the number of eggs obtainable from a single clam would not suffice for a complete assay of an uninseminated and inseminated set, the eggs from several females were pooled for each assay. Homogenates of each of several pools of uninseminated and inseminated eggs were incubated aerobically for one hour at 37.5° C. in media containing INT and substrate or appropriate controls. Formazan (reduced tetrazolium) was extracted and the quantity measured spectrophotometrically. The substrate-dependent dehydrogenase activity was expressed as micrograms of formazan per milligram of protein.

Of the seventeen substrate-dependent dehydrogenase systems assayed, two of the Krebs cycle were most active: namely malate which ranked first and isocitrate, second. Succinate was in about position 7 and alpha-ketoglutarate was at 6. However, the latter was the least consistent of the group in that its activity was positive in most cases but not in every case tested. Thus succinic dehydrogenase activity, though lowest of this cycle, did not rank as low as was seen earlier (Biol. Bull. **119**, 1960) for *Asterias*. With respect to the pentose cycle, the rank of activity of the glucose-6-phosphate-dependent dehydrogenase system and that of 6-phosphogluconate were fourth and fifth, respectively.

The relative activity was in general similar for inseminated as for uninseminated eggs.

Hormonal activity of sinus glands in Carcinus maenas from different geographic localities. L. H. Kleinholz.

Differences in distal retinal pigment hormone (DRPH) activity had been observed when sinus gland extracts of *C. maenas* were tested at several marine laboratories. To insure that such differences are not attributable to the species used as test animal in each locality, standardized preparations and testing methods were necessary. Sinus glands, eyestalks from which the sinus glands had been removed, and whole eyestalks of *C. maenas* from Woods Hole (WH), Plymouth (P), Roscoff (R) and Naples (N) were dried for 5 hours at 105° C. Sets of the three tissues were collected within two weeks. These, as well as similar tissues prepared at Kristineberg (K), were subsequently tested for activity on dark-adapted *Palaemon adspersus*. Extracts of sinus glands, in a concentration of 10 per 1.0 ml. of water, when injected into dark-adapted test animals, produced the following retinal response indices: WH, 0.086; R, 0.087; N, 0.098; P, 0.171; K, 0.170; retinal indices for uninjected dark-adapted control animals averaged 0.029. Similarly injected extracts of eyestalks from which the sinus glands had been removed effected the followed indices: WH, 0.165; P, 0.162; R, 0.173; N, 0.174; K, 0.174. Extracts of entire eyestalks gave these indices: WH, 0.178; R, 0.171; N, 0.174; K, 0.184. The observed results indicate that sinus gland extracts from *C. maenas* of three geographic regions, even though injected in relatively high concentrations, contain little light-adapting DRPH; similar extracts from *C. maenas* of Plymouth and of Kristineberg, however, produce nearly maximum responses. It is also evident, from the light-adapted responses produced in the test animal by extracts of sinus glandless eyestalks, that light-adapting DRPH is present in tissues of the eyestalks other than the sinus gland.

This study was aided by a grant from the National Institutes of Health, B–2606.

Enzymatic inactivation of crustacean distal retinal pigment hormone. L. H. Kleinholz and Frances Kimball.

An enzymatic mechanism has been indicated as inactivator of pigmentary effector hormones from the crustacean eyestalk; such inactivation of the light-adapting distal retinal pigment hormone (DRPH) was examined. Standardized assay determinations show that aqueous extracts of eyestalks of *Palaemonetes* (20 eyestalks per 1.0 ml.) produce average DRP indices of 0.20 when injected into dark-adapted test *Palaemonetes*, compared with average indices of 0.05 in uninjected dark-adapted controls. Progressive decline in DRPH activity occurs in aliquots taken at intervals from eyestalk extracts kept at 25° C.; at this temperature, DRPH activity may be completely absent after 12 to 18 hours. On the other hand, DRPH activity remains essentially unaltered over a 24-hour period if a freshly-prepared eyestalk extract is heated at 100° C. for two minutes. Addition of antibiotics to unheated eyestalk extract did not prevent marked decrease in hormonal activity. The pH optimum for enzymatic inactivation of DRPH was examined in appropriately buffered mixtures of eyestalk extract, from which DRPH had been removed by dialysis, and of partially-purified hormone as substrate. Buffered experimental and control mixtures were incubated for 6 hours at 38° C.; control mixtures contained enzyme extract denatured by heating for two minutes at 100° C. The pH of each mixture was verified at the end of each incubation period. DRPH content was assayed by determining DRP indices in dark-adapted *Palaemonetes* after injection of the buffered preparations. Enzymatic inactivations of DRPH at pH 5.1, 6.0, 6.7, 7.3, 8.0 and 9.0 are, respectively, 6%, 15%, 43%, 65%, 53% and 5%. Optimum for the inactivating enzyme is about pH 7.5. The enzyme occurs also in crustacean nerve cord and in hypodermis; its specificity is being examined in further studies.

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Anodal inhibition of the regeneration of Tubularia crocea within an electric current. Stephen Levin.

With an apparatus similar to that described by Barth (1934), constant currents were passed through 10-mm. regenerating stem sections of *Tubularia crocea* oriented parallel to the direction of ion flow. Fresh sea water was constantly circulated through the current chamber at an exchange rate of approximately 450 ml./minute. The stems were left in a continuous curent density of 80 δ ($\delta = \mu$amperes/mm.^{2}, cross-sectional area of sea water carrying current) for 24 hours, at the end of which time they were removed and placed in a chamber supplied with circulating sea water for two days. The polarity of regeneration was then observed.

Of the stems oriented with their distal ends closest to the anode, 18% regenerated hydranths at the original distal end only; 25% exhibited reversal of polarity, with a hydranth regenerated at the proximal end only; 48% of the stems regenerated hydranths at both ends, and 9% failed to regenerate at all.

Seventy-two per cent of the stems oriented with their proximal ends closest to the anode regenerated hydranths at the distal end only; 1.5% of the stems regenerated at the proximal end only (*i.e.*, reversals); 3.5% regenerated hydranths at both ends; 23% failed to regenerate at either end.

Barth (1934) has reported a 7.3% frequency of polarity reversals for stems oriented toward the anode, using a current density of 62.5 δ. The higher frequency of reversals obtained in our laboratory (25%) may have been the result of the higher δ value.

An attempt was made to determine the period of critical effectiveness of the current. Batches of stems were placed in the current for the first 12 hours after cutting, and others for the second 12 hours of regeneration. After removal from the current, the stems were allowed to regenerate for three days in a water chamber. No significant difference could be found among the data obtained from the two sets. This may have been due to the wide variability of rate of regeneration in the stems in a single batch, since the current thus acts on different stages in the regeneration process of different stems.

Experimental missed abortion and missed labor. M. A. Lloyd-Jacob and C. B. Kovacsics.

Csapo's theory that these functional disorders of the pregnant uterus are due to an imbalance of three major controlling parameters, namely estrogen, progesterone, and volume, was tested experimentally. Missed abortion and labor were produced predictably by the method of Csapo and Lloyd-Jacob, who maintained pregnancy with progesterone in rabbits whose ovarian and placental endocrine functions had been suspended by surgery. Delivery of the dead litter during progesterone treatment was determined by the dose administered and by the uterine volume (at the day of surgery). Increased progesterone balanced the facilitating effect of volume on the evacuation of the uterus.

The ovariectomized and placental dislocated animal not only failed to complete delivery *during* prolonged progesterone treatment but also *after* such treatment in case of a small uterine volume. However, the animal did deliver during progesterone treatment if it was inadequate (too low, or of short duration), or after the discontinuation of effective progesterone treatment if it was combined with or followed by estrogen therapy.

These observations, together with the findings of Takeda and Csapo on the electrophysiological properties of the myometrium under similar experimental conditions, suggest that the delivery of a dead litter is facilitated by the withdrawal of progesterone, by an increase in uterine volume, and by estrogen therapy. These joint studies predict that missed abortion and labor in women occurs when placental progesterone production continues after fetal death *in utero*. Since the uterine volume is static or is decreasing after fetal death and placental estrogen production greatly diminishes, little progesterone maintains pregnancy. The estrogen support of the myometrium being insufficient, uterine activity is inadequate when progesterone withdrawal permits the evacuation of the uterus. The experiments described suggest a rational therapy.

Oviparity versus ovoviviparity in the brine shrimp, Artemia. J. H. Lochhead.

The ovisac of *Artemia* is surrounded by unicellular shell-glands. Sometimes these glands discharge a copious brown secretion and the animal then lays hard-shelled eggs which may not hatch for several years. Sometimes the shell-gland secretion is scanty and forms only a thin membrane around the eggs. These then develop rapidly to naupliar larvae which emerge from the parent after hatching in the ovisac. Females release either eggs or larvae about once every four days. Which type of reproduction will occur presumably depends on external factors, but the literature dealing with such factors has been conflicting.

Experiments were set up in which animals of known age were subjected to different salinities, oxygen tensions, and foods. In some cases individual animals were observed daily over a period of several weeks. Where possible water was changed daily, to minimize differences which might develop between control and experimental cultures. None of the factors tested was found to exert an absolute control. Oviparity was most frequent in animals fed on algae or on liver. Ovoviviparity was most frequent in animals fed on yeast or kept at a high salinity (about six times that of sea water). However, both types of reproduction occurred under all the conditions tested and in animals both with and without hemoglobin in the blood. Individual animals sometimes changed, in either direction, from one type of reproduction to the other. An animal having once changed, tended to maintain the new type of reproduction in successive instars, although other animals under identical conditions continued reproducing by the other method. Age of the individuals did not appear to be a critical factor, and both types of reproduction were observed even in primiparous animals.

The lamprey eel blood clot. L. Lorand.

The blood plasma of higher vertebrates (*e.g.* human and bovine) contains a globulin, called fibrin stabilizing factor (FSF), whose apparent physiological function is to cement together the fibrin units of a blood clot (Physiol. Rev., 30, 742, 1954). A clot consisting of fibrin alone is mechanically weaker than the natural plasma clot and, unlike the latter, it can be dispersed by a number of solvents such as 30% urea or 1% monochloroacetic acid. This difference in solubilities between the two types of clot allows for easy recognition of the func-

tioning of FSF in blood. Similarly to fibrinogen, FSF appears to be activated by thrombin and full potency is preserved only in the presence of sulfhydryl compounds. Pathologically, there may be a relative (Proc. Soc. Exptl. Biol. Med., **89**, 45, 1955) or total (Thromb. et Diath. Haemorrhagica, **5**, 179, 1960) lack of FSF in plasma and, in spite of normal clotting and bleeding times, the latter results in grossly impaired wound healing. Since FSF seems to be as important a component of blood plasma as fibrinogen itself, it was of interest to ask where in the phylogenetic scale FSF makes its appearance. This paper reports that stored citrated plasma of *Petromyzon marinus*, when clotted by recalcification in the presence of 25 m*M* cysteine and homologous skin thromboplastin (Doolittle), developed clots which were insoluble in 30–40% urea or 1% monochloroacetic acid. Further, similarly to bovine fraction I (Armour), ethanolic fraction I (Cohn) of lamprey plasma is contaminated with FSF. Such lamprey fibrinogen preparations, after clotting with bovine thrombin in the presence of 25 m*M*. cysteine and calcium chloride, give rise to urea- and monochloroacetic acid-insoluble clots.

This work was aided by NIH grant H–2212. Thanks are due to Warner Love for providing the plasma and to Russell Doolittle for advice regarding lamprey blood.

The effect of cobalt, ribonucleic acid, and ribonuclease on the development of Arbacia punctulata. G. M. Mateyko.

It has been reported that cobalt may bring about persistent nucleoli during mitosis. Moreover, it has an inhibiting action on ribonuclease and reacts with sulfhydryl groups. Accordingly, the development of fertilized eggs of *Arbacia punctulata* was followed in sea water containing varying concentrations and combinations of cobalt chloride, ribonucleic acid, and ribonuclease, and the patterns of cleavage through the pluteus stage were studied.

At concentrations below 0.01 mg./ml. for $CoCl_2$, 5 μg./ml. for RNA, and 0.01 mg./ml. for RNA-ase, no morphologically detectable developmental retardation was evident, nor was there any acceleration. Above these levels growth was retarded. A specific response to cobalt, elicited in addition to the depression in the rate of cleavage, was the distortion of the prism and young pluteus to a form simulating a pollen grain. In 0.02 mg./ml. cobalt, deformed prisms occurred in 48 hours, and at 0.04 mg./ml., bloated prisms with swollen arms were characteristic. In 0.06 mg./ml. cobalt, the retardation and bloating to the *pollen grain* stage is characteristically reached by 36 hours. Internal organization is lost and isolated cells fill the sac-like arms and body in 96 hours. Although skeletal development proceeded in 0.04 mg./ml. cobalt and higher, the spicules were deformed and incomplete. Separation and dispersion of blastomeres was striking in cobalt (0.08 mg./ml.) especially after 8 hours, in that morulae hatched out of the fertilization membrane, and subsequently the blastomeres disaggregated.

Neither RNA-ase nor RNA in physiological amounts, nor in higher concentrations (0.1 mg./ml. and 50 μg./ml., respectively), minimized or reversed the cobalt effect. In fact, each, especially ribonuclease, depressed still further the rate of cleavage.

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Digestion of the Fundulus chorion by the hatching enzyme. Roger Milkman, Barry F. Wood and Delbert E. Philpott.

The *Fundulus heteroclitus* egg chorion has previously been shown to consist of two layers. The thick inner layer is tough and digestible by the hatching enzyme, while the thin outer layer is weak but resistant to the enzyme.

The changes in the chorion during hatching have been studied. Electron micrographs of cross-sections of intact chorion show a uniform membrane about 6–8 microns thick, within which are suggestions of 7–8 roughly defined layers. There is a thin outer layer present (⅙ to ⅓ micron thick), which remains undigested by the hatching enzyme. Three progressive changes are apparent in cross-sections of chorions removed at successive times during hatching. First, the inner edge becomes rough and fibrous. Second, the digestible layer's density is reduced. Third, holes appear in this layer. In some places the layer is completely digested away. The roughened edge is seen after two minutes' exposure to the enzyme; the holes

begin to appear after four minutes; after about seven minutes, the process is complete. The weak, indigestible outer layer is all that remains, and the fish breaks out of it with a lash of its tail.

Experiments have shown that enzyme from recently hatched eggs will attack the chorions of intact eggs wherever the protective layer has been filed away. If this filing is done in a ring around the egg, the chorion comes apart into two hemispheres and can easily be removed, leaving the embryo alive and intact.

This work supported by a grant from NIH (Milkman) and an NSF Cooperative Summer Fellowship (Wood).

Oxygen uptake in short pieces of Tubularia stems. JAMES A. MILLER, JR., LORALEE L. PHILPOTT AND C. LLOYD CLAFF.

Reconstitution in *Tubularia* is initiated by the presence of a cut surface. However, if two cut surfaces are close to each other (*i.e.*, in stems 1½ mm. or less in length), reconstitution may fail completely and if it does take place proceeds slowly and produces very small hydranths. These results suggest that influences which emanate from the cut surfaces inhibit reconstitution. Since the perisarc is relatively impermeable and metabolic exchange is chiefly through the cut ends, the influence of the cut ends on oxygen uptake of short pieces was determined. Measurements were made with Cartesian divers on stems at 18° C. which had been treated with a solution of streptomycin in filtered sea water. Using the oxygen uptake of 3-mm. stems as a standard of reference it was found that doubling the surface area (two 1½-mm. pieces) or tripling the surface area (three 1-mm. pieces) without altering the mass of tissue failed to produce comparable increases in oxygen uptake. Comparisons between the oxygen uptake of one 1-mm. piece and three 1-mm. pieces in 2 μl. showed that the single 1-mm. piece consumed over one-half that of three 1-mm. pieces in the same volume of sea water. Since in all these experiments CO_2 was absorbed by the NaOH in the diver it is concluded that some metabolic depressant other than CO_2 is liberated from the cut surface. Although it is tempting to correlate the observed metabolic depression and inhibition of reconstitution in short pieces, as yet a cause-effect relationship between these two phenomena remains to be demonstrated.

Malic dehydrogenase isozymes of Asterias forbesi and Arbacia punctulata ovary. RICHARD O. MOORE AND CLAUDE A. VILLEE.

The relative reaction rates of certain dehydrogenases with several pyridine nucleotide analogues have been implicated in the discernment of evolutionary relationships. In 1960 Villee reported that the malic dehydrogenases of comparable tissues, *e.g.* ovary, of related marine animals were more similar in their DPN-analogue activities than those of different tissues, *e.g.* ovary, gut, muscle, from the same species. To determine whether a single enzyme was present in each tissue with varying properties among tissues, or rather that each tissue contained several malic dehydrogenases with different pyridine nucleotide specificities so that the observed species and tissue patterns could be accounted for on the basis of varying concentrations of several malic dehydrogenases, an attempt has been made to demonstrate the presence (or absence) of multiple molecular forms of this enzyme in the ovary of *Asterias* and *Arbacia*. The tissue was homogenized, centrifuged and the proteins of the supernatant fluid fractionally precipitated with $(NH_4)_2SO_4$. The fraction with highest malic dehydrogenase activity was dialyzed and placed on a DEAE-cellulose column. The malic dehydrogenase did not adsorb to the DEAE-cellulose. The DEAE-cellulose elulate was placed on a carboxymethyl-cellulose column and 1.3- or 2.6-ml. fractions collected over a gradient of 100 ml. tris-succinate-mg-acetate buffer, pH 5.4, and 150 ml. 1.0 *M* KH_2PO_4. Malic dehydrogenase activity with DPN, 3-acetylpyridine-DPN (APDPN), deamino-DPN, thionicotinamide-DPN, and 3-pyridine-aldehyde-DPN was followed spectrophotometrically at critical points throughout the procedure and on individual fractions eluted from the carboxymethyl-cellulose column. Supernatant fractions of the original homogenates were also subjected to zone electrophoresis on starch. The data from these experiments indicate that three molecularly distinct malic dehydrogenases exist in ovaries of *Asterias* and *Arbacia*, each with a different DPN-analogue pattern. The further

observation has been made that the enzyme with major APDPN activity decreases during the breeding season from early June to August, and that this same enzyme is more labile to laboratory manipulation (*e.g.* frozen storage).

Studies on the isolated islet tissue of the toadfish (Opsanus tau): glucose-6-phosphate and 6-phosphogluconate dehydrogenases. JOSEPH F. MORAN, JR.

As part of a systematic study of the isolated islet of the toadfish, and because of the known effect of glucose on the release of insulin from the beta cell, we have been investigating the enzymes involved in the metabolism of glucose by islet tissue. In the present study the glucose-6-phosphate (G-6-PD) and 6-phosphogluconate (6-PGD) dehydrogenase contents of toadfish tissues were determined by measuring the rate of reduction of triphosphopyridine nucleotide (TPN) at 340 mμ in a Beckman spectrophotometer under standardized conditions in the presence of cyanide and magnesium. Weighed samples of islet (1–4 mg.) and other tissues were homogenized in glycylglycine buffer and aliquots (5–20 μl) were added to the assay system; the final volume was 300 μl. The enzyme activity was expressed as the change in the optical density per minute per mg. wet weight of tissue added.

The average G-6-PD content of the islet tissue of 10 animals was 0.023; this value is 33–50% of that found in liver, ovary, testis, gill and brain, and 8–15% of that found in the heart and kidney. The 6-PGD content of islet (.023) was the same as that found in the other tissues, except for kidney which had twice the activity. Skeletal muscle had little, if any, of either of these enzymes. The ratio of the G-6-PD activity to that of 6-PGD was lower in islet (1.00) than in any other tissue studied. In liver this ratio was 1.8, in brain 2.1, ovary 2.3, gill 2.5, kidney 2.9, testis 4.2, and heart 4.4.

The relatively low G-6-PD activity observed in islet tissue is not due to the presence of an inhibitor, since the addition of islet tissue homogenate to kidney did not inhibit the activity of the latter.

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Effects of cobaltous chloride on the eggs and embryos of Ilyanassa obsoleta. JOHN B. MORRILL.

In the course of a study of the effects of the animalizing agent, cobalt, on protein differentiation in mollusc eggs, *Ilyanassa* eggs and embryos were treated with sea water solutions of cobaltous chloride (0.001–0.05 *M*) for 2–24 hours at 20–23° C. Treatment was begun at the trefoil stage and at later stages up to and including 4-day embryos with foot and shell gland anlagen. Eggs were either killed during the treatment period or developed into one of several types of abnormal larvae or into normal veligers. The main types of abnormalities included: (1) irregularly lobed, ciliated bodies filled with a mass of yolk cells; (2) veligers with a reduced body, a swollen eyeless velum filled with a mass of yolk, and rudimentary foot, shell gland and cerebral ganglia; (3) veligers with deformed but not swollen velum, eye malformations, and rudimentary cerebral ganglia, shell and mantle fold; (4) veligers with reduced velum and eye malformations. Most of the eye malformations consisted of the absence of either the left eye (100 cases), the right eye (76 cases) or both eyes (101 cases). Occasionally veligers with supernumerary eyes occurred (3 cases). The absence of an eye from one side was associated with an abnormal but not necessarily reduced cerebral ganglion. The greatest number of veligers with eye malformations developed from eggs treated at the beginning of the trefoil stage with 0.0015 and 0.002 *M* cobaltous chloride for 24 hours.

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Effect of lithium chloride on the number of eyes in Ilyanassa obsoleta veligers. JOHN B. MORRILL.

Ilyanassa eggs and embryos were treated with sea water solutions of lithium chloride (0.01–0.05 *M*) for 2–24 hours at 20–23° C. Various aspecific malformations resulted, including

shell-less veligers with reduced velar lobes and everted stomodaea. But the most striking malformations were seen in relatively normal veligers in which one of the paired eyes was absent or in which one or more supernumerary eyes were present on the right or left side or on both sides of the velum. These eye malformations were obtained frequently when eggs in the trefoil stage were treated for 24 hours with 0.02–0.03 *M* lithium chloride. Of ninety veligers with eye malformations 44% had two eyes on the right side of the velum and one on the left side; 15% had two eyes on the left side and one on the right; 16% had two eyes on one side and none on the other; and 25% had four or five eyes distributed in various combinations between the two sides. Supernumerary eyes were twice as frequent on the right side as on the left side of the veliger.

Microscopical examination of whole mounts of normal veligers revealed that the eyes with their mass of pigment granules and hyaline vesicles were closely associated with the lateral margins of the cerebral ganglia. Supernumerary eyes were similar structurally to normal eyes and were either next to the regular eyes in the region of the cerebral ganglia or else located in the region of the cerebral commissure. Regardless of their position, supernumerary eyes were always next to cerebral nerve cells. When a regular eye was absent from one side, the velar lobe and cerebral ganglion on that side were reduced.

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Uptake and transport of L-leucine by intestinal segments of Ameiurus nebulosus and Stenotomus versicolor. X. J. Musacchia, S. S. Fisher and D. Westhoff.

A preliminary investigation of absorption and transport of amino acids by segments of fish intestine was made. The *in vitro* everted intestinal sacs, previously described, were incubated in teleost Ringer's containing L-leucine (2.5, 5 and 10 millimolar) at 22° to 25° C. In catfish (50 animals), the middle intestinal segments showed the greatest level of uptake from the mucosal fluid and increased concentration in the serosal fluid. This held consistently for the three concentrations used, *i.e.*, 2.5, 5 and 10 millimolar L-leucine. In scup (33 animals), the uptake appeared greatest in the upper intestinal segment only with 5 and 10 millimolar L-leucine. In terms of transport the greatest increases in concentrations in the serosal compartment of both upper and lower intestine (21 animals) were obtained with the 2.5 millimolar L-leucine.

When the incubation media for catfish (12 animals) intestine were made anaerobic by gassing with nitrogen, uptake of 5 millimolar L-leucine was greatly inhibited only in the middle and lower segments. Nitrogen gassing also inhibits uptake of 5 millimolar L-leucine in both upper and lower segments of scup (9 animals) intestine.

When the intestine of catfish (6 animals) was treated with 0.1 *M* ethionine, as previously described, there was an enhanced uptake of the 5 millimolar L-leucine in all three intestinal areas. Histological studies of these segments are underway.

Each experimental series was paralleled with "blank" runs (6 to 12 animals), *i.e.*, incubation in Ringer's minus the test compound. Final values are adjusted by taking into consideration the varying amounts (non-detectable to trace amounts) of endogenously produced amino acid or sugar.

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A comparative study of D-glucose uptake and transport by intestinal segments of Stenotomus versicolor and Ameiurus nebulosus. X. J. Musacchia, D. Westhoff and S. S. Fisher.

Previous studies showed that D-glucose was absorbed and actively transported across intestinal wall of catfish, *A. nebulosus,* whereas in scup, *S. versicolor,* intestinal transport did not occur. Re-investigation with improved *in vitro* methods showed that D-glucose was taken up and actively transported by inverted intestinal sacs made from upper and lower sections of scup (26 animals) mid-gut. Segments from catfish (15 animals) mid-gut were also studied:

upper (adjacent the pylorus), middle, and lower (adjacent the rectum). Thus, characteristics of absorption and transport of various intestinal areas were evaluated. Evidence for active transport was taken as movement of sugar from the mucosal fluid to the serosal fluid against a concentration gradient when the initial concentrations on both sides are equal. Serosal/mucosal ratios of D-glucose were calculated: micromoles/gm. dry wt. gained in the serosal fluid divided by micromoles/gm. dry wt. lost from the mucosal fluid. In catfish preparations with starting concentrations of 5 mg.% and 10 mg.%, S/M ratios were highest for upper intestinal segments. Measures of uptake (absorption) only, showed that middle intestinal areas were most efficient.

Because catfish are subject to variation in environmental temperatures a series of 13 specimens were maintained at low temperature (10° C. at least one week) and intestinal segments were incubated with starting concentrations of 5 mg.% D-glucose at 10° C. Absorption in the upper segments was reduced about 70% and at least 95% in middle and lower segments.

In scup, using starting concentrations of 5 mg.% (found optimal for uptake and transport) and 10 mg.% D-glucose, S/M ratios were lowest in upper segments.

When incubation media were gassed with nitrogen, active transport of D-glucose was inhibited in intestinal segments of scup and catfish.

Preliminary studies of effects of carcinogenic agents were made by flushing the entire intestine of catfish with 0.1 *M* ethionine. Five days later the three intestinal areas from 15 catfish showed enhanced uptake and transport of D-glucose.

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Environmental temperature change and blood sugar change in the toadfish, Opsanus tau. Paul Foley Nace and Joseph E. Schuh, S.J.

Following the observation of seasonal change in blood sugar levels of toadfish, groups of experimental animals were kept in the laboratory at temperatures maintained above and below the seasonal harbor temperatures. A large group of animals was kept at 15° C. while the sea water supply rose to 21.8° C., and smaller groups were kept within the ranges of 0–3° and 4–5° C. In winter, aquarium temperatures were raised gradually from 2° to 22° C. Blood samples were drawn from the gill arches and analyzed by modified Folin and Glucostat procedures.

In early summer, fish kept at low temperatures showed the blood sugar levels appropriate to the season of corresponding low environmental temperature. In mid and late summer, increasing numbers of fish failed to show hyperglycemia at reduced temperature. Variation in osmotic strength and photoperiod did not conclusively restore the hyperglycemic response to reduced temperature.

In May, fish kept at 5° C. attained blood sugar levels of 440 and 467 mg.% (Glucostat) without alloxan. Mean values were 171 mg.% (F) and 211 mg.% (G) at 0–3° in May, and 108 mg.% (F) and 138 mg.% (G) at 4–5° C. Controls at 10–15° averaged 42 (F) and 16 (G) in 1960, and 61 (F) and 38 (G) in 1961. June means were comparable, but July means fell to 97 mg.% for both Folin and Glucostat at 0–3° C. Through July and August, some fish maintained levels as high as 350 mg.% at low temperature, but increasing numbers showed levels equivalent to the warm water controls. In winter, fish taken from Eel Pond at –1.7° C. showed means of 152 (G) and 123 (F) after one day at 16° C. and 104 (G) and 67 (F) after two days at 21° C.

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Mechanical and electrical properties of some invertebrate visceral muscles. T. Nagai, R. A. Nystrom and C. L. Prosser.

Non-striated muscles of invertebrates in which conduction has previously been examined conduct mainly by intrinsic nerves, rarely by stretch, whereas vertebrate visceral muscles

conduct electrically from fiber to fiber. Conduction was studied in the intestines of *Venus, Spisula, Busycon, Loligo, Thyone* and *Libinia*. Average velocities in moist air were: *Venus* 1.1 cm./sec., *Spisula* 2.3 cm./sec., *Busycon* 9 cm./sec., *Thyone* 5.9 cm./sec., *Libinia* 33 cm./sec. Conduction occurred when mechanical pull was prevented in *Thyone, Venus, Busycon* and *Spisula*. Intestinal conduction was blocked by procaine (or tetracaine) in *Libinia,* not in the molluscs or holothurian. In *Spisula,* and to a less extent in *Venus,* the intestines showed both phasic and tonic contractions, both spontaneously and in response to shocks. In *Spisula* the electrical threshold for the tonic contraction was lower than for the phasic one and action potentials were associated with the phasic, not with the tonic contraction. Phasic contraction time for *Venus* intestine was 2 to 3 seconds, time for half-relaxation 15 to 40 seconds; tonic contractions persisted for many minutes. Contractions of the *Libinia* intestine were fast (0.2- to 0.4-second contraction time) and spontaneous contractions were often tetanic with an electrical spike corresponding to each rhythmic contraction. At times the electrical record consisted of a volley of fast spikes. Acetylcholine was relatively ineffective on the crab intestine but 5-hydroxy-tryptamine elicited trains of large synchronized spikes. Squid intestine and esophagus showed continuous asynchronous spike-like electrical activity. Conduction in the *Libinia* intestine appears to be by nerves and the spontaneous contractions neurogenic, while conduction in the clams, snail and holothurian appears to be from muscle fiber to fiber much as in vertebrate smooth muscle.

The glass micropipette as a mechano-transducer. M. Ogato.

If current is applied through a conventional glass micropipette, filled with 3 *M* KCl, and its tip is in contact with the surface of a rubber or polyethylene film, or a muscle (suspended in frog Ringer solution), the resistance of the electrode changes in approximate proportion to the extent of movement at the contact. This change of resistance was measured by means of a Wheatstone bridge circuit. When the polarity of the pipette is reversed, the change of resistance displayed on the CRO also reverses its sign. The exact nature of the resistance change of the pipette induced by the movement of the material in contact with its tip is under investigation.

The promptness of this resistance change provides us with a new method for the study of the relationship between membrane activity and the early phase of the mechanical response. Inserting an intracellular electrode into the frog sartorius at a point close to the mechano-transducer pipette, and recording the action potential together with the mechanical response after direct stimulation, the latency period (the time lapse between the peak of the spike potential and the onset of mechanical activity) is accurately determined. Such measurements show that the latency period of the frog sartorius at 20° C. is 1.9 msec.

Supported by the Health Research Council of the City of New York.

Pigmentation in Cordylophora lacustris. Edward E. Palincsar and George Szabó.

Cordylophora in nature is dark brown. When cultured by Fulton's technique, the pigmentation is lost and the morphogenetic pattern of the colony changes. The nature of this brown pigment was investigated by the following tests. The pigment was insoluble in acetone, chloroform, ether, xylene, ethanol, methanol and water. It bleached within 48 hours in 10% hydrogen peroxide. There was no reversible redox color change, nor a violet color in concentrated sulphuric acid, nor a change in ammonia gas. The pigment was soluble in 0.5 N sodium hydroxide, concentrated ferric chloride and slightly soluble in formic acid and in 1 N HCl. A positive test for tyrosinase (darkening of the perisarc) was obtained with dihydroxyphenylalanine at pH 7.4 and pH 6.3. Boiled controls were negative. A weak positive reaction occurred with tyrosine incubation at pH 6.8. Homogenates of *Cordylophora* tanned gelatin with catechol and a brown pigment appeared. This homogenate also considerably speeded up the rate of melanization of DOPA (pH 7.4). Non-pigmented *Cordylophora* in Fulton's medium with the addition of tyrosine (2.5×10^{-4} *M* to 10^{-3} *M*) developed brown pigmentation *in vivo* in the perisarc after 3–5 days. No brown pigmentation appeared during

parallel treatment with phenylalanine (5×10^{-4} *M* to 10^{-3} *M*), but in thienylalanine (10^{-3} *M*) the perisarc developed a dark brown pigmentation and the morphogenetic appearance of the colony changed, approaching the pattern found in nature. The data suggest that the pigment is not a carotenoid, ommochrome or flavone. It appears that the pigment is melanin or sclerotin involved in the tanning of the perisarc. Failure of this sclerotinization results in the laboratory pattern of colonies. The possibility of natural inhibitors from the food source, *Artemia,* is being investigated. A more detailed analysis of the pigment is now in progress.

Fixing agents and the echinochrome granules of Arbacia punctulata. ARTHUR K. PARPART, EARL VAN NORMAN AND JOHN C. BERNHARDT.

Arbacia eggs were observed during the action of various fixing agents by means of the television microscope at screen magnification of 5000×. Alterations in the yolk granules, echinochrome granules, cortical granules, and nucleus were observed when the egg, fixed in view on a wedge slide, had its environment rapidly changed from sea water to a fixative solution.

Osmic acid solutions in 0.5, 1 and 2% concentrations, prepared in sea water or 0.5 *M* NaCl, all stopped protoplasmic streaming rapidly (15 to 30 seconds). Until egg opacity increased (2 to 4 minutes), the yolk, cytoplasmic, and cortical granules and the nucleus appeared well fixed. However, the echinochrome granules all exploded violently after one to three minutes.

Bouin's, Susa's, Zenker's, Zenker-formol, and Dalton's solutions, as well as 0.03, 0.3 and 3% $KMnO_4$ in sea water, caused major disruptions and explosions of cytoplasmic and cortical granules. The echinochrome granules all exploded. The egg volume increased.

Five per cent formalin in sea water and 1% $HgCl_2$ in sea water each gave good preservation of cytoplasmic and cortical granules, but after 10 to 15 minutes the echinochrome granules exploded. One per cent osmic acid in sea water added any time up to 10 minutes after this solution also caused explosion of echinochrome granules.

Uranyl nitrate (4%) dissolved in sea water stopped cytoplasmic streaming rapidly, and cytoplasmic, cortical, and echinochrome granules and nucleus were in excellent condition. Subsequent fixation by any method produced explosions of the echinochrome granules.

During the first few seconds after addition of 10% acrolein in sea water, the fixation of the egg was excellent. After 30 to 60 seconds, cytoplasmic and echinochrome granules began to explode, and at two to four minutes the cytoplasm appeared granular, with surface blebbing.

A new method of making photographic films for electron microscopic autoradiography. DELBERT E. PHILPOTT AND CLAIR E. FOLSOME.

Photosensitive films were produced by a combination of shadow-casting and vapor application. The thickness of the resulting film can be controlled by calculating the amount of silver to be used for shadowing. This amount of silver is shadow-cast directly down at 90° on a collodion filmed electron microscope grid at an estimated thickness of 50 Å. The resulting silver film is not continuous and when viewed under the electron microscope is seen to be made of very fine, individual crystalloids of silver, averaging several hundred Å. This silver is then converted to the halide, in the dark, by blowing iodine or bromine vapors over the silver surface. The resulting films are extremely thin, compared to ordinary photographic films, and since sections or other material containing radioactive tagging can be placed on the grid before this process takes place, the photosensitive film can be formed in contact with the surface of the specimen.

Electron diffraction has been used to follow the production of the silver films, their conversion to the halides, and reconversion to silver upon exposure, as well as electron microscopy of the resulting products.

Higher resolution should be possible for two reasons. The silver particles, and thus the resulting halide, are about ten times as finely dispersed as ordinary photographic emulsions and the thinness of the resulting film reduces the effect of scatter as the radioactive decay passes out through the film. Preliminary experiments with tritium-labeled bacteria indicate that this method can be used for autoradiography.

Effects of distal and proximal hydranth extracts and stem extracts from adult Tubularia on the regeneration of isolated proximal ridge regions. JOSEPH A. POWERS.

Twenty pieces from the respective adult *Tubularia* regions were homogenized in an ice bath. These homogenates were then brought to 10-ml. volumes with filtered, pasteurized sea water which contained 125 mg. of chloromycetin per liter. Centrifugation at 3400 r.p.m. for about six minutes followed. The respective supernatants were decanted into stender dishes and refrigerated for two days. The control was 10 ml. of filtered, pasteurized, chloromycetinated sea water given similar treatment. The test pieces were isolated proximal ridge regions of regenerating *Tubularia,* approximately stage 6 (Berrill and Davidson, 1948). The extracts and control were removed from the refrigerator and placed in an ice bath. Ten test pieces were then placed in each of the four solutions, *i.e.,* distal, proximal, stem and control supernatants. These stender dishes were then placed in a water bath (13° C.–16° C.). Observations were made 24–30 hours after treatment. Eighty-three per cent of the 30 pieces treated with the supernatants of distal homogenates lacked distal parts, though in most cases the gonophores and proximal tentacles were quite normal. Treatment with the proximal solutions caused 97% of 30 test pieces to lose their structure, with the coenosarcs becoming completely opaque. The latter was also true of 63% of the 30 assay pieces treated with stem homogenate supernatants. Only 17% of the control pieces failed to regenerate all of their parts normally.

Contractile responses of marine and fresh-water sponges. C. L. PROSSER AND T. NAGAI.

Oscula and "veins" of sponges contract in response to mechanical, not to electrical, stimulation. Contracting myocytes are scattered and are less than 5 μ in diameter. Contractions of *Microciona* showed a latency of 0.3 to 1.0 second, contraction time of 4 to 5 seconds, time for half-relaxation 15 to 20 seconds. Contractions of *Spongilla* were slower. Responses recorded with electrolytically tapered silver leads were of similar time-course to mechanical recordings with a transducer, whether recorded in sea water (*Microciona*) or in pond water (*Spongilla*). All contractions were local and non-propagated, but they showed summation up to a maximum response at 3 to 4 taps within one second. When calcium was omitted from artificial sea water bathing *Microciona,* the mechanical responses decreased and sometimes disappeared after 10 to 15 minutes; recovery was complete when calcium was restored. Increases in potassium in the medium to 10 times that in normal sea water for *Microciona* and to 12 m*M* in pond water for *Spongilla* did not impair the responses to mechanical stimulation. Such concentrations of potassium must have depolarized the cells. It is concluded that the contractile responses of sponge myocytes are non-propagated, that they may not involve membrane action potentials but that they involve a calcium-releasing mechanism.

The use of permanganate as a fixative for electron microscopy of invertebrate eggs. LIONEL I. REBHUN.

A variety of procedures using permanganate as a fixative for invertebrate eggs was tried. Various concentrations from .5% to 5% were used, temperatures were varied from 0° C. to room temperature, formalin or osmium in sea water were used as postfixatives, sodium or potassium permanganate was used, the permanganate was dissolved in distilled water or sea water, and finally, the pH was either uncontrolled or maintained at about pH 7.5, using either veronal-acetate or dichromate as buffers. The final mixture used was 5% potassium permanganate plus 1% potassium dichromate at about pH 7.5. Little variation was seen with different procedures as long as the concentration of permanganate was at 5%. Fixation was for ½ to 1 hour at room temperature, dehydration was with acetone or ethylene glycol and Araldite 502 or Epon 812 were used as embedding media.

The final procedure yields very uniform results, giving excellent preservation of endoplasmic reticulum, Golgi bodies, mitochondria, etc., that is, all membrane systems within the egg. The surface membrane is not as well preserved and occasionally small vesicles (blebs)

can be seen arising from *Spisula solidissima* vitelline membranes, which vesicles are not seen by osmium fixation or by freeze-substitution. Surfaces of fertilized *Arbacia* eggs are occasionally disrupted by the fixative. However, as a fixative which is consistent and easy to use, yielding excellent preservation of internal membrane systems, permanganate is superb.

Polarized control of regeneration in Tubularia by charged particles. S. Meryl Rose.

When a distal graft of a regenerating *Tubularia* primordium is made to the distal end of a whole primordium, one is combining two differentiated, but not fully developed, distal parts and one proximal part, DDP. If this is done during the labile proximally striate stage, reorganization occurs. The first D retains its organization and continues to develop as D. The second D loses all visible signs of differentiation and becomes a P. Part of the original P after considerable loss of structure may become the base of the hydranth and the upper part of the stem. The result is DPS. The work has now been repeated but with the addition of a direct current of 25 ma and 12–30 volts across a plastic tray containing 2% agar in sea water, with a cross-sectional area of 280 mm.^{2} from distal to proximal or in the reverse direction. The current is applied for 4–5 hours and then the combinants are transferred to standing sea water at 15°–16° C. In all cases when the primordia were in the proper stage and faced the anode, (+) DDP, the result was well organized single hydranths, DPS. There were 19 cases. When the arrangement was (—) DDP, the result was always DDP. There were 18 cases. In this arrangement the distal part at the end did not control the distal part behind it and a hydranth with two sets of distal tentacles and two mouths arose. It is suggested that differentiation-controlling substances in *Tubularia* are positively charged and move from distal to proximal. When an electric current is applied in the direction that would prevent the motion of these particles along the already polarized pathway, more distal parts can no longer suppress differentiated and partially developed parts lying behind them.

DNA synthesis and cell division in elasmobranch lenses maintained in vitro. Howard Rothstein.

Investigations of lens epithelium employing autoradiographic procedures have indicated that it is possible to maintain both DNA synthesis and mitosis *in vitro*. If the isolated lens of the dogfish, *Mustelus canis*, is perfused at 1.25 ml./hr. with tissue culture medium (M-199, 18° C.) which has been modified by adding 2.16 gms. urea/100 ml. and 0.79 gms. sodium chloride/100 ml., the epithelial cells incorporate thymidine-H^3 after having been *in vitro* for 48 hours. In such lenses, which have been completely isolated from the surrounding tissues, mitosis ceases almost completely. Mitotic activity can, however, be maintained to a certain degree if the anterior portion of the eye is left attached to the lens during incubation. There is no apparent difference between lenses incubated in M-199 and those in elasmobranch Ringer. Parallel findings have been obtained with the skate, *Raja erinacea*. Isolated lenses incubated *in vitro* for 24 hours at 18° C. in either elasmobranch Ringer or modified M-199 manifest active incorporation of thymidine but show virtually no mitosis. There is, therefore, an apparent dissociation of cell division from DNA synthesis under these conditions. However, as in the dogfish, *in vitro* lenses which remain attached to the anterior portion of the eye continue to show mitotic activity for a long period (in this case, through 48 hours). Preliminary experiments indicate that upon incubation of the lens within the whole eye, in which a corneal window has been dissected, the lens epithelium loses its mitotic activity within 24 hours. Thus, there is an indication that the loss of mitotic activity in the isolated lens is not due to the effects of physical manipulation during the process of isolation, but rather to the withdrawal of factors which stimulate, or aid in the maintenance of normal mitotic activity. In the dogfish, cells undergoing mitosis and DNA synthesis are more numerous near the equator, whereas the skate does not show such localization.

Cytoplasmic radiation damage in Arbacia punctulata. Ronald C. Rustad.

Numerous examples of cytoplasmic radiation damage are known. In general, structures associated with the nucleus are more sensitive than purely cytoplasmic systems. Two studies

on anucleate *Arbacia* eggs revealed no radiation-induced mitotic delay following insemination with normal sperm. However, the anucleate cells were prepared by centrifugation, which stratifies various cytoplasmic components.

In the present study anucleate half-eggs were prepared by cutting the cells with a fine glass needle. Nucleate half-eggs divided at the same time as uncut eggs. The anucleate controls cleaved between five and ten minutes later. Irradiation of anucleate cells with x-ray doses between 17 and 60 Kr. produced significant mitotic delay. At high doses most of the embryos lysed before hatching.

These data show that the cytoplasm of *Arbacia* eggs is x-ray-sensitive, although the site of damage is unknown.

Seasonal changes in blood sugar and related phenomena in the toadfish, Opsanus tau. Joseph E. Schuh, S. J. and Paul Foley Nace.

Through 18 months, some 800 toadfish were assayed for normal blood sugar (Folin and Glucostat), hyperglycemic and lethal doses of alloxan and magnitude of hyperglycemic response to alloxan. In this period, sea water temperatures varied from —1.7° C. (January) to 21.8° C. (August). Blood samples were drawn from heart and gill arches. Intracardiac injection of 10% aqueous alloxan ranged from 200 to 850 mg./kg. Simultaneous Folin and Glucostat determinations were run on each sample. The difference between the two values (expressed as mg.% glucose reducing equivalents) measured reducing substances other than glucose. Principal islet, pituitary, liver and kidney of each fish were fixed for histological study and tritiated thymidine autoradiography was used to examine variations in mitotic rates of these tissues and of skin and intestine.

As water temperatures declined in autumn, both Folin and Glucostat values rose, from August mean values of 30 mg.% (Folin) and 20 mg.% (Glucostat) to February levels of 118 mg.% (F) and 140 mg.% (G). From November through April, many fish assayed above 200 mg.% by both procedures. Only in this period have individual Glucostat values exceeded Folin values. In winter, alloxan dosages of 300 mg./kg. were hyperglycemic, while 700 mg./kg. was necessary in early summer. Doses of 850 mg./kg., which were lethal without hyperglycemia in late summer, produced no deaths in winter. Post-alloxan levels as high as 440 mg.% (G) and 417 mg.% (F), found in winter, represented increments two to three times the greatest increments common in summer.

This work was conducted with the assistance of Costante Ceccarini and Henry Laufenberg. It was supported by grant A–1129, NIAMD, NIH, USPHS, grant T–93 of the National Research Council (Ottawa), the Brown-Hazen Fund of The Research Corportion and by AEC–MBL contract.

Heat-induced local furrow rupture in Arbacia eggs. Allan Scott.

A heat treatment of one minute at 39.0 C., applied at about 40 minutes after fertilization, causes "spontaneous" breaks in the furrow cortex during cytokinesis. An analysis of the behavior of the echinochrome granules in the cortex of heated eggs shows that the sudden local out-thrust of the furrow results from the yielding of a sector of the furrow cortex. When the furrow gel parts, the granules in that area of the cortex move rapidly to the edges of the area and the endoplasm flows out to fill that sector of the furrow. The granules involved move, in one or two seconds and collect at the two boundaries of the ruptured furrow band. There is no movement of granules in directions other than around the equator. Granule patterns in the adjacent subfurrow cortex remain unaffected. The normal furrow cortex is viewed as being under a circumferential tension, perhaps due to the contraction of fibrils oriented around the equator. The rupture may involve an area only a few micra in diameter or it may involve a third of the furrow cortex. It is not a simple solation for this would not explain the directed movement of the granules. The rapid granule movement is considered to result from the parting of fibrils in one region of the furrow band and a sudden contraction of the gel on either side of the break. The repaired area is immediately integrated into the furrowing. One burst may be followed by another in the same or in another sector of the furrow, yet cells often divide successfully after several ruptures. The evidence that the

furrow is under contractile tension seems indubitable and the nearly instantaneous formation of new furrow cortex seems especially noteworthy.

Surface changes during cytokinesis in flattened eggs. ALLAN SCOTT.

It is possible to follow the shrinkage of the furrow cortex and the expansion of the non-furrow cortex during the early cleavages of the *Arbacia punctulata* egg by photographing the changing patterns of echinochrome granules. *Arbacia* eggs cleave well although flattened if they are in 70% sea water. The geometry of the flattened egg surface in relation to the mitotic apparatus is greatly altered from that obtaining in the spherical egg, since the two flat surfaces are brought close to the mitotic apparatus whose long axis is forced into the plane of the slide.

We have shown previously that the ring of furrow cortex around the equator of the spherical egg, shrinks two-dimensionally at the onset of cytokinesis. The first cortical areas to shrink during mitosis in the disc-shaped egg lie on either side of the spindle. Two triangular areas of concentrating granules are joined at their apices across the spindle equator. The area beyond the base of each triangle and lying in the presumptive furrow path, shows no sign of shrinkage at first. Cortical shrinkage proceeds outwards from the triangular areas to the edge of the disc, after which a deepening furrow cuts in from the disc margin. Granule-pattern-expansion occurs in the areas of flattened cortex covering the asters but there is no obvious expansion of the cortex that would represent the polar cortex in unflattened cells. Flattened polyspermic eggs always show an expansion of cortex in the areas covering the several asters and a shrinkage of the cortex between them. These observations are consistent with Wolpert's concept of astral relaxation and with the view that a furrow inducing substance arises from the mitotic apparatus.

Reaggregation of regions of Amaroecium zooids following their independent culture in vitro. SISTER FLORENCE MARIE SCOTT AND PRISCILLA CROWE.

This experiment was designed to test the stability of tunicate tissues in their ability to recognize, in synthetic combinations, tissues of the same histogenetic character. Young adult zooids of *Amaroecium constellatum* were divided into two regions, branchial basket and abdomen. Both regions were washed in several baths of sterile sea water (and penicillin) and then placed separately in a culture medium consisting of filtered, autoclaved sea water, dextrose, penicillin and streptomycin, with phenol red as an indicator. They were cultured for periods of twelve and twenty-four hours. They were then combined in either of two patterns: one branchial basket and one abdomen; or two branchial baskets and one abdomen, the combination of parts being minced together in the tunic of an older *Amaroecium*. The minced mass was held in place in the host test by a plug of tunic of the day-old experimental animals.

The parts that were cultured for twelve hours reaggregated and formed a single zooid in double combinations, or Siamese twins in triple combinations, in all cases where the injured surfaces of contributing parts were brought into close contact. The series of unions, whether in double or triple combination, from the twenty-four cultures reaggregated in a very low percentage of cases. Triple aggregates of such fragments minced together immediately upon dissection form Siamese twins with persisting regularity. Conclusive evidence depends upon the analysis of serial sections but it seems that, by maintaining fragments of tunicates independently *in vitro,* something is lost either from the cells themselves or from the intercellular substance that enables tissues to recognize, grow toward and fuse with tissues that share their histogenetic character.

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Osmotic regulation in the estuarine isopod Cyathura polita. EARL SEGAL AND KAY MCDONALD.

Cyathura polita were collected at low tide in the Pocasset River marsh on Cape Cod, Mass. During a daily tidal cycle the salinity of the waters flowing over the animals may vary from $\triangle$ 0.02 to $\triangle$ 0.95 (approximately 1–60% sea water based on 100% s.w. = $\triangle$ 1.70).

All animals were maintained in 25% s.w., with the natural substrate, for at least 24 hours before being placed at the experimental salinities—distilled water, natural river water at low tide (2.5% s.w.), 1%, 3.5%, 25%, 50%, 75%, 100%, 125%, and 150% s.w. Blood was picked up in 0.4-mm. diameter blood coagulating tubes through an incision made in the dorsal carapace behind the heart. Samples were taken at 3-, 24-, and 48-hour intervals. All samples were quick-frozen on dry ice and the osmotic pressure of the blood determined by a modification of the comparative freezing point technique suggested by Gross. The experiments were carried out at 22° C.

In the range of salinities to which it is normally subjected, *Cyathura polita* is a superb regulator of its blood osmotic concentration. In external salinities of 75%–1% s.w. the blood concentration falls but only within the relatively narrow limits equivalent to 75%–65% s.w. In distilled water the blood concentration is maintained at 60% s.w. for at least 48 hours and undoubtedly longer, since we have been able to keep *C. polita* in distilled water without ill effects for one week. *C. polita* does not maintain an osmotic gradient between its blood and the medium in external salinities of 75% to 150% s.w. However, in 100% s.w and above, isotonicity is not evident at the three-hour sampling although the blood of these animals is more concentrated than that of animals in 75% s.w. at the same time. By 24 hours the blood of all animals at the higher salinities is isotonic with the medium.

Animals placed in 100% s.w., 25% s.w., and river water, then weighed 1, 2, 3, 4, 8, 24, 48, and 72 hours thereafter showed no systematic weight changes. This suggests that both hypoosmotic regulation and hyperosmotic isotonicity are accomplished by the gain or loss of ions, rather than the gain or loss of water.

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Influence on the early development of three varieties of wheat of varied sea water-tap water mixtures. B. P. Sonnenblick.

Concurrent with low dose radiation studies, the influence of water (tap, distilled, sea, and sea-tap water mixtures) on early wheat growth (Dual, Thorne and Pennoll varieties) was tested in replicate experiments. Seeds, dormant or variously pretreated, were germinated on filter paper in polystyrene dishes in which daily changes of test waters were made. Experiments continued for 6–8 days, with temperature continuously recorded, light intensity measured, dishes daily rotated according to a pattern, and pH of all fluids periodically taken. The sea water-tap water mixtures ranged from 1 : 1 to 1 : 9 dilutions. Observations were made on some 2000 seedlings, each of which was finally cut and measured from the kernel to the tip of the first leaf; root measurements were made in many instances.

The following comments may be made: (a) the mixtures form a series of diminishing alkalinity, the least alkaline favoring growth, (b) germination begins in all mixtures, from 1 : 1 to 1 : 9, but not in sea water, (c) leaf growth in 1 : 9 mixture may occasionally approximate that of controls, but controls usually average slightly longer length, (d) influence of mixtures on first leaf growth may be summarized, those from 1 : 6 to 1 : 9 sea-tap waters maintain good to very good growth, those from 1 : 1 to 1 : 3 result in non-appearance of first leaf to poor growth, while those in the 1 : 4 and 1 : 5 categories give intermediate results. This latter group of sea-tap water mixtures strongly merits further systematic study under varied conditions and with material carried as far to maturity as possible, (e) a test series made in flats with a vermiculite-peat-soil combination or vermiculite alone suggests that growth in 1 : 4 and 1 : 6 mixtures is superior to that with the same mixtures in test dishes, (f) roots of control seedlings were elongate and straight, but those developing in mixtures range from twisted, shriveled filaments (1 : 1 to 1 : 3) to wavy, long roots (1 : 9).

Time-lapse motion pictures of deviations in density, viscosity, and cleavage in Arbacia derived from various fertilization combinations of irradiated gametes. Carl Caskey Speidel and Ralph Holt Cheney.

Eggs and sperm were irradiated with either x-rays or 2537 Å ultraviolet rays. They were then mixed with one another or with normal gametes. Eight different fertilization combinations of irradiated gametes were thus obtained. During the first hour or two many zygotes

developing from these combinations exhibited conspicuous deviations in density and viscosity from the normal. One type of deviation was characterized by formation of irregular dense areas in both central and peripheral zones. The peripheral zone often exhibited progressive crenation. A second type was featured by sudden protoplasmic readjustments as the time for first (or later) cleavage approached. As tensions changed near the incipient furrow the relatively stiff gel zone gave way sufficiently to allow the more fluid cytoplasm to break through and bring about a different arrangement. Several adjustments of this sort might occur in quick succession. These appeared as *popping* adjustments in time-lapse motion pictures which showed *movements accelerated 32 times.* The ensuing cleavage was usually irregular, often resulting in the formation of several blastomeres instead of two. A third type was induced by strong x-ray or ultraviolet irradiation. This type was characterized by violent general internal upheaval with profound gel-sol changes accompanied by temporary peripheral bulges. The integrity of the cell was destroyed as rapid churning of cytoplasmic constituents took place with drastic alteration of intracellular organization. This was followed by massive central pigment concentration and cell death. In some instances severe irradiation of gametes effectively prevented normal junction of the egg and sperm pronuclei.

Time-lapse cinephotomicrographs of specific cases from the moment of insemination on, vividly illustrate the progress of density and viscosity changes in the three types described above. They also reveal the sequence of protoplasmic events that culminate in abnormal cleavages.

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Heavy water as a parthenogenic agent. WILLIAM SPINDEL AND PAUL R. GROSS.

The effect of D_2O upon dividing cells is delay or arrest of mitosis, depending upon the concentration of deuterium. Thus, cleavage of the fertilized sea urchin egg is blocked by > 70% D_2O-sea water, and the effect is independent of the mitotic stage. The unusual properties of D_2O as an antimitotic are (1) the catholicity of the effect with regard to mitotic stage, extending as it does even to cytokinesis, (2) its reversibility, (3) stabilization, rather than destruction, of the achromatic figure, (4) a sharp and reversible process by which the cytoplasm and the mitotic machinery become rigid, and (5) the appearance of numerous cytasters during prolonged immersion in D_2O. These properties may help to explain our recent finding that D_2O evokes parthenogenic division of eggs stored therein prior to fertilization. When unfertilized sea urchin eggs are stored in 99+% D_2O-sea water for 1–2 hours, then washed and returned to normal sea water, they cleave, in remarkably high percentages, at about 35 minutes after removal from D_2O (21° C.). These are cleavages without benefit of sperm, and they continue for many hours, most cells becoming disorganized "blastulae." The first cleavages are always multiple and irregular in such experiments, and the furrowing pattern is closely related to the distribution of the numerous cytasters which remain and grow after removal of the cells from D_2O. When the period of immersion is shorter, or the concentration of D_2O reduced, the time required for the appearance of the first parthenogenic cleavages increases rapidly; hence, for a ten-minute storage period, the first cleavage interval is 3–4 hours, and the final yield of divided cells is smaller. Parthenogenesis cannot be produced if the concentration of D_2O falls to 70% or less. The striking effects of prolonged storage in D_2O are not results of aging alone, since controls stored for the same intervals in normal sea water do not divide. The effect appears to depend, in short, upon the formation of stable cytasters, which begins in 99+% D_2O-sea water after 10 minutes of storage for unfertilized eggs. Thus, eggs stored in D_2O and then fertilized show normal cleavage in the absence of cytasters, but multiple cleavage when cytasters persist in the cytoplasm after removal of D_2O.

In vitro studies on thymidine incorporation and cell division in skate (Raja erinacea) lens. B. DOBLI SRINIVASAN AND CLIFFORD V. HARDING.

The use of the whole-mount technique and labeled DNA precursors in *in vivo* and *in vitro* studies on cell division in lens epithelium has been described. By allowing sufficient time between exposure of the epithelium to H^3-thymidine *in vivo* and fixing, it is possible to obtain

radioactive nuclear pairs in rabbit lens. These pairs represent the daughters of the originally labeled cells, all of which are visible in the whole-mount. It is thus possible to determine the time and per cent of division of all originally labeled cells. The present study represents an attempt to obtain such pair formation in an *in vitro* system, which offers the advantage of a more direct analysis.

Freshly isolated skate lenses incubated *in vitro* with elasmobranch Ringer for 24 hours at 18° C. had essentially no mitotic activity. However, when whole skate eyes were incubated, with a portion of the posterior side cut out, for 24 hours at 18° C., mitotic activity was evident. Such "half eyes" were used for the rest of this study. Mitotic figures were seen after 14, 24, 48 hours of incubation at 18° C.; after 14, 24, 48, 72 hours of incubation at 5° C. and 0° C. Freshly enucleated half eyes were exposed to H^3-thymidine in elasmobranch Ringer (Sp. Act. 3.0 C./m*M*.; conc. 5 μc./ml.) at 18° C. for two hours, rinsed with elasmobranch Ringer and incubated at 18° C., 5° C., and 0° C. Although labeled cells were seen, no radioactive pairs or radioactive mitotic figures were seen at the end of 24 hours at 18° C. and up to 72 hours at 5° C. and 0° C. In most preparations, the diameter of the nuclei undergoing DNA synthesis appeared larger than that of the unlabeled nuclei.

It is probable, at least at lower temperatures, that a long period of time is required between the onset of DNA synthesis and the initiation of cell division.

Uterine function in experimental missed abortion. H. Takeda and A. I. Csapo.

Experimental missed abortion is produced in ovariectomized and placental dislocated 24 days pregnant rabbits by prohibiting the evacuation of the uterus with a prolonged and decremental progesterone treatment. When progesterone administration ceases, the myometrium often fails to complete delivery.

Recording the electrical and mechanical activity of the pregnant uterus in the intact animal, we studied the properties of the myometrium immediately after ovariectomy and placental dislocation and during and after progesterone treatment, for a period of about two weeks. The intrauterine pressure and the electrical activity of the myometrium at three different portions were recorded by a new method. Immediately after surgery, when the uterus is strongly dominated by progesterone the electrical activity was irregular, asynchronous and its relationship to mechanical activity obscure. The active intra-uterine pressure was small and irregular. The uterus did not respond to oxytocin.

As the daily progesterone dose was decreased gradually, the electrical activity improved somewhat. However, 3–5 days after operation both the electrical activity and the intrauterine pressure diminished; they became less regular and their relationship was obscure. This decrease in uterine activity continued after progesterone withdrawal.

However, activity improved gradually during decremental progesterone treatment if estrogen was given to the animal or if the animal was treated with estrogen after progesterone withdrawal. This improvement of uterine activity facilitated the evacuation of the uterus.

These observations confirm earlier findings, namely that the excitability and contractility of the uterus is dependent on estrogen and that progesterone blocks the myometrium, resulting in asynchronous uterine activity. They also suggest an explanation for missed abortion in women and provide the basis for a rational therapy.

The nuclear apparatus of a new species of Tracheloraphis (Protozoa, Ciliata). Reuben Torch.

Many Trachelocercidae contain Feulgen-negative macronuclei. Raikov found that macronuclei of three species of *Trachelocerca* contain primarily RNA, although originating from DNA-containing micronuclei. A new species of *Tracheloraphis* (Dragesco, 1960) was collected from Nobska Pond (salinity 10‰) in Woods Hole. Bright-field, phase, polarizing and interference microscopy were used to study nuclei in living organisms or in temporary mounts stained with aceto-orcein (0.4% in 70% acetic acid) or with chloroform-extracted methyl green (1% in 1% acetic acid). Other organisms were fixed in Bouin's, Schaudinn's, Zenker's or Champy's and stained with dilute (1:3) Delafield's hematoxylin, the Feulgen reaction, or gallocyanin-chrome alum.

The single complex nucleus of *Tracheloraphis* sp. contains four Feulgen-negative spheres (rarely 6 or 8), each surrounded by a clear area and a membrane. Each subnucleus (sphere + clear area + membrane) measures 6–10 μ in diameter; the spheres measure 4–6 μ. Lying between the subnuclei are two Feulgen-positive micronuclei, each 2–4 μ in diameter and surrounded by a membrane. A membrane surrounding the group of subnuclei is sometimes visible in Champy's fixed specimens, but never in living organisms. Surrounding the nuclear components is an extensive area (20–40 μ) of granular cytoplasm composed of 1–3 μ Feulgen-negative spheres, which stain with hematoxylin and gallocyanin-chrome alum. The sharp delineation of the granules from the remaining cytoplasm suggests that they belong to the nuclear complex. In most organisms, the subnuclear spheres produce weakly birefringent, plate-like crystals, generally rectangular but sometimes dumbbell-shaped or irregular. The crystals occur in matched pairs; if an arrow-shaped crystal is found in one subnucleus, an identical crystal is present in another subnucleus. During nuclear division, the micronuclei divide, forming four. Two of these, together with two of the subnuclei, migrate posteriorly. Prior to cytokinesis, the micronuclei divide again, two becoming functional micronuclei and two forming subnuclei. These observations and preliminary studies with DNase and RNase indicate this to be a direct transformation of DNA into RNA.

Differentiation of species of Diophrys by means of silver staining. Reuben Torch and Linda Hufnagel.

During a systematic survey of the hypotrichous ciliates of Woods Hole, five species of *Diophrys* were collected, three marine (salinity of 31‰) and two from brackish water (salinity 10‰). Routinely, the organisms were fixed in Schaudinn's and stained with dilute (1:3) Delafield's hematoxylin or by means of the Feulgen reaction. Except for differences in size and minor morphological characteristics, the animals were essentially similar: all had 7 right ventral, 5 anal, 2 left ventral and 3 caudal cirri. One species contained numerous macronuclei, while all the others had two with their associated micronuclei. While morphological characters suggested that the organisms represented separate species, species differentiation based on external morphology is difficult. Therefore, we resorted to the Chatton-Lwoff silver technique for the infraciliature which has proved important in taxonomic studies on other ciliates. The technique employed was essentially identical to that used by other workers, except that rinsing the organisms in dilute sea water (1:2) before embedding in saline gelatin gave enhanced results. (The saline rinse also improved the staining of other ciliates which are normally difficult to impregnate with silver.) Silver-impregnated slides were exposed either to sunlight or to ultraviolet light (GE H-4 lamp). Exposure time was determined by microscopic examination of the specimens and was generally around 30 minutes.

In *Diophrys,* silver staining reveals a dorsal infraciliature which is constant within each species. Of the five species studied, one has 9 kineties, one has 5, two have 4, and in the fifth species, each kinety is reduced to two or three widely separated groups of two or three kinetosomes in a row. Fibrillar connections between kineties were not observed in any of the species.

In addition to providing a means for identifying species of *Diophrys* (when used in conjunction with other morphological criteria), variations in number of kineties and kinetosomes gives insight into evolutionary relationships within the group.

A method for determination of oxygen uptake and respiratory quotients in carbon dioxide-air mixtures. David M. Travis.

This method employs simple apparatus for simultaneous determination of oxygen uptake and carbon dioxide output in the presence of wide ranges of carbon dioxide in air. The principle involves the sampling of gas from a reaction chamber of known volume. A 30-ml. syringe fitted with a three-way stopcock or a rubber stopper provides a vessel of variable volume which is convenient for gas sampling by pipette or syringe and needle. It will contain a variety of marine invertebrates. *Golfingia gouldii, Thyone briareus* and *Mytilus edulis* have been used. The simplest procedure with only a gas phase is described, although both liquid and gas phases can be employed. The syringe is filled with the animal and immersed in a water bath at constant temperature. Gas is sampled initially and at suitable intervals. Analysis can be done for

carbon dioxide and oxygen using the ½-cc. method of Scholander. The changes in concentration of these gases and the initial and final gas volumes are used to calculate oxygen uptake and carbon dioxide output. Initial volume is set at a calibrated mark with gas brought to the temperature of the bath. Changes in gas volume occur during an experiment which may seriously affect results. This difficulty is met by measuring changes in concentration of nitrogen or "inert" gas in the system. In this way the final volume is estimated indirectly. A formula can be used to correct the final oxygen and carbon dioxide concentrations. Equilibration of the animal with the gas may require repeated gassing over several hours. Great care must be exercised in the sampling and analysis of gases to avoid contamination or loss. The method is relatively simple in theory and is adaptable to a variety of biological materials and analytical techniques.

Factors affecting germinal vesicle breakdown in Pectinaria (Cistenides) gouldi. KENYON S. TWEEDELL.

Prior to shedding, the eggs of *Pectinaria* are tightly packed but freely suspended in the coelomic fluid. When shed into sea water, mature eggs continue maturation with breakdown of the germinal vesicle (G.V.), nucleolus, and formation of the first maturation metaphase. G.V. breakdown begins from six to ten minutes post-shedding, the time varying with the season, individual animals, temperature, light and other factors. The process lasts 2.5 to 3 minutes and is completed in all eggs by 15 to 20 minutes post-shedding. Since breakdown may be caused by a release from the internal environment or reaction with the external environment, several possibilities were investigated.

Animals were carefully dissected from their sand tests in Moore's calcium-free sea water, washed repeatedly and induced to shed into Ca-free, artificial (MBL formula) and natural sea water. Nuclear breakdown occurred in all solutions, although it was retarded in both artificial solutions.

To test for the possible release of calcium from the egg as the activating agent, eggs were shed into an isotonic (0.30 *M*) sodium citrate solution as well as hypo- and hypertonic solutions. Germinal vesicle breakdown occurred concurrent with controls (+ 9 minutes) and the eggs remained stable for 30 minutes in the isotonic solution.

Eggs were next shed into hypertonic or hypotonic artificial sea water (plus or minus 100 millimoles NaCl), artificial sea water and natural sea water. Nuclear breakdown again took place in all solutions but was delayed in the artificial solutions.

Animals were again dissected from their tests, washed, and blotted dry with bibulous paper. The coelomic fluid containing eggs was then either collected from the posterior gonopores or removed with a hypodermic needle. The remaining eggs were shed into natural sea water. Normal nuclear breakdown of eggs in the extracted coelomic fluid took place repeatedly as in the controls. Thus, removal of eggs from the coelom rather than external environmental factors appears to trigger nuclear breakdown. Possible causes to be tested are CO_2/O_2 ratios and hydrostatic pressure.

Studies on the site of action of alloxan: effect of alloxan on the permeability of the toadfish islet cell membrane to mannitol. DUDLEY WATKINS, S. J. COOPERSTEIN AND ARNOLD LAZAROW.

Previous studies have suggested that alloxan does not enter the cell but that it may exert its diabetogenic effect by acting on the beta cell membrane. The present investigation was undertaken to determine the effect of alloxan on the permeability of the cell membrane *in vitro*. Since C^{14}-mannitol does not enter most cells, it was used as an indicator of the integrity of the cell membrane.

Toadfish islets were removed, decapsulated, cut in half, and incubated at 0° C. in an isotonic medium containing C^{14}-mannitol, with and without added alloxan ($2.5 \times 10^{-4} M$). (The diabetogenic dose of alloxan in the rat, 40 mg./kg., is equivalent to 2.5×10^{-4} moles/kg.) The islet slices were removed after various incubation periods (0, 5, 10, 15, 30, 60, 120, and 180 minutes), and rinsed momentarily in a medium containing non-radioactive mannitol in

order to minimize surface contamination. The slices were weighed and homogenized in water, and the C^{14} content per mg. of tissue was determined.

The results showed that at maximum equilibration the C^{14}-mannitol content of the tissue was 30% of that in the medium. With the amount of tissue used, equilibration was almost complete at 10 minutes, whereas at 5 minutes the C^{14} content was 22% of that in the medium. Alloxan markedly increased the penetration of C^{14}-mannitol; at 5 minutes the C^{14}-mannitol content of the tissue was 40% of that in the medium, at 10 minutes it was 49%, and at maximum equilibration it was 53%. The same concentration of alloxanic acid (a non-diabetogenic decomposition product of alloxan) did not influence the penetration of C^{14}-mannitol. In contrast to islet, the permeability of heart, kidney, brain, gill, muscle, and liver was not affected by this concentration of alloxan. These data are therefore consistent with the hypothesis that alloxan produces its diabetogenic effect by damaging the beta cell membrane.

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Organismic responses to differences in weak horizontal electrostatic fields. H. Marguerite Webb, Frank A. Brown, Jr. and Thomas E. Schroeder.

Snails, as they emerged from a south-directed exit to an asymmetrical field, black to left and white to right, were subjected to changes in electrostatic field. The field was modified by large vertical copper plates four inches to right and left, either shunted together by direct wire connection (control) or subjected to a 45-volt difference, +L −R or −L +R, yielding about 2 volt/cm. addition or subtraction from whatever ambient terrestrial E-W vector obtained at the time. Experimental series were run at various times of day. About half the series comprised C (control), +L, +R, C, +L, +R, C, +L, +R, C and in the remainder the experimental fields were in the reversed order (C, +R, +L, . . .). The average differences between paths in the experimental fields and the average of the two adjacent controls were determined. The +L field produced, in 52 such series, 18% greater deviation ($P < .02$) from controls than +R. The effect of the +L field was stronger for those series +L, +R (+31%) than for the alternative series, +R, +L (+4%), suggesting a persistent effect of the fields on the snails.

In a shorter set of comparable experiments conducted concurrently, in which the snails were directed east, the +R field possessed a stronger influence, 13% more than the +L field, and comparable, when the +R field directly followed a control it was more effective. In the C +R, +L series the +R was 20% more effective than +L, and in the C +L, +R series 5% more so. The data of east-directed snails, though still too few to establish good statistical probability, do, however, clearly suggest an interrelationship between magnetic and electrostatic orientational responses.

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Allometric growth in fish. Charles G. Wilber.

In an attempt to ascertain more precisely the mechanism of action of selected psychotropic drugs, studies of the effects of some of them on normal growth and development in fish were initiated. Previous work showed that lysergic acid diethylamide in various concentrations modified the development of the heart in *Fundulus*. This fact suggested a more detailed study of a number of species. In the toadfish *Opsanus tau*, ranging in weight from 30–600 grams, the heart weight varied linearly with body weight. The coefficient of correlation was 0.9. If all the data were combined to get mean body weights and mean heart weights, it was calculated that in *Opsanus* the heart weight accounts for about 0.1% of the body weight. Comparisons were made with other marine and fresh-water fish. In the glut herring, for example, heart weight accounts for 0.18% of the total body weight. The average value for heart weight, as per cent of body weight, based on the 12 species available in this study, was 0.11. A similar average for mammals is about 0.84%. Comparisons of the same sort are being calculated for brain, eyes, gut and liver. With this basic information available, it should then be possible

to evaluate the biological effects of various chemical agents in terms of the modifications induced in organ weight: body weight ratios in fish.

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The effect of actinomycin D on the development of Arbacia eggs. ALEXANDER WOLSKY AND MARIA DE ISSEKUTZ WOLSKY.

The substance, known to be carcinostatic in some cases (kindly supplied by Merck, Sharp & Dohme), was used in 2.5 mg. per 100 ml. concentration. Treatment of sperm suspensions with this solution before fertilization for 3 to 4 hours and subsequent transfer to sea water produces no ill effect on fertilization and development. A similar pre-treatment of unfertilized eggs, although it apparently does not interfere with the subsequent fertilization in sea water (membrane elevation occurs promptly), delays the formation of the first cleavage furrow by 10 to 18 minutes in comparison to controls. (Normal time interval between fertilization and first cleavage under the conditions of observation: 40 minutes.) This marked delay has no influence on the formation of the furrow, which is in most cases normal. (Abnormalities were discarded, as they occur occasionally also in controls.) Later development of the pre-treated eggs is also sluggish and they never recover completely. Tetrahedric plutei are formed but the growth of the arms is stunted. Fertilization of eggs in actinomycin without pre-treatment has no immediate effect (except a much slighter delay of the first cleavage) but keeping them afterwards continuously in the solution leads to retardation and abnormalities of the cleavages and death before the gastrula stage. On the basis of these observations and earlier (unpublished) studies on the effect of actinomycin on onion root tip cells, it is suggested that the substance produces abnormal conditions in the nuclei, which manifest themselves both in chromosome aberrations (elongations and breakages, observed in onion root tips) and delayed cleavage divisions (delay of the fusion and amphimixis of pronuclei?). However, an effect on cytoplasmic constituents (kinetic apparatus) must be also assumed as the contrast between pre-treatment of sperm and eggs indicates. A microscopic study of fixed eggs to clarify these questions is in progress.

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Effect of certain adrenocorticoids on blood sugar and liver glycogen of skate. PAUL A. WRIGHT.

Apparently the only attempt to relate adrenocorticoids to carbohydrate metabolism in elasmobranchs was by Hartmann and co-workers in 1944. They showed that liver glycogen fell somewhat following interrenalectomy in skates (although the enormous range of values for intact animals made this difficult to prove statistically), but could not demonstrate any consistent lowering of blood glucose in interrenalectomized animals.

Our studies during the past two summers have indicated that certain adrenocorticoids, active in carbohydrate metabolism in mammals, at least, are of only minimal importance in carbohydrate metabolism in *Raja ocellata*. In 2 of 6 animals stunned by a blow on the head, intra-arterial injection of cortisone (2 mg./kg.) raised the blood sugar by only 32 mg.% in 30 minutes, and this value dropped to +17 mg.% by 60 minutes. Corticosterone, prednisolone, and hydrocortisone, in the same dosages, did not raise blood glucose levels significantly.

Daily intramuscular injection (5 or 10 mg.) of hydrocortisone in normal fasting animals raised the blood glucose level by only 10 mg.% in 24 hours, but cortisone in the same dosages was without effect. Liver glycogen, determined by a modified anthrone procedure 48 hours after the first of two daily 5- or 10-mg. intramuscular doses of hydrocortisone or cortisone, was not altered from that of saline-injected controls.

This study was aided by a grant (A–2986 C-1) from NIH, USPHS. Thanks are due to Dr. P. L. Perlman of Schering Corporation for steroid hormones, and to Mrs. Ann R. White for technical assistance.

Optic nerve responses of squid, Loligo pealii. ROBERT WURTZ.

Recordings from squid optic nerve by MacNichol and Love were verified and extended, and a more stable method of restraining the intact squid was developed. In the present method

only the head was restrained, leaving the mantle entirely free to perform respiratory movements. Under .3% urethane the squid was placed, back down, on cork board, and four pins were inserted into the head: one on each side just anterior and medial to the eye, one on each side lateral to the optic lobe in the lateral extension of cartilage. Posterior pins were not so tight as to pressure-block the underlying respiratory nerves. The squid was then submerged in freely running sea water at 20° C. and remained vigorous, often for 24 to 36 hours. With steel microelectrodes inserted through a separately held syringe needle, optic nerve responses were held even during siphon squirts. During light presentation initial slow oscillations and a sustained background discharge of impulses occurred. Bright light (500 mμ) produced clear oscillations of approximately 40-msec. latency and 1.4-mV. amplitude while light in a more physiological range produced very slight oscillations with latency above 55 msec. and .4-mV. amplitude. Number of oscillations varied both with light intensity and position of electrode. With D.C. coupling, oscillations were seen to occur on the rising phase of the ERG. Section of the optic nerve between the electrode and the optic lobes did not eliminate the oscillations. The oscillations later disappeared, usually within 30 minutes, as did the ipsilateral but not contralateral ERG. A knife cut between the electrode and the eye abolished immediately all nerve responses to light. Prussian blue marks left by microelectrode lesions were found to lie in the optic nerve. It is concluded that background discharge and oscillations arise in afferent optic nerve fibers. The oscillations possibly arise from partial synchronous discharge of large numbers of afferent fibers.

The nucleic acids of transparent tissues. S. Zigman, B. Carlsson, J. Kielich, E. J. Allaway and William Stone, Jr.

In this study, the distribution of RNA and DNA; the uptake of P^{32} (phosphate) into the nucleotides of RNA; and the nucleolytic enzyme activities of several transparent tissues were examined. Since nucleic acids are important in protein synthesis, it is possible that their nature and metabolism influence the formation of collagen, producing transparent—rather than opaque—tissues.

Sections of embryonic and adult dogfish corneas and shark corneas, stained by Feulgen technique (for DNA) and methyl green-pyronin (for RNA) showed that most of the RNA and DNA was localized in the epithelium of the cornea, very little appearing in the stroma.

Paper electrophoresis was employed to separate the nucleotides of RNA extracted from corneal epithelium and squid skin by aqueous phenol. A nucleotide pattern similar to that of phenol-extracted yeast RNA was found, whereas no such pattern appeared for nucleotides of corneal stroma.

P^{32} (phosphate) was found to be incorporated into all four nucleotides of corneal epithelium and squid skin RNA. These tissues were incubated at 32° C. in Ringer-bicarbonate medium (pH 7.4) containing 5 μc. of P^{32} (phosphate) for two hours. However, positively identified nucleotides containing radioactivity were not found in stromal tissue.

Ribonuclease and deoxyribonuclease activities of stromas and epithelia were measured by the release of ultraviolet absorbing materials at 260 mμ from yeast RNA. The results of these experiments are as yet incomplete.

This work shows that the epithelium of the normal cornea contains nearly all of the nucleic acids of the whole tissue. RNA metabolism, as measured by P^{32} (phosphate) incorporation into nucleotides, occurs only in the epithelium. Further work to compare the nucleic acid metabolism of these normal transparent tissues with opaque, chemically treated, and irradiated tissues is planned for the future.

Structural and functional recovery of the mitotic apparatus following disorganization by high pressure. Arthur M. Zimmerman and Douglas Marsland.

The mitotic apparatus (spindle-aster-chromosome complexes) were isolated from *Arbacia* eggs subsequent to treatment with high pressures (2000–10,000 lbs./in.^{2}) applied at metaphase of the first cleavage division. Subsequent to the compression period (1–10 min.) the eggs were fixed in cold 35% ethanol, either immediately, or after a 1–10-minute recovery period. Later, isolations were effected by selective solubilization in 1% digitonin.

As reported previously, pressure treatments of appropriate intensity and duration effect a drastic disorganization of the linear and radial structure of the spindle-aster complex and a severe dislocation of the chromosomes. One minute of compression in the range of 8000 to 10,000 lbs./in.^{2} suffices to abolish all traces of fibrous structure and to cause a clumping of the chromosomes near the center of the spindle remnant. With longer treatments, the spindle area becomes shorter and the astral remnants are eroded. At the 4000 lbs./in.^{2} level, fairly normal structure endures for some 2–3 minutes, whereas at 2000 lbs./in.^{2} an approximately normal structure persists for 10 minutes (perhaps longer), although the chromosomal movement is definitely retarded.

Post-compression recovery, as judged by a study of isolations fixed at various times after treatment, is longer with more drastic degrees of disorganization. An apparently normal fibrous structure in the spindle and astral regions and a normal metaphase arrangement of the chromosomes reappears within 5 minutes after a one-minute compression at 6000 lbs./in.^{2}, and there is a corresponding (6 minutes) delay in onset of furrowing in the treated eggs. At a pressure of 10,000 lb./in.^{2}, maintained for one minute, only partial recovery is observed at 5 minutes, but full recovery appears to occur at 10 minutes. Moreover, such eggs begin furrowing just 10 minutes later than untreated controls. This indicates that structural recovery must occur before function can be resumed.

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LALOR FELLOWSHIP REPORTS

Fine structure of an unusual spermatozoan in the flatworm Plagiostomum. A. Kent Christensen.

Spermatozoa from the alloeocoel flatworm *Plagiostomum morgani* Graff were studied by light and electron microscopy. The sperm are spindle-shaped, tapering at each end into slender processes which are respectively about 40 μ and 10 μ long. The fusiform nucleus contains a dense core of unknown nature which protrudes from the nucleus at that end of the sperm which bears the shorter slender process. The entire surface of the sperm is covered by a pellicle consisting of the plasma membrane closely underlain by parallel fibrils or tubules, about 200 Å in diameter and spaced 700 Å apart, which pursue a spiral course up the body of the sperm. This pellicle closely resembles that described by others in trypanosome flagellates, where it may be responsible for characteristic body contractions. The cytoplasm around the middle portion of the nucleus contains whorls of flattened cisternae, resembling endoplasmic reticulum (without ribosomes), which extend out to insert along every other or every few pellicular fibrils. The rest of the cytoplasm contains mitochondria, but otherwise is homogeneous in appearance. No evidence was seen of a flagellum or of undulatory membranes.

Sperm received in copulation are transported into the general connective tissue, in which all body structures, including the ova, are embedded. The sperm wander in this tissue throughout the body, and many congregate around the eggs to effect fertilization. Since the connective tissue is a continuous mass of fixed parenchymal cells, without intercellular spaces (Pedersen, confirmed here), the sperm are wedged in between cells during these travels, and must presumably proceed by some sort of wriggling motion. Flagella or undulatory membranes would seemingly be useless under these circumstances. It is suggested that the specialized pellicle may be the basis of motility, perhaps through differential contractions of the pellicular fibrils over the surface of the sperm.

Further investigation of egg jelly dispersal by Arbacia sperm extract. R. R. Hathaway and L. Warren.

We have described previously how *Arbacia* sperm can be extracted with sodium lauryl sulphate to yield a solution which causes egg jelly-coats to disappear in 15 to 60 minutes. The evidence suggested that the jelly-coats were being dispersed rather than being precipitated

to the cell surface. We have now employed S^{35}-labelled *Arbacia* eggs to obtain additional evidence for the solubilization of the egg jelly-coat.

Arbacia eggs labelled with S^{35} were mixed with the active sperm extract. The subsequent disappearance of the jelly-coats of the eggs was accompanied by the appearance of radioactive materials in solution. Two other mixtures served as bases for comparison: (1) labeled eggs treated with acidified sea water in order to dissolve the jelly-coats completely, (2) labeled eggs treated with sea water. In four experiments, supernatant solutions of eggs treated with sperm extract contained radioactivity equal to 74%, 126%, 101%, and 111% of the radioactivity released by the eggs in acidified sea water. Since the jelly-coat contains a large proportion of sulphate groups, the appearance of S^{35}-labeled materials in solution would seem to confirm that these sperm extracts cause the solubilization of the gelatinous material. Two of the above values (126%, 111%) suggest that sulphur-containing materials from within the egg may be released following exposure to sperm extract. S^{35} in dissolved materials did not diffuse through cellophane.

After one hour there were small amounts of radioactivity in the control supernatant solutions of eggs mixed with sea water. When labeled eggs and sperm extract were kept for one hour at 0° or 11° C., the jelly-coats of the eggs did not disappear, nor did the supernatant solutions contain more radioactivity than did control solutions. Sperm extracts were inactivated when heated for two minutes at 80° C. The jelly-dispersing agent in sperm extracts can be recovered after precipitation by 30% ethanol at 0° C.

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THE EFFECT OF SALINITY ON THE AMINO ACID CONCENTRATION IN RANGIA CUNEATA (PELECYPODA)

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Simpson *et al.* (1959) reported that the amino acid taurine was in high concentration in marine molluscs. Taurine could not be detected by means of paper chromatography in extracts of fresh-water and terrestrial molluscs. Allen and Awapara (1960) studied the metabolism of S-35 methionine in the mussel, *Mytilus edulis,* and the brackish-water clam, *Rangia cuneata.* Taurine was not detectable in the tissues of the latter. Following injections of radioactive methionine, both species were found capable of degrading methionine to taurine and sulfate. In *R. cuneata* taurine was formed but was rapidly metabolized and excreted as an unknown compound. Taurine was retained in high concentration in the tissues of *M. edulis.*

These results suggested that the high concentration of taurine and perhaps other amino acids may be associated in some way with the salinity of the environment. To investigate this possible relationship, *R. cuneata* was gradually moved from water of 3‰ to water of 25‰. Amino acid extracts were made from specimens at several salinities within the above range, and four individual amino acids were measured. The results of this study showed that taurine was not retained in the tissues of *R. cuneata* regardless of salinity, but that the amino acids, particularly alanine, increased with the higher saline environments.

Materials and Methods

R. cuneata was collected in the San Jacinto River, Houston, Texas. Following collection, the clams were transported to the laboratory and kept at approximately 25° C. in large aerated 50-gallon aquaria. The clams were allowed at least three days to adapt to the aquarium before being used in experimental work.

A series of five-gallon aquaria were set up in consecutive order of increasing salinities which ranged from 3‰ to 25‰. Filtered sea water was diluted to the appropriate volume with distilled water. Salinities were determined by the Mohr

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technique, as described in Standard Methods (1955). Several tests were made on *R. cuneata* to determine how rapidly they could be moved from one salinity to another. It was found that if the animals were allowed to remain in a particular tank for two days, they could be transferred without fatalities.

In order to insure correct controls for starvation a group of clams was started together in the fresh-water tank. After two days all but a dozen of the original group were moved to the next tank of higher salinity. This was repeated until all the tanks contained a dozen clams. After the final tank was reached, two days were allowed to elapse prior to making amino acid extracts from representatives of each tank. The effects of leaving clams in one tank over a period of time were checked, and up to 20 days there was no measurable change in the amino acid concentration.

The extraction and fractionation of amino acids were carried out as reported by Allen and Awapara (1960). The amino acids were analyzed by two-dimensional ascending paper partition chromatography. The amino acid extracts were spotted on Whatman filter paper #4. Solvent mixtures were 72% phenol and 65% lutidine. Both solvents were redistilled prior to use.

The quantitative estimation of alanine, aspartic acid, glutamic acid and glycine was carried out by the method of Awapara *et al.* (1950) with the following modification from Moore and Stein (1948). After localizing the amino acids on the chromatogram the spots were outlined with pencil and the paper sprayed with 1% KOH in methanol. After drying the sprayed papers in air, the spots were cut out and placed in test tubes. The tubes were put in a vacuum desiccator over concentrated sulfuric acid for three hours. Color was developed with the reagent of Moore and Stein (1948). The tubes were read at 570 mμ on a Coleman Junior Spectrophotometer.

The dry weight was determined by drying the total animal minus shell at 100° C. until the weight remained constant. The inorganic constituents (ash weight) were estimated by incinerating the dry tissues in a muffle furnace at 1000° C. The difference in dry and ash weights was expressed as per cent ash weight.

Results and Discussion

In view of the possible relationship between amino acid concentration and the hydration of tissues, the dry weight and ash weights were determined on specimens of *R. cuneata* from environments of different salinities. The results are shown in Table I.

From the dry weight studies, it is obvious that as the clams progress from a dilute to a more saline environment there is a loss of water. The ash weights, on the other hand, increase slightly in the higher salinities, reflecting an increase of inorganic constituents. These results are in agreement with earlier investigations of Fredericq (1904), Krogh (1939), and Fox (1941). These investigators demonstrated that euryhaline molluscs establish an equilibrium with their environment with respect to inorganic ions. This equilibrium is observed by a weight-volume change and a fluctuation of ion concentration depending on the salinity of the environment.

The effect of increased salinity on the amino acid concentration in *R. cuneata* is shown in Table II and Figure 1. All four amino acids increased in concentration

until the animals reached a salinity of 17 ‰. In the two higher salinities the amino acids decreased in concentration. From the data on per cent dry weights and ash weights it appears as if the loss of tissue water and gain of inorganic ions reaches a maximum in salinities of 17–20‰. This leveling off of water loss and the decrease in inorganic ion exchange in *R. cuneata* suggests a shift in osmotic control. The concurrent decrease in amino acid concentration may be associated with this phenomenon. This possible relationship between amino acids, the control of tissue water, and inorganic ions needs further investigation. These three factors may prove to be in dynamic equilibrium in euryhaline molluscs when these animals are

TABLE I

Per cent dry weights and ash weights of R. cuneata *from different salinities*

Salinity		Per cent dry wgts.	Per cent* ash wgts.
3‰	Range	18.18–20.38	2.56–4.54
	Mean	19.34	3.43
	S.D.	0.36	0.36
6‰	Range	19.21–21.40	3.54–4.43
	Mean	20.36	3.95
	S.D.	0.41	0.13
10‰	Range	21.06–24.00	3.75–4.97
	Mean	23.18	4.41
	S.D.	0.51	0.24
17‰	Range	23.54–25.76	4.31–5.97
	Mean	24.41	5.13
	S.D.	0.95	0.35
20‰	Range	25.03–26.82	5.14–6.31
	Mean	25.75	5.84
	S.D.	0.33	0.22
25‰	Range	24.62–26.86	4.63–6.12
	Mean	25.70	5.27
	S.D.	0.32	0.23

* Per cent ash weight expressed in terms of dry weight of animal.

moved from one environment to another. If such is the case, the inclusion of amino acids in osmotic control broadens considerably ideas encompassed in the process of osmoregulation.

Whether or not this increase in alanine is in response to an osmotic imbalance cannot be determined from these results. However, some studies have shown indirectly that amino acids play a role in osmoregulation. Camien *et al.* (1951) and Duchâteau *et al.* (1952) have shown that marine invertebrates have a higher concentration of free amino acids than fresh-water forms. This fact was the basis for investigating the effect of salinity change on euryhaline crabs by Duchâteau and Florkin (1955) and Shaw (1958a, 1958b, 1959). These investigations showed

that the amino acid concentration fluctuated in direct proportion with the salinity of the environment. Likewise, Duchâteau *et al.* (1961) moved the polychaet worm, *Arenicola marina,* from 100% sea water to 50% sea water, and observed a decrease in the amino acid concentration. The amino acids most affected by the change were glycine and alanine. The total decrease in amino acids was more than what would be accounted for on the basis of tissue hydration. Potts (1958) reported the effect of changing salinity on two molluscs, *Mytilus edulis* (marine) and *Anodonta cygnea* (fresh-water). The amino nitrogen decreased in *M. edulis* as this animal was moved into water of lower salinity. The reverse was true when

TABLE II

Amino acid concentration in R. cuneata *from different salinities (expressed as μmoles/gm. tissue dry weight)*

Salinity		Alanine	Glycine	Glutamic	Aspartic
3‰	Range*	3.2–9.6	3.0–6.7	3.2–6.2	1.1–3.4
	Mean	6.1	5.1	4.3	2.1
	S.D.	1.1	0.7	0.4	0.4
6‰	Range	14.3–24.3	14.5–26.4	10.1–14.7	2.3–6.0
	Mean	18.4	18.9	13.3	3.5
	S.D.	1.9	2.1	0.8	1.1
10‰	Range	82.9–108.6	29.0–38.4	19.9–22.8	5.7–9.5
	Mean	92.6	34.8	21.4	7.7
	S.D.	4.3	1.7	0.6	0.7
17‰	Range	205.7–249.2	45.9–58.2	29.5–35.8	14.5–17.4
	Mean	225.4	51.6	32.3	16.2
	S.D.	7.6	2.4	1.3	0.5
20‰	Range	179.5–209.3	26.6–36.0	21.0–29.2	8.9–12.8
	Mean	192.6	32.1	24.4	10.3
	S.D.	5.1	1.5	1.6	0.8
25‰	Range	156.5–186.7	20.8–25.6	18.3–21.6	5.0–9.0
	Mean	170.2	23.7	20.0	6.9
	S.D.	5.1	0.8	0.6	0.8

* Range represents distribution of amino acid concentrations from five separate individual animals from each salinity.

A. cygnea went into higher salinities. There is an effect of salinity on the amino acid concentration in euryhaline invertebrates. The exact relationship between amino acids, water balance and inorganic constituents needs to be worked out.

The source of alanine as well as the other measured amino acids cannot be explained readily. They may arise from the increased hydrolysis of protein. However, the high concentration of one amino acid such as alanine suggests that there may be more involved than just increased protein breakdown. A process whereby one amino acid could reach such high values could be explained by the interaction of the amino acids from protein and keto-acids from carbohydrate

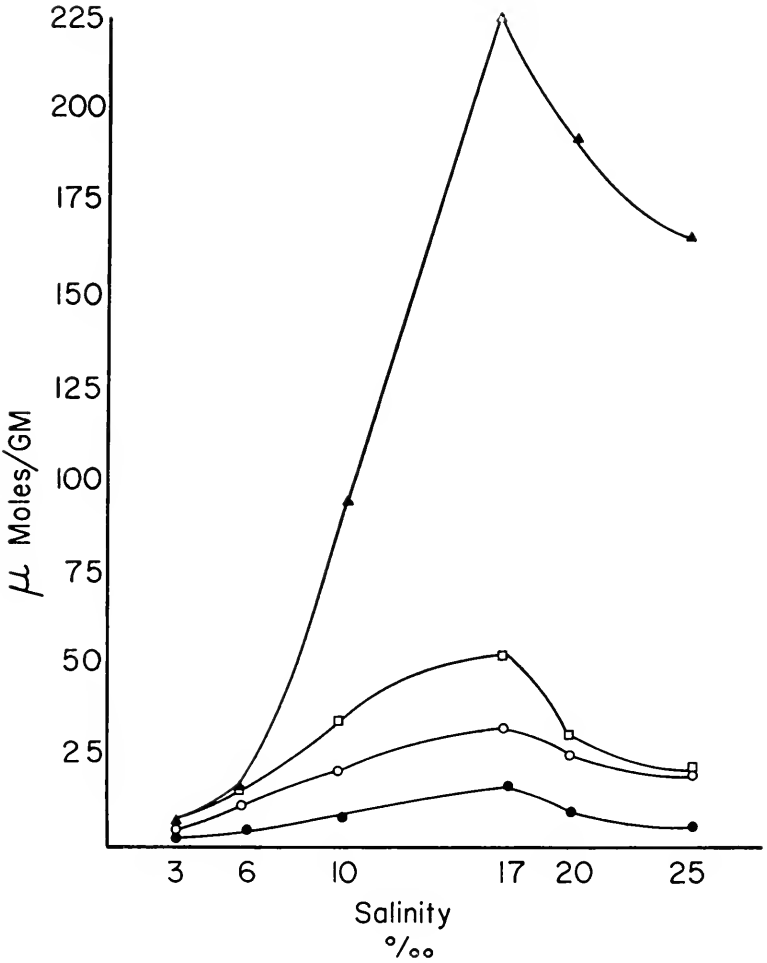

FIGURE 1. Amino acid concentration in *Rangia cuneata* taken from different salinities. Alanine ▲; aspartic acid ●; glutamic acid ○; glycine □.

metabolism via transamination. There is little evidence for this. If such were the case one might expect to observe an increase of respiration, a decrease in glycogen and an increase in one or more amino acids. Pieh (1936) and Maloeuf (1938) observed an increase in the respiration of *M. edulis* as this mussel was placed in dilute sea water. This was also true when the animals were returned to 100% sea water. Preliminary studies in this laboratory have shown a decrease in glycogen as *R. cuneata* is moved into higher salinities. These results may be interpreted as an indication of work being done in order to maintain osmotic equilibrium. At the same time carbohydrate is being used for energy purposes, the production of keto-acids provides a source of carbon chains for the formation of amino acids via transamination. Only experimental evidence will verify the latter.

Summary

1. The amino acids alanine, glycine, glutamic acid and aspartic acid were quantitated in *Rangia cuneata* taken from different salinities.

2. The individual amino acids increased in concentration as the salinity increased.

3. The increased concentration of individual amino acids followed a definite pattern which was already established prior to the change into higher salinities. The pattern was: alanine > glycine > glutamic acid > aspartic acid, regardless of environment.

4. The possible role of amino acids in an animal withstanding changes in salinity is discussed.

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STUDIES ON THE ISOLATED ISLET TISSUE OF FISH. IV. IN VITRO INCORPORATION OF C^{14}- AND H^3-LABELED AMINO ACIDS INTO GOOSEFISH ISLET TISSUE PROTEINS [1, 2]

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Elucidation of the factors involved in the biosynthesis of insulin is essential for a better understanding of the etiology and development of diabetes mellitus. To this end, *in vitro* studies of the incorporation of labeled amino acids into islet cell proteins have been undertaken. In mammalian species the islet tissue is dispersed into a million or more individual islets of Langerhans, whose total mass approximates only 1% of the pancreatic mass. This makes it very difficult to separate the islet from the acinar tissue. In teleost fish, however, the islet tissue is concentrated into one or more discrete bodies called the principal islets (Diamare, 1905; Rennie, 1905); the acinar tissue, in contrast to that of mammalian species, is dispersed throughout the mesentery and located along the bile duct and within the liver. The goosefish, *Lophius piscatorius,* a marine teleost of wide distribution, was chosen because of its large and discrete aggregations of islet tissue, which are rich in extractable insulin and relatively free of exocrine tissue (Macleod, 1922). The *in vitro* study of insulin biosynthesis, using isolated islet tissue and radioactive amino acids, is based on the assumption that this process is closely analogous to the natural biosynthetic pathway. Net synthesis of specific proteins from labeled amino acids, *in vitro,* has been established for serum albumin (Peters and Anfinsen, 1950) and cytochrome *c* (Kalf *et al.,* 1959).

Previous *in vitro* studies of insulin biosynthesis using labeled amino acids and mammalian pancreas have been carried out by Pettinga and Rice (1952), Vaughan and Anfinsen (1954), and Light and Simpson (1956). Since the ratio of exocrine to islet tissue is large in mammalian pancreas, it was necessary to use large samples of tissue (weighing 10 to 100 gm.). In addition, the presence of large amounts of non-insulin proteins required the use of exacting purification procedures.

The present report deals with our studies on the *in vitro* incorporation of C^{14}-labeled amino acids into proteins using the isolated islet tissue of the goosefish. A preliminary report of these studies has been published (Bauer and Lazarow, 1961).

[1] These investigations were initiated in the summer of 1959 by the late Austin Lloyd Yates, a brilliant young graduate student at the University of Minnesota. Mr. Yates had successfully demonstrated that C^{14}-labeled amino acids, added to toadfish islet tissue *in vitro,* were incorporated into the alcohol-soluble fraction. Because of his tragic accidental death in the summer of 1959, and because all of the original protocols were lost, these studies were interrupted; they were resumed in June, 1960.

[2] This investigation was supported by research grants A-1659 and A-1887 from the National Institute of Arthritis and Metabolic Diseases, and 2G-114 from the Division of General Medical Sciences, National Institutes of Health, United States Public Health Service.

Methods

Incubation procedure. Goosefish were obtained periodically during the summer months in the vicinity of Woods Hole, Massachusetts, and maintained in refrigerated sea water tanks. The large principal islet, located in the mesentery adjacent to the cystic duct, was removed, and the connective tissue capsule dissected away. Occasionally, the smaller secondary islet, located near the pyloric stomach, was also used. One to three pieces of islet tissue, weighing between 2 and 6 mg., were incubated at 25° C. in a micro-homogenizer tube (Fig. 1). The incubation medium

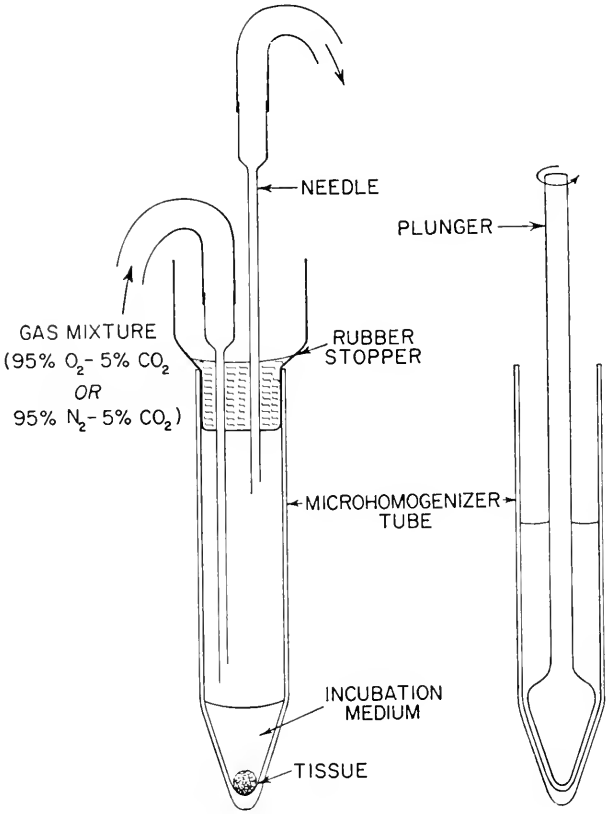

Figure 1. The incubation and extraction vessel.

consisted of 50 μl. of Krebs' Ringer bicarbonate buffer (Krebs and Eggleston, 1940), containing leucine-C^{14} + valine-C^{14}, or leucine-H^3 (initial pH 7.4). In experiments using C^{14}-labeled amino acids, the amino acids were dissolved in water at 11 times the desired final concentration. One part of this mixture was added to 10 parts of mammalian Krebs' Ringer bicarbonate buffer to give a final electrolyte concentration of 0.14 *M*; this is isotonic to fish blood. (Since there are 6 leucine and 5 valine residues per mole of insulin, a mixture of leucine-C^{14} and valine-C^{14} was

used in order to obtain high specific activities of the proteins synthesized.) When leucine-H^3 was used, the desired amount of amino acid was dissolved in the appropriate volume of 0.14 *M* Krebs' Ringer bicarbonate buffer.

The concentrations of amino acids used in these studies were the following: DL-leucine-1-C^{14}, 2.1 m*M*/l. (specific activity = 8.75 mC./m*M*), or 2.2 m*M*/l. (S. A. = 7.80 mC./m*M*); DL-valine-1-C^{14}, 1.6 m*M*/l. (S. A. = 10.5 mC./m*M*), or 3.0 m*M*/l. (S. A. = 6.05 mC./m*M*); DL-leucine-4,5-H^3, 0.43 m*M*/l. (S. A. = 3,570 mC./m*M*).

The micro-homogenizer tubes, containing the incubation medium, were equilibrated with the appropriate gas mixture and stoppered. They were re-equilibrated after introduction of the tissue and intermittently during the incubation period. Mixtures of 95% O_2-5%CO_2 were used in aerobic experiments, or 95% N_2-5%CO_2 in anaerobic experiments. Before introduction into the vessels, the gas mixtures were saturated with water vapor by bubbling them through a series of flasks containing water, thus minimizing evaporation of medium from the incubation tubes. The micro-homogenizer tubes were shaken periodically throughout the incubation period.

Extraction procedure. The incubation period was terminated by adding an equal volume (50 μl.) of 10% trichloroacetic acid (TCA). An additional 100 μl. of 5% TCA were added. The plunger of each micro-homogenizer was inserted and the tissue homogenized. The plunger was removed and the tubes centrifuged for 4 minutes at 3000 r.p.m. in a clinical centrifuge, and the supernatant decanted (by capillary pipette). Subsequent washings and extractions were carried out in the original micro-homogenizer tube, the plunger being used to re-suspend precipitates. The tubes were cooled in an ice bath during the extraction and purification procedures.

The precipitate was washed with 200 μl. of 5% TCA (5 times) in order to remove free amino acids. The washed residue was extracted with 200 μl. of 95% ethanol; this was repeated once, and followed by a 95% ethanol-ether (1 : 1), and an ether wash. The residual protein, designated as the trichlor-precipitable protein residue (TPR), was suspended in 500 μl. of acetone, and aliquots of this were immediately transferred to planchets for counting. The term "protein" includes nucleoprotein precipitated by cold TCA.

Purification procedure. The supernatant of the first alcohol extraction, designated as the first alcohol-soluble fraction (ASF), was transferred to a new micro-homogenizer, and the alcohol removed by evaporation under a stream of nitrogen. The residue was washed twice with acetone (100 μl.), twice with ether (200 μl.), and then dissolved in 100 μl. of acid alcohol (75% ethanol acidified with HCl; pH < 1.0). After centrifugation, the supernatant was removed and the residue re-extracted with 100 μl. of acid alcohol. The acid alcohol supernatants were combined and transferred to a new micro-homogenizer. Four volumes of acetone were added; following centrifugation and decantation, the precipitate was redissolved in acid alcohol and aliquots transferred to planchets for counting.

The acetone and ether supernatants obtained were pooled and plated *in toto;* this fraction is designated as the lipid solvant fraction (LSF). The acetone supernatant was also plated *in toto*. The radioactivity in this fraction represents unprecipitated proteins and soluble peptides.

Insulin-I^{131}. The I^{131}-labeled insulin used in the recovery experiments was obtained from Abbott Laboratories; it had a specific activity of 2 to 5 mC./mg.

Radioisotope counting. In the experiments using C^{14}-labeled amino acids, triplicate 25-μl. aliquots were transferred to planchets and air-dried. The samples were counted to an accuracy of 5%, using a gas flow counter (Nuclear Chicago) under conditions in which the counting efficiency was 31%. In the studies using tritium, triplicate 25-μl. aliquots were pipetted into the counting vessels; 1 ml. of hyamine and 10 ml. of toluene containing 0.4% PPO (2,5-diphenyloxazole) were added. The samples were counted to an accuracy of 5% in a Packard Tri-Carb liquid scintillation counter at an efficiency of about 4%.

Results

The distribution of radioactivity recovered in the various fractions during the washing and extraction procedures is illustrated in Table I. The counts re-

Table I

Distribution of radioactivity in various fractions isolated from goosefish islet tissue incubated 2 hours in C^{14}-labeled leucine and valine

Step	Fraction	cpm†	%*	%**
A	TCA supernatant	1,460,000	96.7	
B	First TCA wash	41,500	2.8	
C	Second TCA wash	2,290	.15	
D	Third TCA wash	192	.01	
E	Fourth TCA wash	83	<.01	
F	Fifth TCA wash	54	<.01	
G	First alcohol-soluble fraction (ASF)	1,860	.12	31.4
H	Second alcohol-soluble fraction (ASF-2)	232	.02	3.9
I	Ethanol-ether fraction	25	<.01	.42
J	Ether fraction	14	<.01	.21
K	Trichlor-precipitable protein residue (TPR)	3,790	.25	64.0

† Expressed as cpm/200 μl. (total supernatant).
* % of total counts recovered.
** % of residual counts remaining after last TCA wash.

maining in the fifth TCA wash, presumably due to the presence of free C^{14}-amino acids, were 50 cpm/200 μl. in this experiment. This is only 3% of the counts recovered in the ASF. The counts in the ethanol-ether and ether extractions were 25 and 14 cpm/200 μl., respectively; these are less than 2% of the counts recovered in the TPR.

Recovery experiments were carried out in which insulin labeled with I^{131} was added to the incubation medium, and the extraction procedure followed. The insulin-I^{131} was added to the incubation medium along with islet tissue; the C^{14}-labeled amino acids were omitted. The results, summarized in Table II, indicate that 79 to 89% of the added radioactivity was recovered in the ASF at 0 hours of incubation (*i.e.*, when TCA was added immediately after the addition of insulin-I^{131} to the incubation medium). When insulin-I^{131} was incubated with islet tissue

TABLE II

Recovery of I^{131}-labeled insulin added to islet tissue

Experiment	Incubation time (hours)	Alcohol-soluble fraction (ASF)		TCA supernatant		Trichlor-precipitable protein residue (TPR)	
		cpm	% of total	cpm	% of total	cpm	% of total
1	0	125,000	89	8,980	6.4	8,100	5.8
2a (Av. of 2 samples)	0	105,000	79	13,500	10	14,500	11
2b	1	79,400	62	26,500	21	19,600	15
2c	2	64,300	49	49,000	37	17,600	13

Each vessel contained approximately .289 μC. of I^{131}-labeled insulin and 1.44 to 1.91 mg. of goosefish islet tissue.

for one and two hours, the counts recovered in the ASF decreased to 62 and 49%, respectively. This was associated with a corresponding increase in counts recovered in the TCA-soluble radioactivity. These data suggest that the added bovine insulin is partially degraded in the presence of islet tissue.

In a similar experiment, not illustrated, the counts in both the first alcohol-soluble (ASF) and second alcohol-soluble (ASF-2) fractions were determined; 80% of the added counts were recovered in the first, and about 9% in the second.

TABLE III

Incorporation of leucine-C^{14} + valine-C^{14} into goosefish islet and liver fractions

Tissue		0-hour control				2-hour incubation				Ratio $\frac{ASF}{TPR}$
		Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		
		No. of Samples	cpm†	No. of Samples	cpm†	No. of Samples	cpm†	No. of Samples	cpm†	
Islet	A*	10	326	10	150	22	5,900	22	7,440	0.79
	B**	2	96	2	131	3	3,860	3	6,480	0.61
Liver	A*	2	0	2	248	3	1,930	3	8,800	0.22
	B**	2	42	2	246	3	1,220	3	6,090	0.20

† The values given represent the average counts per minute/10 mg. of tissue.
* The concentration of leucine-C^{14} was 2.1 m*M*/l., S. A. = 8.75 mC./m*M*.
The concentration of valine-C^{14} was 1.6 m*M*/l., S. A. = 10.5 mC./m*M*.
** The concentration of leucine-C^{14} was 2.2 m*M*/l., S. A. = 7.80 mC./m*M*.
The concentration of valine-C^{14} was 3.0 m*M*/l., S. A. = 6.05 mC./m*M*.

TABLE IV

Effect of incubation time on leucine-C^{14} + valine-C^{14} incorporation into protein fractions of goosefish islet tissue

Time of incubation (hours	cpm/10 mg. of tissue: Alcohol-soluble fraction (ASF)	cpm/10 mg. of tissue: Trichlor-precipitable protein residue (TPR)
0	390	210
½	2,740	3,030
1	3,870	6,650
2	10,400	9,450
4	15,200	25,400
6	25,400	35,800
9	23,500	50,500
12	25,300	46,000

The concentration of leucine-C^{14} was 2.2 m*M*/l., S. A. = 7.80 mC./m*M*.
The concentration of valine-C^{14} was 3.0 m*M*/l., S. A. = 6.05 mC./m*M*.

On the basis of these recovery experiments, it was presumed that insulin present in islet tissue would likewise appear in the ASF. This assumption is consistent with the known solubility of insulin in acid alcohol (the pH of the ASF was approximately 2; this acidity is due to the presence of TCA extracted from the precipitate by alcohol). In similar experiments, where C^{14}-labeled amino acids were added to the incubation medium, the majority of the counts extracted by alcohol were likewise found in the first ASF.

A comparison of C^{14}-amino acid radioactivity incorporated into the various fractions of islet and liver is shown in Table III. The total number of counts incorporated into the ASF of islet tissue was approximately equal to that found in the TPR; the ratios of counts in the ASF/TPR were 0.79 and 0.61. By

TABLE V

Effect of aerobic and anerobic conditions on the incorporation of leucine-C^{14} + valine-C^{14} into goosefish islet tissue protein fractions

Experiment	Time of incubation (hours)	Alcohol-soluble fraction (ASF): No. of samples	ASF: cpm/10 mg. tissue: Aerobic	ASF: cpm/10 mg. tissue: Anaerobic	ASF: % inhibition	Trichlor-precipitable protein residue (TPR): No. of samples	TPR: cpm/10 mg. tissue: Aerobic	TPR: cpm/10 mg. tissue: Anaerobic	TPR: % inhibition
A*	0	2	113	113		2	383	383	
	2	3	2,900	239	91.8	2–3	4,500	1,450	67.8
B**	0	1	124	124		1	44.5	44.5	
	2	3	3,820	814	78.7	2–3	7,130	2,260	68.2

* The concentration of leucine-C^{14} was 2.1 m*M*./l., S. A. = 8.75 mC./m*M*. The concentration of valine-C^{14} was 1.6 m*M*./l., S. A. = 10.5 mC./m*M*.

** The concentration of leucine-C^{14} was 2.2 m*M*./l., S. A. = 7.80 mC./m*M*. The concentration of valine-C^{14} was 3.0 m*M*./l., S. A. = 6.05 mC./m*M*.

contrast, the total number of counts incorporated into the ASF of liver was much smaller than that in the TPR (ASF/TPR ratios of 0.20 and 0.22). Thus, islet tissue appears to incorporate a much greater fraction of the added counts into the protein component which is soluble in alcohol.

The effect of incubation time on the incorporation of amino acid radioactivity into the ASF and the TPR fractions is shown in Table IV. The counts incor-

TABLE VI

Effect of varying C^{14}-amino acid concentration on incorporation into goosefish islet fractions

Experiment	Sample	C^{14}-amino acid concentration m*M*/l.†	mg. of tissue	Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)	
				cpm/10 mg.	%*	cpm/10 mg.	%*
A	1	leucine 2.2 valine 3.0	5.84	4,110	100	6,380	100
	2	leucine 1.1 valine 1.5	8.58	2,220	68	4,200	71
	3		6.36	3,320		4,780	
	4	leucine 0.55 valine 0.75	5.94	3,690	101	5,160	83
	5		4.64	4,620		5,430	
B	1	leucine 2.2 valine 3.0	5.50	4,600	100	8,150	100
	2		4.99	5,480		9,450	
	3	leucine 0.22 valine 0.30	6.33	1,880	48	2,910	45
	4		5.04	2,920		5,010	
	5	leucine 0.11 valine 0.15	4.84	1,700	31	2,860	30
	6		6.56	1,340		2,380	

* Calculated as % of counts incorporated using maximum C^{14}-amino acid concentrations in each experiment (av. of duplicates).

† The specific activities were: leucine-C^{14}, 7.80 mC./m*M*; valine-C^{14}, 6.1 mC./m*M*.

porated into the ASF increased progressively with time of incubation (*i.e.*, at ½ hour, it was 2,740 cpm/10 mg. of tissue; at 2 hours, 10,400; and at 9 hours, 23,500). A similar increase with time was also observed in the TPR fraction. These results clearly indicate that the progressive increase in counts in the ASF is a result of a metabolic incorporation of the added amino acids. They also suggest that few, if any, of the contained counts are due to the adsorption of C^{14}-amino acids.

When the incubations were performed in the absence of oxygen (Table V), incorporation of radioactivity into the ASF was markedly diminished; the inhibition was 80 to 90%. Incorporation into the TPR was also inhibited in the absence of oxygen. Since the conditions of incubation and subsequent fractionation were identical, except in the substitution of 95% N_2-5% CO_2 for the oxygen mixture, these studies clearly indicate that the incorporation of amino acids is oxygen-dependent, and therefore related to an energy-utilizing process.

The original C^{14}-amino acid concentrations selected proved to be greater than that required for maximal labeling of the protein fractions. The effect of varying the concentrations of amino acids is shown in Table VI. When they were decreased to 0.55 m*M*/l. leucine-C^{14} + 0.75 m*M*/l. valine-C^{14} (*i.e.*, one-quarter of the original C^{14}-amino acid concentrations), the level of incorporation into the pro-

TABLE VII

Incorporation of amino acids into protein fractions of goosefish islet tissue: Comparison of leucine-C^{14} + valine-C^{14} of varying specific activities with leucine-H^3

Amino acid concentration (*m*M/l.) Sp. act. (mC/*m*M)	Leucine-C^{14}+Valine-C^{14} 2.1 8.8		Leucine-C^{14}+Valine-C^{14} 1.6 10.5		Leucine-C^{14}+Valine-C^{14} 2.2 7.8		Leucine-C^{14}+Valine-C^{14} 3.0 6.1		Leucine-H^3 .43 3,570			
Fraction	Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		ASF		TPR		ASF		TPR	
Time of incubation (hours)	0	2	0	2	0	2	0	2	0	2	0	2
Number of samples	10	21	10	20	11	33	11	32	1	5	1	5
cpm/10 mg. tissue ± S.E.*	211 ±63	5,320 ±681	106 ±25	6,540 ±1,040	305 ±48	5,720 ±420	67 ±18	8,560 ±466	2,200	48,100 ±4,000	204	41,500 ±2,150
Ratio ASF/TPR (2-hour values)	0.81				0.67				1.2			

* Standard errors were calculated using the equation: S.E. = $\sqrt{\frac{\sigma^2}{N}}$ where σ = standard deviation.

tein fractions was essentially unchanged. When they were decreased to 0.22 m*M*/l. leucine-C^{14} + 0.30 m*M*/l. valine-C^{14} (*i.e.*, one-tenth of the original C^{14}-amino acid concentrations), the level of incorporation was decreased by only 50%.

Table VII summarizes the results obtained using mixtures of leucine-C^{14} + valine-C^{14} of varying specific activities with those obtained using leucine-H^3. The counts incorporated per 10 mg. of islet tissue were dependent upon both the specific activities and the concentrations of the amino acids used. The incorporation into the ASF, using tritiated leucine, was 7- to 10-fold greater than that obtained with C^{14}-labeled amino acids. Considering that the counting efficiency of tritium was about 4%, whereas that of C^{14} was 31%, the theoretical number of counts incorporated into the ASF using leucine-H^3 was 60 to 80 times greater than that obtained using C^{14}.

TABLE VIII

Effect of added glucose on C^{14}-amino acid incorporation into goosefish islet tissue protein fractions

Sample no.	Glucose conc. mg./100 ml.	Experiment A					Experiment B				
		mg. tissue	Alcohol-soluble fraction (ASF)		Trichlor-precipitable protein residue (TPR)		mg. tissue	ASF		TPR	
			cpm†	%*	cpm†	%*		cpm†	%*	cpm†	%*
1	0	5.13	5,580	100	14,200	100	4.26	4,600	100	9,050	100
2	12.5	3.39	6,910	124	13,800	97.2	3.79	2,860	84.0	10,600	117
3	25	4.23	5,330	95.6	11,700	82 4	4.76	2,300	50.0	5,720	63.2
4	50	5.25	4,480	80.3	6,790	47.8	4.14	1,780	38.7	4,300	47.6
5	100	4.82	3,260	58.5	3,120	22.0	4.64	1,500	32.6	3,840	42.4
6	200	3.88	2,730	49.0	5,210	36.6	4.54	1,360	29.6	3,530	39.0

The concentration of leucine-C^{14} was 2.2 mM/l., S. A. = 7.80 mc./mM.
The concentration of valine-C^{14} was 3.0 mM/l., S. A. = 6.05 mC./mM.
* Calculated as % of counts found in sample No. 1 without added glucose.
† Expressed as cpm/10 mg. of tissue.

When the counts incorporated into the ASF and TPR fractions were compared, it was observed that the ratio of counts in the ASF/TPR was greatest when tritiated leucine was used, and lowest when the valine-C^{14} concentration exceeded that of leucine-C^{14}. These results suggest that the rate of incorporation of specific amino acids into protein differs for the ASF and TPR fractions.

The effect of adding glucose to the incubation medium is shown in Table VIII. The incorporation of C^{14}-amino acids into both the ASF and TPR fractions decreased progressively with increasing glucose concentration. At a concentration of 100 mg./100 ml., the incorporation was decreased by 40 to 70% of that obtained in samples incubated without glucose.

TABLE IX

Recovery of I^{131}-labeled insulin, added to acid alcohol, followed by precipitation with acetone

Sample no.	Unlabeled insulin μg./100 μl.	Insulin fraction (acetone precipitate)		Acetone supernatant	
		cpm†	% of recovered counts	cpm†	% of recovered counts
1	100	2,420	68.0	1,150	32.3
2	50	1,620	43.9	2,080	56.4
3	20	512	13.8	3,200	86.3
4	10	315	7.8	3,700	92.4
5	5	201	5.1	3,740	95.0
6	2	149	3.9	3,680	96.3
7	1	154	4.0	3,720	96.1
8	0.5	120	3.1	3,760	96.9
9	0	266	7.5	3,290	92.6

† Expressed as cpm/10 mg. of tissue.

Purification of the ASF was accomplished by employing a method based on the solubility of insulin in acid alcohol, and its precipitation by acetone. The ASF was dried and extracted with acetone and ether in order to remove lipids. The residue was dissolved in acid alcohol, centrifuged, and the supernatant transferred to a new tube. It was then precipitated by 4 volumes of acetone, and redissolved in acid alcohol. The efficacy of this procedure was tested by adding tracer amounts of insulin-I^{131} to various concentrations of non-radioactive insulin. It was found that in the presence of 100 μg. of unlabeled insulin per 100 μl. of acid alcohol, approximately 68% of the I^{131} radioactivity was recovered by this purification procedure (Table IX). When smaller amounts of unlabeled insulin were used, a corresponding decrease in recovery was obtained.

Upon purification of the ASF, illustrated in Table X, 65 to 90% of the counts recovered were found in the purified alcohol-soluble fraction; of the total counts recovered, the lipid solvant fraction (LSF) accounted for less than 10%, whereas the acetone supernatant accounted for approximately 10%. The total number of counts recovered in these three fractions varied, in different samples; they averaged 65.2% of the counts added: incomplete recovery would be expected when small amounts of insulin were present.

The protein contents of the purified ASF and the TPR fraction were determined; these values are expressed as μg. of protein per 10 mg. of tissue. The specific activities were expressed as cpm per mg. of protein. The results are given in Table XI.

Approximately 30 μg. of protein were recovered in the purified ASF isolated from 10 mg. of islet tissue. The specific activity of the purified ASF averaged 128,000 cpm per mg. of protein, whereas the specific activity of the TPR averaged 43,000 cpm per mg. of protein. Thus, the specific activity of the purified alcohol-

TABLE X

Purification of the alcohol-soluble fraction obtained from goosefish islet tissue incubated in C^{14}-leucine + C^{14}-valine†

Sample no.	Initial cpm/ 10 mg.	Total counts recovered cpm/ 10 mg.	% recovery	Purified alcohol-soluble fraction		Lipid solvant fraction (LSF)		Acetone supernatant	
				cpm/ 10 mg.	%*	cpm/ 10 mg.	%*	cpm/ 10 mg.	%*
1	7,630	2,920	38.1	2,480	85.8	230	7.9	171	6.43
2	10,500	5,260	50.2	3,440	65.5	1,350	25.6	469	8.92
3	5,890	6,160	105	4,770	77.5	646	10.5	760	12.3
4	4,590	4,740	103	3,900	82.3	448	9.43	400	8.45
5	4,880	3,400	69.8	2,600	76.6	462	13.6	336	9.86
6	4,440	3,520	79.3	2,840	80.6	269	9.48	418	11.9
7	9,740	4,000	41.2	3,580	89.8	269	6.75	146	3.66
8	8,870	4,740	53.5	4,140	87.0	340	7.16	280	5.89
9	13,300	6,750	50.8	5,360	79.5	454	6.74	920	13.7
10	8,660	5,280	61.0	4,320	81.9	520	9.85	435	8.25

* % of total counts recovered.

† The concentration of leucine-C^{14} was 2.2 mM/l., S. A. = 7.80 mC./mM. The concentration of valine-C^{14} was 3.0 mM/l., S. A. = 6.05 mC./mM.

TABLE XI

Specific activities of the purified alcohol-soluble fraction† and trichlor-precipitable protein residue

Sample	mg. tissue	Purified alcohol-soluble fraction			Trichlor-precipitable protein residue (TPR)		
		cpm*	μg. protein / 10 mg. islet tissue	Specific activity cpm/mg. protein	cpm*	μg. protein / 10 mg. islet tissue	Specific activity cpm/mg. protein
1	3.62	7,630	26.8	93,000	9,670	—	—
2	4.40	10,500	20.5	167,000	13,000	144	39,700
3	4.06	5,890	35.4	134,000	8,670	80.0	44,000
4	5.70	4,590	29.0	135,000	8,950	152	33,600
5	4.96	4,880	33.2	78,100	4,800	119	20,000
6	4.46	4,440	34.6	82,400	5,430	113	21,400
7	5.16	9,740	29.4	121,000	10,300	—	—
8	5.38	8,870	30.9	134,000	12,700	122	56,500
9	2.92	13,300	28.0	191,000	19,900	72.3	80,400
10	3.88	8,660	30.0	145,000	10,700	79.3	52,200
Averages ± S.E.**	4.45	7,850	29.9	128,000 ±10,800	10,400	110	43,500 ±6,590

† The purified alcohol-soluble fractions used were the same as shown in Table X.

* The incorporation of amino acid into the protein fraction during a two-hour incubation period is expressed as cpm/10 mg. of islet tissue.

** The standard errors were calculated as in Table VII.

soluble fraction is approximately three times greater than that of the trichlor-precipitable protein residue.

DISCUSSION

The results obtained clearly indicate that C^{14}- and H^3-labeled amino acids, incubated *in vitro* with islet tissue, are incorporated into the protein fractions. Since added I^{131}-labeled insulin is recovered in the purified alcohol-soluble fraction, it is presumed that insulin synthesized *in vitro* by islet tissue will similarly be found in this fraction. Although the total counts incorporated into the alcohol-soluble fraction are approximately equal to that incorporated into the trichlor-precipitable protein fraction, the specific activity of the alcohol-soluble fraction is three times greater than that of the trichlor-precipitable protein residue. The incorporation of C^{14}-amino acids into the alcohol-soluble fraction is increased with increasing periods of incubation. It is markedly inhibited when the incubations are carried out in the absence of oxygen, thus suggesting that the incorporation is dependent upon an active metabolic process which requires energy. The incorporation is also dependent upon the amino acid concentration; however, maximal values are reached at leucine and valine concentrations of about 0.25 m*M*/l.

The addition of glucose decreases the counts incorporated into both the alcohol-soluble fraction and trichlor-precipitable protein residue. A similar effect of glucose on the incorporation of alanine-C^{14} into TCA-insoluble protein was observed by Sinex *et al.* (1952). Since hyperglycemia stimulates the release of insulin from the β-cell (Lazarow, 1960), it would have been expected that glucose might

increase the rate of insulin secretion. This apparent inhibitory action of glucose might be explained by the appearance of intermediates (derived from glucose) which can be used for the synthesis of insulin; this hypothesis requires further study.

The specific activities of the ASF proteins obtained in the C^{14} studies (averaging 128,000 cpm/mg. of protein) and in the tritium studies (400,000 cpm/mg. of protein) are many times greater than those previously reported using mammalian pancreas incubated *in vitro*. For example, Pettinga and Rice (1952) isolated insulin containing 1600 cpm/mg. of protein; in the studies of Light and Simpson (1956), the specific activities were less than 1000 cpm/mg. of protein.

In order to further characterize the radioactive protein found in the ASF, additional studies have been done using a specific immuno-precipitation method. Under the appropriate conditions, serum prepared from guinea pigs previously immunized to bovine insulin will form a specific insulin-antibody complex. Using the method of Skom and Talmage (1958), this soluble complex may be precipitated by reacting it with anti-guinea pig serum obtained from rabbits immunized to normal guinea pig serum. Studies carried out by Carl R. Morgan (personal communication) using insulin-I^{131} as a marker, have indicated that approximately 85% of added insulin-I^{131} (1 μg.) combines with the specific insulin-antibody and is precipitated by the anti-guinea pig serum (rabbit). Control studies, identical to the above, except for the substitution of normal guinea pig serum for the anti-insulin guinea pig serum, yielded insignificant counts in the precipitated complex.

Similar immuno-precipitation experiments have been carried out using the tritium-labeled goosefish alcohol-soluble fraction (specific activity = 400,000 cpm/mg. of protein); these strongly support the thesis that a significant fraction of the tritium label found in the alcohol-soluble fraction represents insulin which has been synthesized *in vitro* from added leucine-H^3.

The counts incorporated into the alcohol-soluble fraction of islet tissue can be expressed as mμ moles of amino acid per gm. of islet tissue per hour. In the leucine-H^3 experiment (where the amino acid concentration was 0.43 m*M*/l., specific activity = 3,570 mC./*M*, and the counting efficiency assumed to be 4%), an incorporation of 48,100 cpm/10 mg. of tissue during a two-hour period is equivalent to 7.5 mμ moles of leucine per gm. of islet tissue per hour. Since 6 of the 51 amino acid residues in insulin are leucine, the maximal rate of insulin synthesis would not be greater than 1.25 mμ moles of insulin per gm. per hour. Converting this to units per day, the maximal rate of insulin synthesis would not be greater than 4.5 units per gm. of islet tissue per day. The human pancreas contains approximately 1 gm. of islet tissue (Lazarow, 1960), and it has been reported that the totally depancreatized man can be maintained with doses of insulin as low as 20 units per day (Rockey, 1943; Goldner and Clark, 1944). It should be noted that the *in vitro* incubation studies using goosefish islet tissue were carried out at 25° C. (whereas insulin synthesis in man takes place at 37° C.) and that the rate of chemical reactions doubles with each 10° increase in temperature. Thus, the calculated maximal rate of insulin synthesis *in vitro*, based on the observed rate of amino acid incorporation into the alcohol-soluble fraction and corrected for temperature, would approximate 10 units per gm. per day; this approaches the order of magnitude of the physiologic rate of insulin synthesis by human islet tissue.

Summary

1. Goosefish islet tissue, incubated *in vitro* with C^{14}- or H^3-labeled amino acids, showed significant labeling of the protein fractions.

2. The purified alcohol-soluble fraction is presumed to contain the insulin which is synthesized *in vitro*.

3. The rate of amino acid incorporation into the alcohol-soluble fraction increases progressively with increasing time of incubation; it is decreased in the absence of oxygen.

4. The addition of glucose decreases the number of counts incorporated into the alcohol-soluble fraction.

5. In the C^{14}-amino acid incorporation experiments, the specific activity of the purified alcohol-soluble fraction (128,000 cpm/mg. protein) is three times greater than that of the trichlor-precipitable protein residue.

6. These studies support the thesis that amino acids, added to the islet tissue *in vitro*, are incorporated into insulin.

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ABSORPTION AND EXCRETION OF COPPER ION DURING SETTLEMENT AND METAMORPHOSIS OF THE BARNACLE, BALANUS AMPHITRITE NIVEUS [1]

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For hundreds of years ships have been sheathed with metallic copper or coated with copper-containing paints to prevent or minimize surface fouling. Sheathing is clearly subject to mechanical failure and once breached exposes the entire structure to attack. Copper-containing paints are effective only as long as enough toxic substance leaches from the coating to establish and maintain lethal concentrations within the barrier layer of water through which members of the fouling complex must approach the structure. These coatings gradually decrease in effectiveness during their service life, because the diffusion gradients necessary to sustained leaching are at best temporary. Millions of dollars annually and countless hours of ship time are devoted to protecting hulls from settling barnacles.

The general biology of the fouling problem, with primary emphasis upon the complex of fouling organisms, has been intensively studied in this laboratory for several years. It is the object of this communication to describe the pathways of copper absorption, circulation, and elimination by settling barnacles, and to discuss the mode of action of this toxic substance.

In a previous paper (Bernard and Lane, 1961) we have described the early stages in metamorphosis of *Balanus amphitrite niveus*. Histological localization of copper in developmental stages was determined by the dithio-oxamide (D.T.O.) method of Okamoto *et al.*, described by Gomori (1952). Specimens representing all the settling stages from normal sea water were fixed in absolute ethanol, stained for copper and then lightly stained with hematoxylin and eosin. Whole mounts, together with serial sections cut 5 and 10 microns thick in various planes of orientation, were prepared. The same procedure was repeated for specimens which had been exposed to 200 mg./kg. of copper ion in sea water for one hour.

Copper Distribution in Barnacles from Normal Sea Water

The concentration of copper ion in Gulf Stream sea water is approximately 0.005 milligrams per kilogram. *B. amphitrite niveus*, both planktonic larvae and attached forms, were collected in Biscayne Bay, where copper concentration is somewhat more variable, and were held in Millipore-filtered Gulf Stream sea water for one hour prior to histological preparation.

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Scientific contribution No. 338 from the University of Miami, Institute of Marine Science.

A. *Planktonic cyprids*

Whole mounts of planktonic cyprids treated with D.T.O. show characteristic greenish-black precipitate of copper in the following sites (Fig. 1):

1. In ovoid to cuboidal vacuoles within the loose connective tissue underlying the postero-lateral carapace.
2. In shapeless masses in the connective tissues of the antero-ventral region.
3. In the epithelium lining the stomach.
4. In the epithelium lining the posterior two-thirds of the hind-gut.

The lateral copper-containing vacuoles of whole mounts fixed in absolute ethanol were quite consistent in pattern and number, varying from 47 to 54 in the eight specimens studied. The size ranged from 2.4–3.5 microns × 4.0–9.5

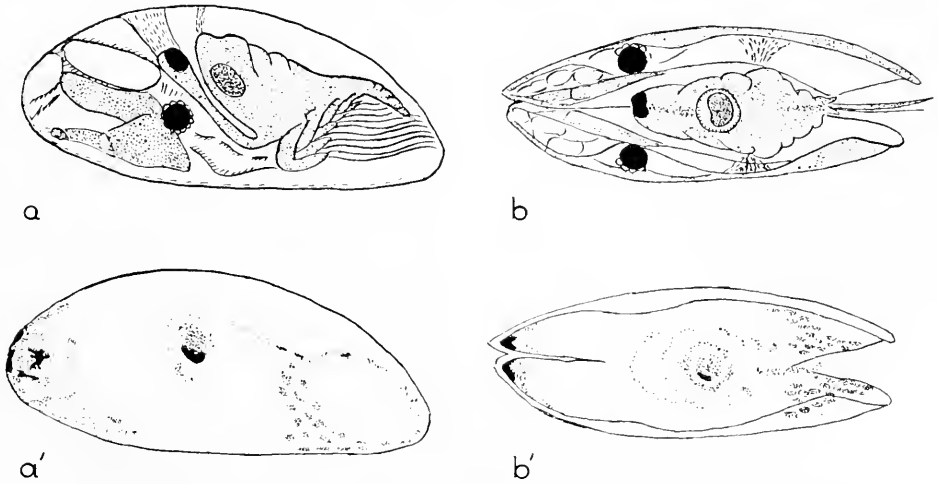

FIGURE 1. Distribution of copper ion in planktonic cyprids from normal sea water. a, a': cyprid morphology, lateral aspect and corresponding areas of copper ion accumulation. b, b': cyprid morphology, dorsal aspect and corresponding areas of copper ion accumulations.

microns. Copper is also concentrated within the loosely organized tissues of the anterior third of the animal, but masses of pigment granules normally present in the integument of the anterior region may mask discrete vacuolar arrangement. The outline of the stomach and of the posterior portion of the hind-gut were clearly marked by copper precipitate in whole mounts of D.T.O.-treated cyprids. A very heavy copper deposit was present in the food mass within the gut, and traces of copper-rich materials were distributed elsewhere in the gut of some animals. No copper was found in the anterior one-third of the hind-gut, in other thoracic structures, or in the digestive caeca.

While copper appears to be uniformly distributed through the stomach epithelium in whole mounts, histological sections reveal that its distribution is not uniform. The epithelial cells of the antero-ventral segment of the stomach contain more copper than cells in other areas. Granular copper deposits occur in the free

edges of the cells, distal to the nucleus and mucous secretions. In cross-section the hind-gut is lined by 5 to 8 low pyramidal cells in which copper is restricted to a semi-lunar cap next to the lumen. Copper is found only in the posterior portion of the hind-gut, although there appears to be no histological difference between these areas and the region adjacent to the stomach.

Clarke (1947) observed that barnacle larvae concentrate copper from sea water and maintain internal concentrations higher than ambient. This is a common occurrence among invertebrates utilizing hemocyanin as a respiratory pigment. Secretion of copper into the lumen of the alimentary canal of barnacle larvae in normal sea water reveals a continual turnover, suggesting that the copper ion may participate actively in the normal physiology of these animals. A functional role is confirmed by the apparent storage of copper in postero-lateral vacuoles and in the loose connective tissue at the anterior of the body.

Since copper ions are moved against the concentration gradient it is reasonable to suggest an active process requiring metabolic work by the cells of the absorbing surface.

B. *Settled cyprids*

The sites of copper concentration in this stage are essentially the same as those of the planktonic cyprid. Anterior deposits of copper disappear with the degeneration and thinning of the connective tissue of this area following attachment. Secretion of copper by the hind-gut epithelium continues even though the entire tubular digestive system is displaced by ventral rotation of the thorax in this stage. Very light deposits of copper appear within the folds of the now inactive thoracic legs. The postero-lateral vacuoles are fewer in number than in the previous stage and are concentrated at the ventral surface. Movement of the

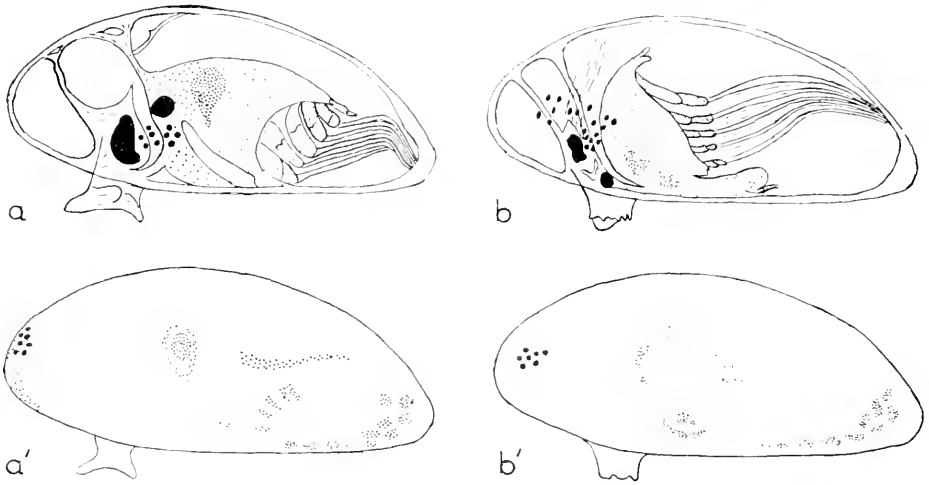

FIGURE 2. Distribution of copper ion in settled cyprid, lateral aspect. a, a′: structure shortly after attachment with corresponding sites of copper deposition. b, b′: structure just prior to decortication with corresponding sites of copper deposition.

general body tissues toward the ventral side during this stage probably accounts for this concentration (Fig. 2).

C. *Decorticated settlers*

Although a few individuals representing this stage contained small amounts of copper in isolated patches in the general parenchyma of the body, there was no consistent pattern of copper distribution.

D. and E. *Young barnacles, without and with shell plates*

No areas of copper concentration were noted, either in whole mounts or in histological sections of specimens taken during these stages.

Distribution of Copper in Barnacles Exposed to Excess Copper

Barnacles in the same metamorphic stages as above were exposed for one hour to Millipore-filtered Gulf Stream sea water to which 200 mg./kg. of cupric ion had been added. Although this concentration is 40,000 times that of normal sea water, it is yet sub-lethal. To dissolve more copper than this in sea water one must reduce the pH.

A. *Planktonic cyprids*

In spite of the great increase of available copper, the size and number of copper-containing vacuoles and the apparent concentration in connective tissues beneath the carapace are not appreciably augmented. These observations suggest either that copper reserves can only be built up over a longer period than the one-hour exposure period employed in this study, or that copper reserves are maximal in normal sea water and can not be increased further.

Deposits of copper in the epithelium of the hind-gut were much more numerous in these animals than in those from normal sea water. In barnacles from untreated sea water, copper deposits were present in approximately half the epithelial cells of the gut. The entire hind-gut epithelium, except for the portion nearest the stomach of specimens exposed to high concentrations of ambient copper, was dark with copper precipitate. Increased copper in the gut epithelium was not directly proportional to the increase in ambient copper concentration, but probably accurately reflected the increased absorption rate (Fig. 4).

In nature barnacle cyprids freely exchange copper with the medium. Established reservoirs within the body insure internal homeostasis in a medium which may fluctuate in copper content. The only sites where significant concentrations of copper normally occur are the copper-containing vacuoles and the epithelium of the hind-gut. In other areas, particularly the possible pathways from absorption sites to point of elimination, concentrations are too low to be revealed by the histochemical technique used.

When ambient copper concentrations were increased 40,000-fold, however, the amount of copper reaching the epithelium of the stomach and hind-gut appeared to exceed the excretory capacity of these cells. Copper accumulated, both in the excretory sites and in the areas of absorption. These sites of copper accumulations,

then, reveal the absorptive surfaces and sites of final elimination from the organism and suggest pathways of distribution.

The surfaces of the cyprid which are covered with a thickened cuticle, *e.g.,* legs and antennae, contained no deposits of copper. If copper were, in fact, absorbed through these regions it was immediately translocated so no local concentration occurred. The surfaces of the thorax covered by thinner cuticle, however, contained particulate copper within the epithelial cells. The flattened epidermal cells covering the posterior part of the thorax were heavily laden with uniformly distributed copper deposits, particularly within dorsal folds. The cuboidal to columnar

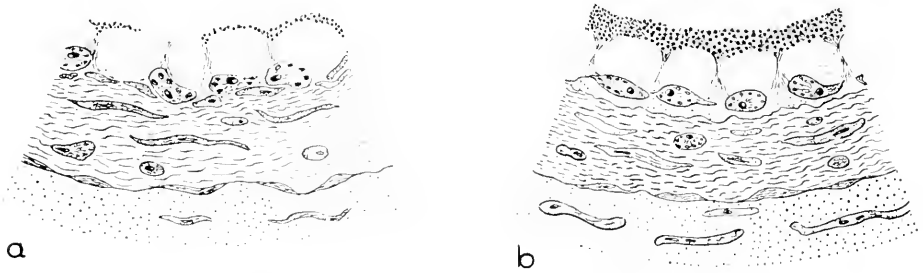

FIGURE 3. Secretion of copper ion by the gastric epithelium. (a) In cyprid from normal sea water. (b) In cyprid exposed to excess ambient copper ion.

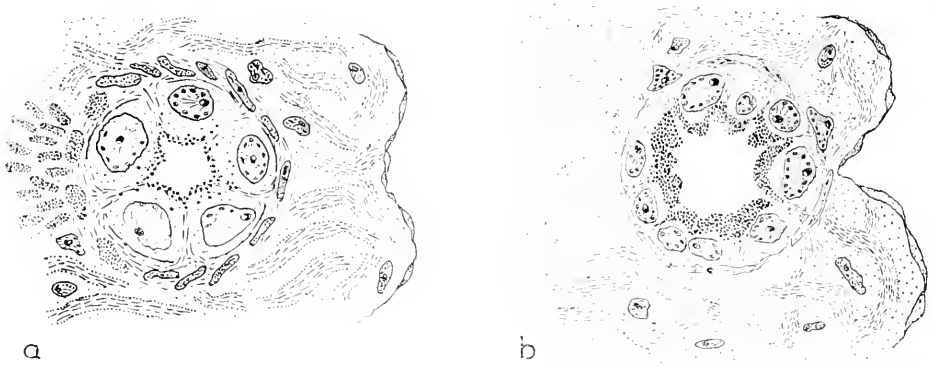

FIGURE 4. Secretion of copper ion by hind-gut epithelium. (a) In cyprid from normal sea water. (b) In cyprid exposed to excess ambient copper ion for one hour.

epidermal cells over the anterior portions of the thorax were quite heavily marked by precipitated copper in treated specimens. Thin projections of the integument with little subepidermal connective tissue, *e.g.,* mouth parts or caudal flaps near the anus, contained copper in the integument as well as in some underlying cells. Diffuse accumulations of copper were also found in the wall of the fore-gut (Fig. 5, a and b).

The internal pathways through which copper circulates from absorptive sites on certain outer surfaces to excretory regions of the gut wall were not identified

in this study. No consistent copper precipitate was observed in nerve or muscle fibers nor in the connective tissue underlying the epithelial layers. This suggests that the circulation of copper occurs in solution in blood and body fluids, where concentrations remain below detectable levels. Excretion is associated with local concentrations of copper.

Failure of the anterior third of the hind-gut to participate in copper exchange probably reflects a functional difference between this segment of the gut and all the rest of the alimentary canal (Fig. 5, c and d).

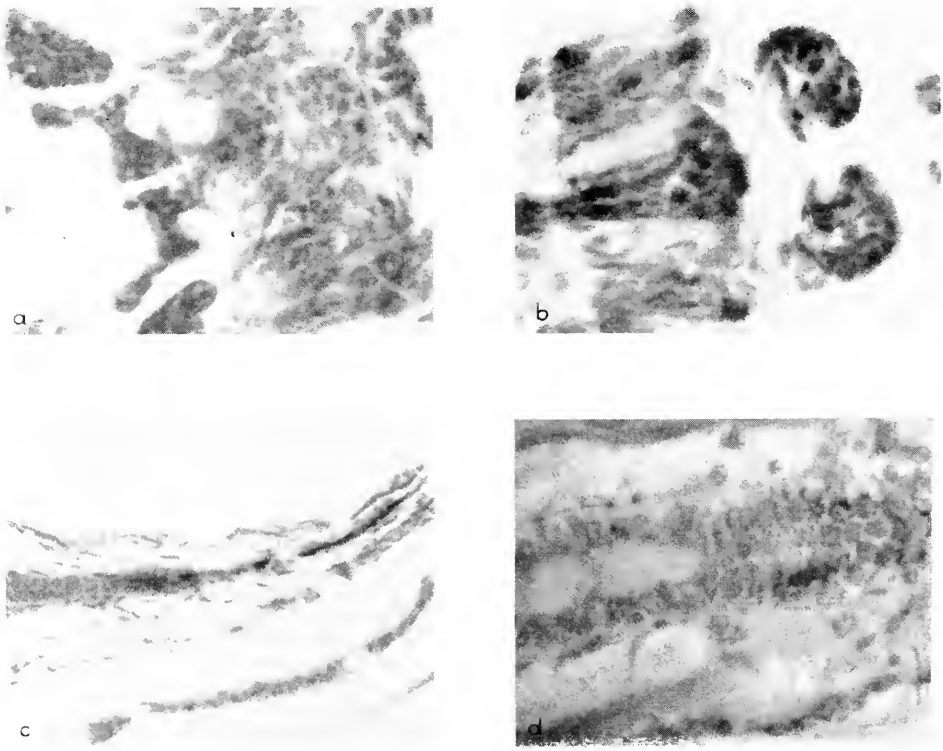

FIGURE 5. Copper precipitate in D.T.O.-treated cyprids exposed to 200 p.p.m. Cu^{++} in sea water. (a) In mouth parts and in epithelium of the fore-gut. (b) In hind-gut epithelium, anus, and anal flaps. (c) Along the posterior two-thirds of the hind-gut but not in anterior region. (d) At the point of transition between Cu^{++}-secreting and non-secreting zones.

No attempt was made to identify the mechanism of copper uptake, *i.e.*, whether it is by active ion transport or simple diffusion. This diffuse distribution of copper within absorbing cells supports the latter supposition. Active transport is suggested, however, by the polar distribution of precipitated copper within the cuboidal epidermal cells of the carapace. If the epicuticle of the carapace is impervious to ions, as is generally supposed, this copper must have been absorbed from water in the mantle cavity through the epithelium of the "mantle" and then accumulated in the epidermal cells of the carapace. The accumulations in mouth

parts and anal flaps substantiate the observation that absorbed copper moves away from the absorbing surface until some obstacle hinders its further movement.

B. *Attached cyprids*

As was described for planktonic barnacle cyprids, little increase was observed in either size or number of copper-containing vacuoles in parenchymatous connective tissue as the medium was enriched in copper ion. A trace of copper precipitate was found near the anterior pigment mass in some specimens where none was observed in animals of the same stage from normal sea water. The addition of copper ion to the medium was accompanied by a marked increase in the number and density of deposits in the hind-gut epithelium similar to that described for planktonic cyprids. Stomach secretion of copper, while increased over that

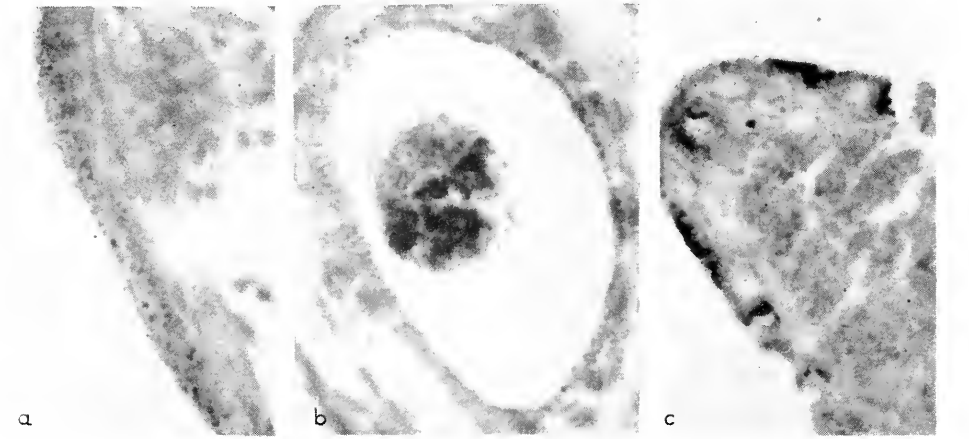

FIGURE 6. Loci of copper accumulation in attached cyprids exposed to excess ambient copper and stained with D.T.O. technique. (a) In the connective tissue underlying the cyprid carapace. (b) Within the food mass and in the secretory region of the stomach epithelium. (c) In the thoracic epidermal cells and in the secretory portion of the hind-gut.

shown by animals from this stage in normal sea water, was somewhat less than had been observed in the previous stage (Fig. 6).

If the rate of absorption of copper were proportional to its concentration within thoracic epidermal cells, then clearly less copper was taken up by cyprids after attachment than before. It is suggested that this reduced absorption results from decreased exposure of thoracic surfaces to sea water because the cirri are inactive during this stage and the mantle circulation is correspondingly reduced.

Sites of heavy copper concentration in the planktonic cyprid, such as the ventrolateral surfaces of the anterior part of the thorax, show somewhat less copper deposition in attached barnacles. The dorsal surface of the posterior half of the thorax, where the gut approaches the surface most closely, contained a fairly heavy concentration of copper (Fig. 6c). Some copper was present in the epithelium lining the fore-gut. The mouth parts and anal flaps contained considerably less copper than in the previous stage. Where precipitated copper was scant in the

planktonic stage, *e.g.*, the mid-thorax and the limb folds, none was found in the attached animals.

The distribution of copper in cells underlying the carapace resembled that already described for planktonic cyprids exposed to a high ambient copper concentration, both in the early phases where epidermal and cuticular layers are in normal contact and later when they become separated (Fig. 6a).

C. *Decorticated settlers*

Isolated particles of precipitated copper were seen in some sections of normal animals in this stage. When the copper concentration of the medium was increased to 200 ppm, there was no increase in detectable copper in the parenchymatous sheath characterizing animals in this stage. In the enriched medium, however, the cylindrical cell mass, previously described (Bernard and Lane, 1961), contained deposits of copper (Fig. 7b). These precipitates were confined to

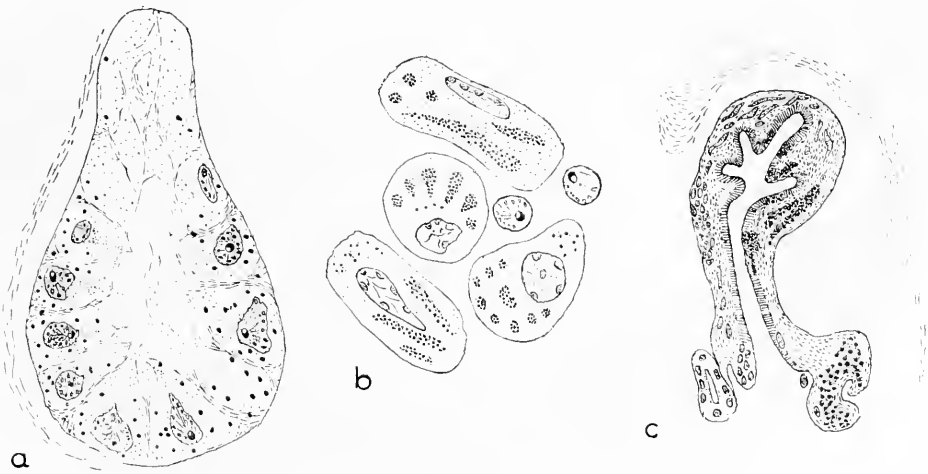

FIGURE 7. Regions of copper ion concentration in later phases of the decorticated stage by barnacles exposed to 200 p.p.m. Cu^{++} for one hour. (a) In the cytoplasm of peripheral organelle cells. (b) In fascicular vacuoles of the cylindrical cells. (c) Within one lobule of the glandular labyrinth.

fascicular inclusions within the cytoplasm of the cylindrical cells and were noted only in the latest phases of this stage when cell orientation is haphazard.

We suggested previously that inclusions within these cells are reservoirs for histolyzed material to be employed in subsequent histogenesis and organogenesis. The deposition of copper in these inclusions implies a dynamic exchange with the ambient sea water and directs attention to a possible role of these cells in concentration of other ions from sea water during morphogenesis. Vital staining experiments also indicated a rapid exchange between parenchyma vacuoles and the medium during this stage.

During the same phase at which these deposits were noted, but not in earlier specimens, some copper was detected in the cytoplasm of the cells in the peripheral

organelles. This material was perinuclear in distribution, but revealed no consistent orientation with respect to the cell surface. (See Figure 7a above.) At the same time copper secretion was observed in a few cells of a tubule connected with the large mucin-secreting organ, believed to be the primorium of the gut.

In the glandular labyrinth, a very small compound tubular gland was found to be actively secreting copper. The D.T.O.-copper precipitate appeared in the gland epithelium and in irregular blotches in the subepithelial connective tissue, as well as in the cells of a finger-like projection which marked the juncture of the gland with the main organ (see Fig. 7c).

In view of the general characters of this stage, *viz.,* no ingestive apparatus and development within a closed gelatinous sheath, it appears that copper absorption occurs only by diffusion from the medium. The lack of localized deposits in specimens from normal sea water suggests that copper ion is not essential to physiological processes characteristic of this stage. The presence of particulate deposits of copper during only one phase of development strongly suggests that free exchange with the ambient medium takes place during a restricted period of development. This period may include concentration of other ions. Activity of certain cells or cell groups, in the absence of a clear general pattern, in either secreting or absorbing copper may be a vestigial functional character from a previous stage, or may presage future activity of these cells.

D. and E. *Young barnacles*

In the transition from the decorticated larva to the young barnacle the parenchymatous sheath becomes a stiff supportive casing for the organism. The homogeneous mass of connective tissue cells and fibers is clothed by epithelial layers on inner and outer surfaces. The inner epithelium consists of squamous cells and the outer covering epithelium is at first cuboidal. The epithelial surfaces

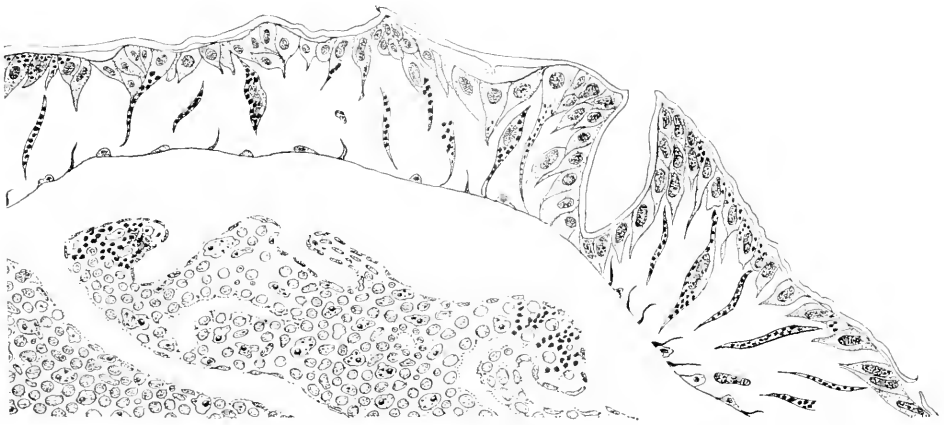

FIGURE 8. Presence of copper precipitate during the early period of shell formation in a specimen exposed to excess copper ion and treated with D.T.O. Note accumulation within the long cells penetrating the forming shell and in certain areas of the glandular region of the thorax.

are interconnected by protoplasmic extension of intermediate cells; the nuclei of these cells generally form a central layer. Within the cytoplasm of these long cells the copper precipitate was found in D.T.O.-treated specimens of the young stage before compartmental plates appeared (Fig. 8). Continued development results in a thickening of these radial cells and formation of lateral extensions from them, until a network of interlacing cytoplasmic strands unites the two epithelia. The copper deposit is no longer present after the strands have thickened. From these facts we assume that these cells are active in the absorption of solutes from sea water into the organism during this last phase of metamorphosis. It is possible that the appearance of copper in these areas at this stage is coincidental with active concentration of other ions; for example, calcium must be concentrated from sea water at this time to form the calcareous plates of the adult barnacle.

The cells in contact with the substrate form a stratified epithelium within whose lowest layer some copper is deposited.

Posterior to the stomach of the young barnacle a series of branching tubules develop which appear to be digestive caeca. In several specimens studied a copper deposit was discovered, but always in only one, or at most two, lobes of this organ. This phenomenon was noted in animals both with and without compartmental divisions.

Summary and Conclusions

1. Sites of copper ion concentration in barnacle cyprids from normal sea water, both planktonic and settled, suggest that the barnacle absorbs copper from the medium through permeable surfaces and eliminates excess copper by excretion through the epithelia of the hind-gut. Reservoirs within which copper storage may occur have been identified. A metabolic role is suggested for copper in normal physiology of barnacle settling stages.

2. Copper deposits are consistently absent from both decorticated settlers and from two stages of the young adults.

3. Exposure of planktonic cyprids and early settled forms to a high-copper medium resulted in increased rate of elimination from the gut epithelium, and identified various surface areas of the thorax as absorption sites. Transport of copper ions in solution from absorption surfaces to points of excretion is probably by way of circulating body fluids.

4. Copper deposits were observed in decorticated settlers only during later phases of this stage and in areas previously identified as active points of exchange with ambient sea water.

5. Absorption of copper from sea water containing excess copper during the stages of shell formation occurs via single-cell extensions through the thickness of the forming shell. Elimination of copper through a lobe of the digestive caecum was also noted during this period.

6. Copper localization studies on post-decortication stages suggest that copper absorption is coincident with absorption of other ions, particularly calcium, which is highly concentrated during these developmental periods.

7. Copper absorption during the cyprid-form developmental stages occurs through respiratory surfaces. It is suggested that the anti-fouling effectiveness of copper is due to interference with normal respiratory exchange.

8. The effect of the copper-ion concentration in the ambient medium on oxygen uptake of cyprids is currently being investigated and will form the substance of a subsequent communication.

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AMINO ACID SENSITIVITY OF THE DACTYL CHEMORECEPTORS OF CARCINIDES MAENAS[1]

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In contrast with the present advanced state of knowledge concerning the physiology of insect chemoreceptors the remainder of the Arthropoda are essentially unknown in this regard excepting electrophysiological observations on the xiphosuran, *Limulus* (Barber, 1956, 1961), and several fresh-water and terrestrial forms (Hodgson, 1958). Although behavioral studies have demonstrated a well developed contact chemical sense generally distributed over the integument in marine crustaceans (Luther, 1930), a physiological analysis of its sensory basis has yet to be reported. Such an analysis is likely to be of more than passing interest since, considering the diet of crustaceans, their chemoreceptors are likely to be responsive to proteins and amino acids. They may consequently be expected to present an array of relationships between molecular configuration and receptor activation unlike any which have been well studied in arthropods.

In this report we describe a simple electrophysiological preparation of crustacean chemoreceptors and present evidence concerning their responses to a number of substances, principally amino acids. A preliminary summary of this work has been given (Case, Gwilliam and Hanson, 1960).

Materials and Methods

While only first walking leg dactyls of male *Carcinides maenas* were used in these experiments, chemoreceptor activity was readily demonstrable in the leg nerves of various other decapods including *Libinia emarginata*, *Callinectes sapidus*, and *Paguris pollicaris*.

The dactyl was prepared for electrical recording by removing all proximal segments and leaving intact approximately a 1-cm. length of nerve consisting at this level of two prominent bundles which both possess chemoreceptor fibers. A bridge of wax was used to support the dactyl with its tip in air and the nerve immersed in sea water. Small bundles of nerve fibers were dissected and arranged on bare platinum electrodes for recording in air or mineral oil, with a recording system consisting of a Grass P5 A.C. amplifier and Tektronix 502 oscilloscope. A loudspeaker monitor was an extremely useful adjunct to photographic recording.

Chemical stimuli were applied to the dactyl as uniform-sized drops of stimulant in filtered, autoclaved sea water. Immediately before application of a test stimulus the dactyl tip was exposed to several rapidly applied drops of sea water in order to

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adapt mechanoreceptors as much as possible. During all experiments the preparation was frequently tested with *Mytilus* extract (prepared by grinding the soft parts of a *Mytilus* in about 25 ml. of sea water) or .001 to .005 *M* l-glutamic acid and discarded once responses to these fell below initial magnitudes. Ordinarily, deterioration of the preparation presented little difficulty. With reasonable care a chemoreceptor bundle remained in satisfactory condition for an hour or longer.

Responses of the dactyl chemoreceptors to test substances were evaluated by comparing the activity induced by a drop of the test substance with the effect of a drop of .001 *M* l-glutamic acid. Data were evaluated from film records and by direct aural and visual comparisons of oscilloscopic activity. For purposes of tabulation these responses were recorded on a scale of 4 in which 0 was equal to a response indistinguishable from the effect of a drop of sea water and 4 was equal to the effect of .001 *M* l-glutamic acid.

Results

1. *General*

In nearly all preparations many nerve fibers could be isolated which mediated responses to *Mytilus* extract. Except in rare instances these were difficult to isolate

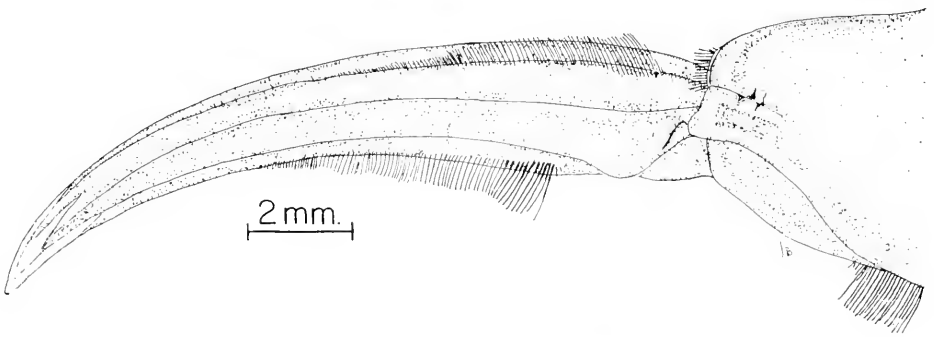

Figure 1. Dactyl of first walking leg of *Carcinides maenas*.

from larger fibers carrying mechanoreceptor impulses. Indeed, there is every possibility that at least some of the chemoreceptor units themselves may respond to mechanical stimulation, although it is clear from our data that not all do. In practice, the nearly unavoidable mixing of the two types of activity was not particularly bothersome since the mechanoreceptor responses were of short latency and adapted rapidly, while the slowly adapting chemoreceptor units had considerably longer latency, ranging from 15 to 250 msecs., measured from the first mechanoreceptor spike and varying inversely with stimulant concentration.

The nature of the sensory endings mediating chemoreceptor activity in the dactyl remains unknown. Application of sufficiently localized stimuli proved so difficult that about all that can be said is that chemoreceptor activity originates from the distal one-third of the dactyl as well as from more proximal regions. While this does not eliminate a chemoreceptive role for the long mechanoreceptor hairs of the proximal two-thirds of the dactyl (Fig. 1), it makes certain that other chemoreceptors must be present. These may be the Büschelorganen of Luther (1930) which

TABLE I

Response thresholds of Carcinides dactyl chemoreceptors

Substance	Number of dactyls tested	Intensity of response	Concentration (*M*)
A. Glutamic acid and related substances:			
l-glutamic acid[1]	18	3	5.0×10^{-5}
d-glutamic acid[6]	7	1	5.0×10^{-4}
		0	5.0×10^{-5}
glycyl-l-glutamic acid[1]	6	1	4.0×10^{-4}
		0	4.0×10^{-5}
l-glutamine[2]	12	1	5.0×10^{-4}
		0	5.0×10^{-5}
n-acetyl-l-glutamic[5]	7	1	1.0×10^{-3}
		0	1.0×10^{-5}
reduced glutathione[1]	7	1	5.0×10^{-3}
		0	5.0×10^{-4}
α-methyl glutamic acid[5]	3	1	3.0×10^{-2}
		0	1.0×10^{-3}
B. Other amino acids:			
glycine[1]	6	1	1.0×10^{-4}
		0	1.0×10^{-5}
l-ornithine[5]	3	1	1.0×10^{-2}
		0	1.0×10^{-3}
d-aspartic acid[6]	6	2	1.2×10^{-2}
l-aspartic acid[1]	6	1	1.2×10^{-2}
d-leucine[6]	7	2	2.5×10^{-2}
l-leucine[6]	7	1	2.5×10^{-2}
l-proline[2]	6	2	5.0×10^{-2}
		0	2.5×10^{-2}
γ-amino butyric acid[1]	5	1	0.2
		0	0.1
C. Organic acids:			
glutaric acid[4]	8	2	3.0×10^{-2}
		0	1.0×10^{-3}
α-keto glutaric acid[1]	5	2	5.0×10^{-2}
succinic acid[3]	6	2	1.0×10^{-3}
		0	1.0×10^{-4}
acetic acid[3]	2	1	0.1
D. Miscellaneous			
sucrose[3]	3	1	0.25
lactose[3]	3	0	0.25
methanol[3]	2	1	0.10
n-propanol[3]	2	1	0.10
n-butanol[3]	3	1	0.50
sea water	2	1	150%

Sources of chemicals: [1] Nutritional Biochemicals Corp.; [2] Matheson, Coleman, and Bell; [3] Fisher Scientific Corp.; [4] Eastman; [5] Sigma Chemical Company; [6] Mann Research Laboratories; [7] Merck and Company.

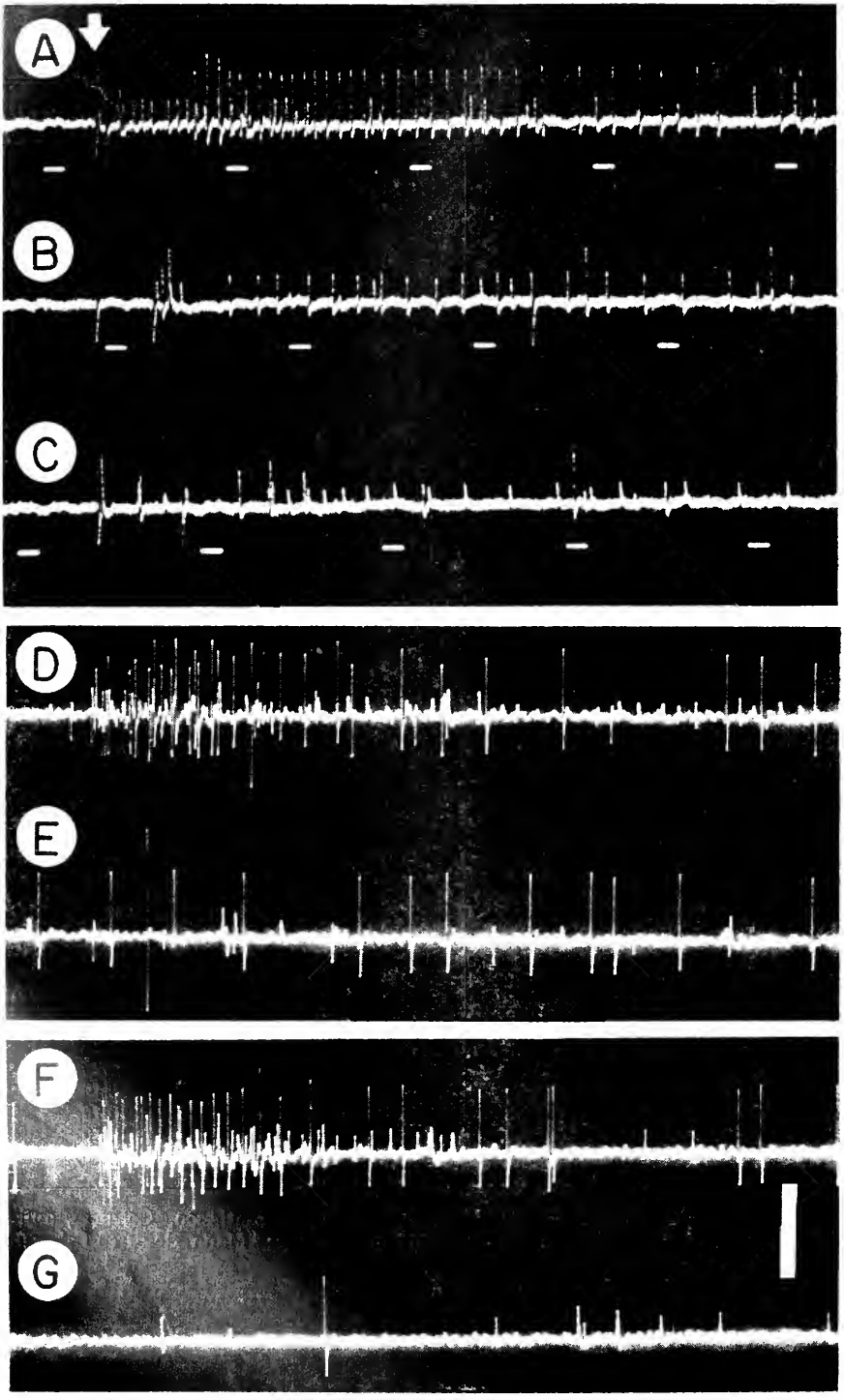

FIGURE 2.

we have also observed extending to the dactyl tip, especially in the grooves of the heavily chitinized cap. Both these and the mechanoreceptor hairs have been shown to be permeable to aqueous solutions of crystal violet in preparations for which we are indebted to Dr. E. H. Slifer, who finds the presence of such permeability in insects to be characteristic of chemoreceptive endings (Slifer, 1960).

2. *Receptor specificity*

In considering these data it must be remembered that our experimental technique has confined the study to a selected category, namely those sensory units which are responsive both to *Mytilus* extract and dilute glutamic acid. It may therefore be presumed that these observations on specificity are not applicable to the total chemoreceptor population of the dactyl.

As shown in Table I, *Carcinides* dactyl chemoreceptors have limited sensitivity to many stimuli, which include various alcohols, sugars, acids, amino acids, and hypertonic sea water. They are moderately sensitive to a few amino acids and exhibit marked sensitivity only to l-glutamic acid among the substances tested. Although these receptors are somewhat responsive to acidity, that property of l-glutamic acid cannot be the primary source of its stimulatory ability since the dilution at effective concentrations is so great that there is a negligible pH change in sea water. At higher concentrations the receptors do not distinguish between neutralized and acid glutamic solutions. Further strong evidence of a high order of specificity in the glutamic response is the fact that all derivatives of glutamic acid which were available to us were no more active than glycine.

Of the three sets of optical isomers tested, those of glutamic acid and leucine were distinguished by preparations of only one to three units and all three by preparations containing many active units. In some instances (Fig. 2, A, B, C) the optical isomers of glutamic acid activate different units. We were unable to detect differences in the responses of single units to d- and l-aspartic acid although this does not preclude the existence of such differences because only a few such preparations have been tested. The characteristic responses to d- and l-leucine are illustrated in Figure 2, D–G, where d-leucine produces a considerably more prolonged burst than its antipode, although the initial phases of the responses to the two isomers are nearly identical in this instance.

In the light of the pronounced specificity which the dactyl chemoreceptors have been shown to have towards glutamic acid it becomes of interest to inquire into the nature of the active element of *Mytilus* extract. Amino acids are almost certainly involved since the extract does not lose potency upon boiling for five minutes and is dialyzable. Moreover, the same receptor population seems to be involved in the response to glutamic acid and to *Mytilus* extract since adapting the dactyl to either abolishes the response to the other.

FIGURE 2. Responses of *Carcinides* dactyl receptors to chemical stimulation. A, 5×10^{-3} *M* l-glutamic acid; B, 6×10^{-4} *M* l-glutamic acid; C, 3×10^{-2} *M* d-glutamic acid; D, 2.5×10^{-2} *M* d-leucine; E, same as D after elapse of 0.5 second; F, 2.5×10^{-2} *M* l-leucine; G, same as F after elapse of 0.5 second. A–C and D–G are from the same preparations. In A–C a larger mechanoreceptor unit is active and indicated by arrow to show chemoreceptor latency. Time base 100 mscs. between markers in A–C and the same throughout. Bar at lower right, 50 μV.

3. *Behavioral correlations*

In confirmation of Luther's observations, blinded *Carcinides* oriented readily to bits of mussel placed near any limb. Before the animal made the orienting movement, the nearest dactyl frequently probed the bit of tissue previous to making a precisely executed turn to present the tissue to the mouthparts. Inert materials were ignored. When *Mytilus* extract or .001 *M* l-glutamic acid was applied to the walking legs of crabs restrained on their backs in water shallow enough to expose all limbs, thus assuring stimulus localization, no sign of recognition could be seen. However, under the same conditions application of either substance to the chelae caused the beginning of feeding movements even before the stimulated claw was touched to the mouthparts. Touching the chelae of an unrestrained crab with either substance was followed by movement of the chelae to the mouth and chewing without orienting movements of the whole animal.

Discussion

The dactyl chemoreceptors of *Carcinides* are considerably more sensitive to amino acids than any arthropod preparation so far described and, indeed, approach in sensitivity the remarkable performance of insects responding behaviorally to sugars (see tabulation in Dethier and Chadwick, 1948, p. 244). Hence, when one considers that feeding responses were induced in *Carcinides* with pure solutions of glutamic acid, together with the high concentrations in animal tissues of glutamic acid, both free and bound (Meister, 1957), it becomes clearly possible that effective localization of food can be made simply on the basis of detection of low concentrations of amino acids.

The poor response of the dactyl preparation to reduced glutathione, known to be a highly specific inductor of feeding responses in at least two coelenterates (Loomis, 1955; Lenhoff and Schneiderman, 1959), reduces the possibility that there is a feeding-inducing substance common to large groups of animals, a suggestion which receives further support from the fact that the major feeding stimulus in the mosquito, *Culex pipiens,* is yet another compound, adenylic acid (Hosoi, 1959). It would therefore seem unwise to venture generalities concerning feeding-inducing substances except that they are likely to be of low molecular weight.

Since the observations of Piutti (1886) the optical isomers of a number of amino acids have been known to produce different tastes in man. The natural isomers may be tasteless, "meaty" in the case of glutamic acid, or bland, while d-amino acids commonly are sweet (Berg, 1953). Occasionally an isomer falls in one human taste modality and its antipode in another: l-leucine and l-isoleucine are bitter and their antipodes are sweet. These relationships, involving radical changes in taste upon subtle structural modification, have never been subjected to neurophysiological investigation despite the long time they have been known. The present findings appear to be the first to extend these observations to organisms other than man and are particularly significant in that they show at least some optical isomers of amino acids are distinguishable strictly in terms of the activity of small chemoreceptor populations. Despite the limited nature of this survey it is already apparent that at least three means exist by which these isomers can elicit differing receptor activity: they may simply activate different receptors, or have different threshold concentrations or adaptation times for the same receptors.

The molecular basis of these possible modes of action obviously cannot be discussed in the light of available direct experimental evidence. However, it is interesting to recall that metabolic systems acting upon d-amino acids are quite rare in multicellular organisms. To mention a pertinent example, active transport of amino acids across the mammalian intestine is specific to the l-isomers (Fridhandler and Quastel, 1955). Such observations lend support to various commonly invoked receptor activation schemes in which stimulant-receptor complexes are envisaged as involving formation on the basis of van der Waal's forces (see discussion by Dethier, 1956). But should more instances appear in which d-amino acids have more long lasting effects than their l-isomers, as appears to be the situation with d- and l-leucine, it may become necessary to consider the possibility of chemical processes functioning in receptor clearance, even though the behavior of the insect sugar receptor argues to the contrary.

Summary

1. Dactyl chemoreceptors were electrophysiologically demonstrated in *Carcinides maenas, Libinia emarginata, Callinectes sapidus,* and *Paguris pollicaris.*

2. In *Carcinides* the receptors respond to a boiled dialysate of *Mytilus,* and to l-glumatic acid at dilutions in excess of 5.0×10^{-5} *M*. Derivatives of glutamic acid and other amino acids are less stimulatory.

3. Optical isomers of glutamic acid, aspartic acid and leucine are distinguished by small populations of *Carcinides* dactyl receptors.

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BEHAVIORAL ASPECTS OF PROTEIN INGESTION BY THE BLOWFLY PHORMIA REGINA MEIGEN[1]

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The problem of specific hungers correlated with unique metabolic requirements has been studied extensively in the vertebrates. The greatly augmented sodium chloride ingestion of persons suffering from adrenal insufficiency is a classical example. Aside from other pathological examples, the most widely studied normal instance of specific hungers is that associated with pregnancy.

It is not altogether surprising that deviations from a standard maintenance diet should occur at times of metabolic stress. Specific hungers have long been known to occur among insects during periods of egg development. Many mosquitoes require a blood meal before they can bring their eggs to ripeness. Similarly, many flies need a meal of protein for oviposition.

While much attention has been directed toward the developmental and hormonal aspects of this phenomenon, there have been few investigations into the behavioral background, specifically in answer to such question as: does the insect actually seek sources of protein? does it ingest protein preferentially? Pospíšil (1958) studied the olfactory responses of a number of saprophilic flies to such odors as skatol, which is associated with decaying proteinaceous material. He found that the fly, during the period of egg development, responded positively to skatol even when it had fed to repletion on carbohydrate; at other times the replete fly was unresponsive. Strangways-Dixon (1959, 1961) reported that the ratio of protein and carbohydrate ingestion by *Calliphora erythrocephala* Meigen varied during the reproductive cycle and that protein was ingested in relatively large quantities during early stages of egg growth as compared to low ingestion during the period of yolk formation. Carbohydrate ingestion followed an inverse course.

The studies reported in this paper were directed toward an understanding of the changes in feeding behavior during the reproductive cycle of the fly *Phormia regina* Meigen and the mechanisms underlying them.

Materials and Methods

Three types of feeding experiments were undertaken. First, flies were placed in individual cylindrical cages constructed of Nylon mesh. Each cage measured 2 cm. in diameter and 5 cm. in length. Through the floor of the cage projected the tips of two 5-ml. volumetric pipettes that had been bent into the shape of a J. The pipettes, which were supported by a clamp and base, served as support for the cage. Every 24 hours the volume of fluid ingested from each pipette was measured by refilling with a hypodermic syringe to the original fluid level and then reading the

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volume directly from the syringe. Evaporation controls were run concurrently (Dethier and Rhoades, 1954).

Second, flies which had been fastened to sticks according to the technique of Dethier and Chadwick (1947) were fed to repletion, and the duration of feeding recorded. Since under given conditions there is a constant relation between the duration of feeding and the volume of fluid imbibed (Dethier, Evans and Rhoades, 1956), the amount of fluid taken could be calculated when desired after sample crops had been removed and weighed for calibration.

Third, flies from which the wings had been removed were allowed to run free on horizontal surfaces where they would encounter lines of fluid drawn in concentric circles around them.

Finally, ingestion was studied by the above-mentioned techniques in flies from which either the ovaries, corpus allatum, or median neurosecretory cells had been removed. Surgical procedures when used were those described by Thomsen (1952) and Dethier and Bodenstein (1958).

In addition to studies of ingestion, preliminary electrophysiological measurements of receptor activity were made by recording through the side wall of the chemoreceptive hairs after the method of Morita (1959). The equipment and techniques were those of Wolbarsht and Dethier (1958).

The flies were from a culture maintained in the laboratory since 1947. Originally homogenized liver was the protein employed for testing. Since similar results could be obtained from a 10% solution of Difco Brain-Heart Infusion, this was eventually substituted in all experiments. It contained, in addition to infusions of calf brains and beef hearts, 2.5 g. disodium phosphate, 5 g. sodium chloride, 2.5 g. bactodextrose, and 10 g. proteose peptone per liter of dry material. For some electrophysiological tests a crystalline bovine hemoglobin was employed. Sucrose was the carbohydrate employed.

Results

Patterns of protein ingestion

Since flies were not able to survive longer than a maximum of four days on a diet of protein alone (*e.g.,* liver homogenate, brain-heart extract), their feeding behavior over long periods could be studied only by providing carbohydrate. It was decided to provide separate sources of protein and carbohydrate concurrently rather than to mix the two. The intake of unadulterated protein could thus be studied.

Under these circumstances the volume of protein consumed daily was found to vary markedly over the lifetime of the fly. There was, furthermore, a difference between the feeding patterns of the sexes. Males, whether mated or not, gradually increased their intake from the time of emergence until the fourth to eighth day (Fig. 1). Thereafter the value reached a low level which was sustained until death. The pattern was similar for virgin females (Fig. 2), but the amount of protein consumed was greater. Mated females, on the other hand, laid eggs sometime between the tenth and fifteenth day, and within 24 hours again increased their protein consumption (Fig. 3). The quantity consumed daily by flies that had not laid eggs was always greater than that consumed by flies at any period after they had laid their first batch of eggs.

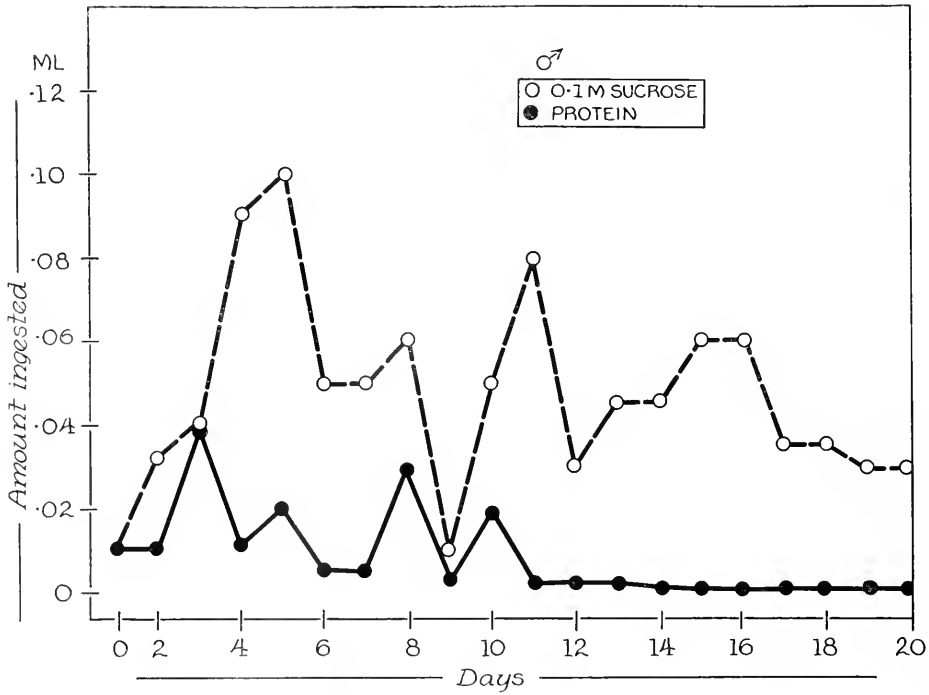

FIGURE 1. Daily intake of 0.1 *M* sucrose and brain-heart extract by a male blowfly in a two-choice situation.

Patterns of carbohydrate ingestion

When carbohydrate (0.1 *M* sucrose) was the only material offered to flies, the pattern of daily intake over the lifetime of the fly was the same for both sexes. Little was taken on the first two days after emergence. Thereafter the intake reached a high value and gradually declined until death some 50 days later for the longest-lived males and 60 days later for the longest-lived females. During this period the daily intake exhibited marked fluctuations, but these were related to differences in activity correlated with variations in the climate of the laboratory. The experiments were conducted under constant lighting but not under constant temperature, humidity, and barometric pressure.

Preferences

If the flies had free access to both protein and carbohydrate at all times from the day of emergence, carbohydrate was nearly always taken in greater volume than protein. If, however, the flies were denied access to protein and maintained on a minimal (0.0001 *M*) carbohydrate diet for the first five days of adult life, the subsequent pattern of carbohydrate and protein intake was quite different. In the case of the males, protein intake remained very low, as before, but carbohydrate intake was very high the first two days (Fig. 4). In the case of virgin females protein

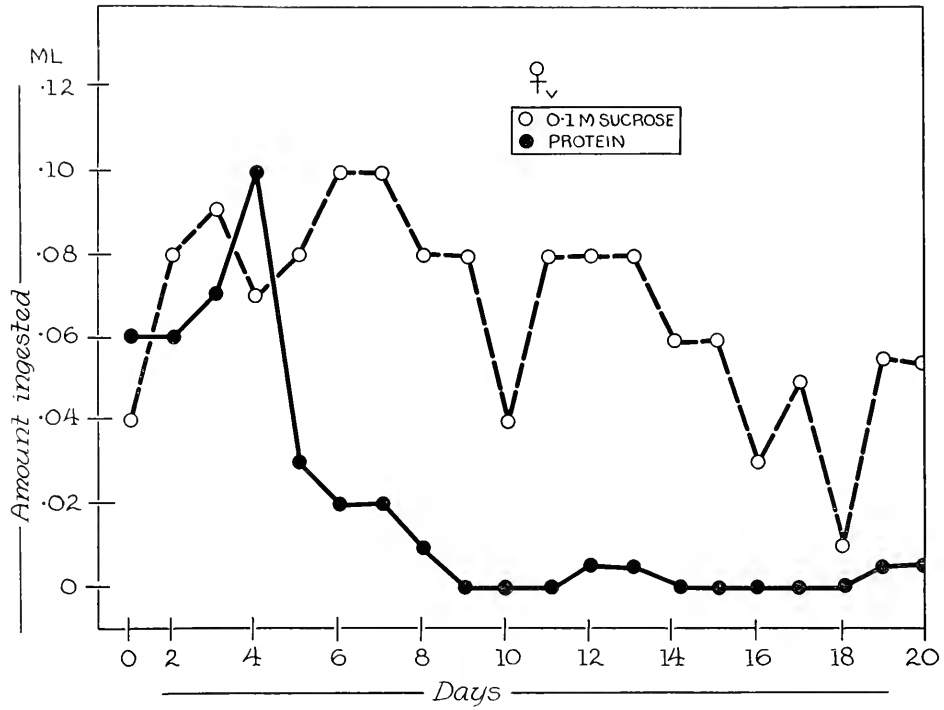

FIGURE 2. Daily intake of 0.1 *M* sucrose and brain-heart extract by a virgin female blowfly in a two-choice situation.

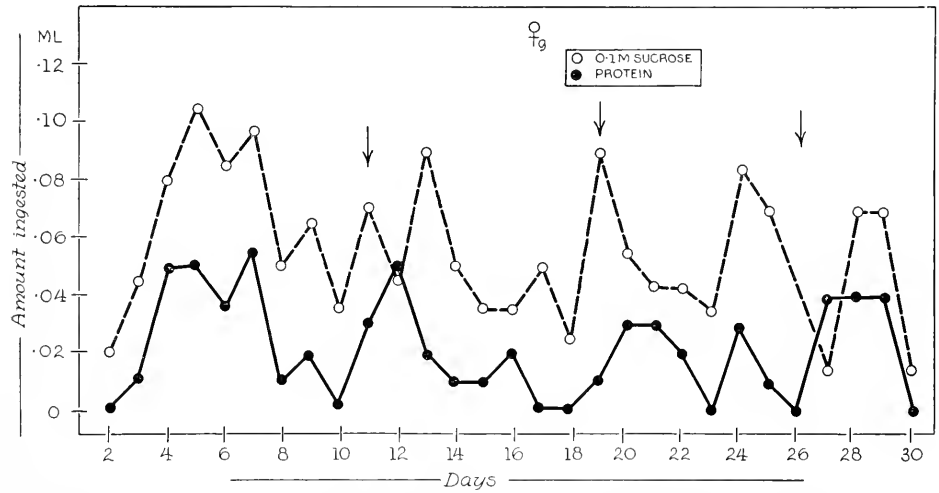

FIGURE 3. Daily intake of 0.1 *M* sucrose and brain-heart extract by a gravid female blowfly in a two-choice situation. Arrow indicate days on which eggs were laid.

intake was very high the first two days and exceeded carbohydrate intake (Fig. 5). If females were denied protein for 10 days, the subsequent preference was even more marked. After 20 days of protein deprivation, a protein preference still existed but was no more pronounced than it had been on the tenth day.

Concentration effects

In the foregoing tests the concentration of sucrose selected (0.1 *M*) was that which flies normally consume in greatest volume over a 24-hour period (Dethier, Evans and Rhoades, 1956). It is not, however, the most highly stimulating con-

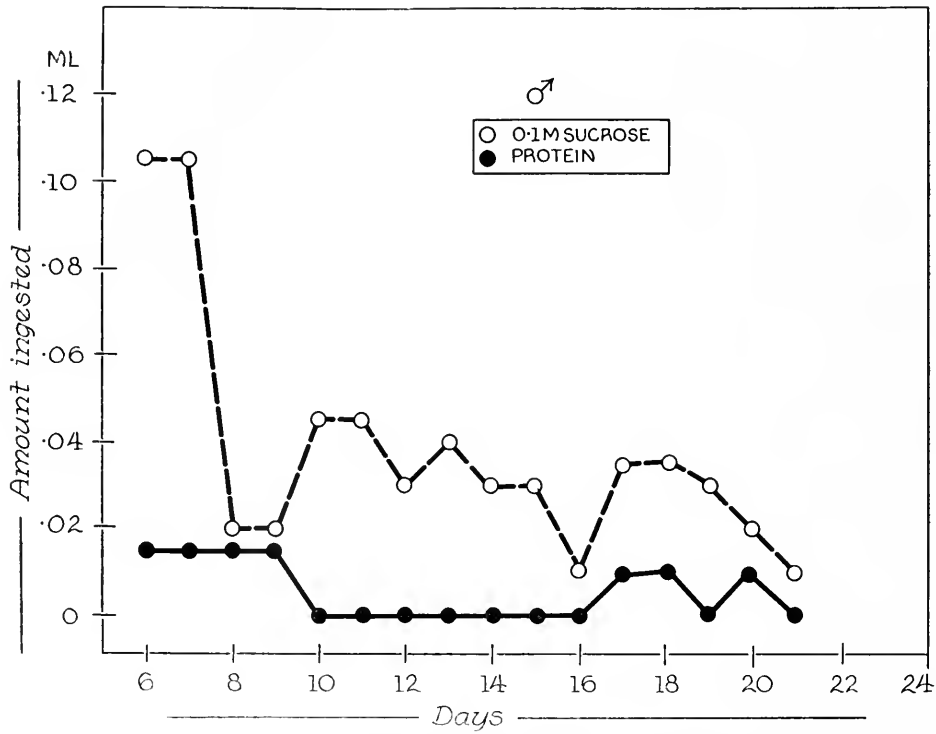

FIGURE 4. Daily intake of 0.1 *M* sucrose and brain-heart extract by a male blowfly in a two-choice situation when it has been deprived of protein for five days.

centration insofar as the sense organs are concerned. Consequently, other series of comparisons were made between protein and various concentrations of sucrose. It is clear from Figure 6 that the availability of a highly stimulating sugar greatly reduced the intake of protein. Conversely, when only a weakly stimulating sugar was available, protein intake was markedly enhanced (Fig. 7).

Initial responses

While the volume consumed has a certain validity as a test of preference, a much clearer insight into the behavior of the fly is obtained by observing its reactions

immediately upon presentation of the different solutions. For this purpose a fly was placed on a sheet of non-absorbent hydrophobic paper and three concentric rings of solution were drawn around it with a camel's-hair brush. In one set of tests the rings from center to outside were in the order water, protein, sucrose; in another set, water, sucrose, protein. Three kinds of flies were tested under these conditions: five-day-old males, starved 24 hours, and previously maintained on 0.1 *M* sucrose; five-day-old virgin females with a similar history; five-day-old virgin females 24 hours starved and previously maintained on protein solution.

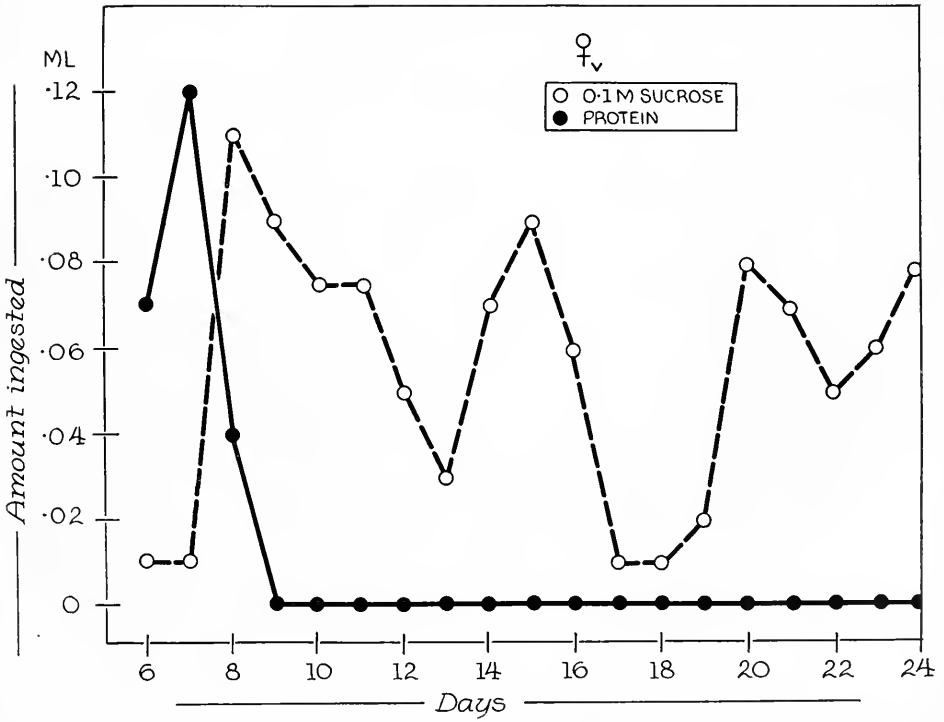

FIGURE 5. Daily intake of 0.1 *M* sucrose and brain-heart extract by a virgin female blowfly in a two-choice situation when it has been deprived of protein for five days.

All flies upon encountering water stopped and drank to repletion; thereupon the water ring became a barrier. Each time a leg encountered the ring the fly turned away where it had previously turned toward the water. This change in response was in itself interesting because it suggested that a solution that was initially acceptable had truly become repellent. Tests in which the water had been absorbed into the paper (thus presenting no unbroken surface) showed, however, that flies after sucking to repletion merely walked across the damp area instead of being repelled. The differences in behavior in the two cases may be explained if we assume that the ring of water stimulated two sets of receptors, the water receptors and mechanoreceptors (the effect of surface tension), the former mediating acceptance, the latter,

rejection. In the thirsty fly the acceptable stimulus overrides the unacceptable one. This balancing of antagonistic stimuli acting on the tarsi and the change in effectiveness with change in internal state is well known (Dethier, 1955; Dethier and Evans, 1961).

Having drunk water, the flies turned away from it. Upon each new encounter they avoided it. After a few minutes, however, their behavior changed. They now waded through and continued until they encountered the next ring of solution. When six-day-old females which had been maintained since emergence on a protein-free diet encountered the protein ring first, they drank the solution avidly, then turned away from it, then followed one of three patterns upon encountering 0.1 *M* sucrose: (1) drank some 0.1 *M*, then ignored it, but would drink 1.0 *M* sucrose if it was presented; (2) ignored 0.1 *M* sucrose, drank 1.0 *M*; (3) ignored all sucrose. If they encountered the sugar ring first, they fed fully on sugar, then drank protein while repeatedly ignoring sucrose. If, however, after drinking protein they were offered 1.0 *M* sucrose, they invariably drank it.

Males maintained on a sucrose diet alone and females which had had protein 24 hours before the test tended to act alike. Encountering protein first they took little or none of it but then drank considerable amounts of sucrose. If they encountered sugar first, they drank a great deal and then virtually ignored protein.

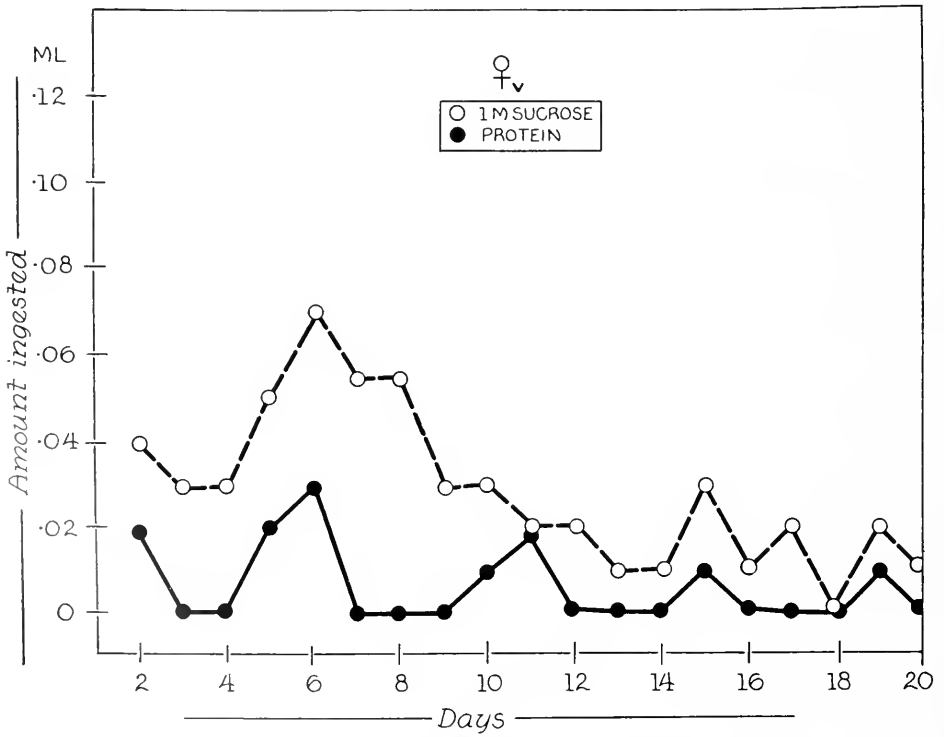

FIGURE 6. Daily intake of 1.0 *M* sucrose and brain-heart extract by a virgin female blowfly in a two-choice situation.

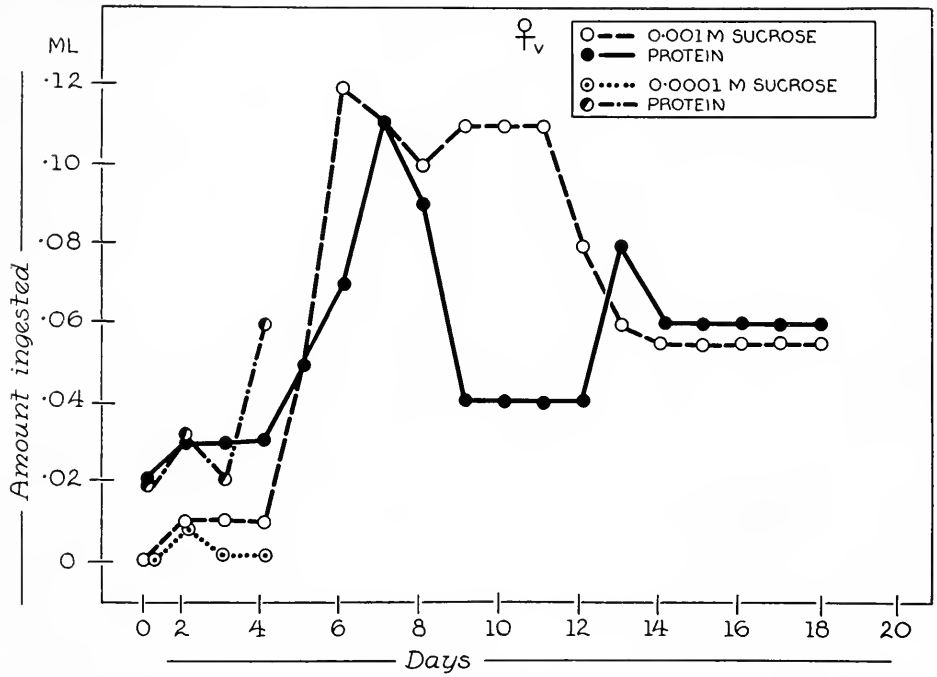

FIGURE 7. Daily intake of sucrose and brain-heart extract by a virgin female blowfly when the protein was paired with 0.001 *M* sucrose and when it was paired with 0.0001 *M* sucrose.

Number and duration of visits in a two-choice situation

In order to assist in correlating the behavior of flies taking single drinks with the measurements of total daily intake, records were made of the number and duration of visits made by flies in the two-choice situation. The records showed that females which had been denied protein made approximately equal numbers of visits to each of the two pipettes but that they took only small nips of the carbohydrate and long draughts of the protein.

Males and females which had had free access to protein also made equal visits to both pipettes but took very few drinks of protein. Drinks of sugar were more hearty.

Anosmic flies

Since all of the protein solutions employed possessed distinct odors, an attempt was made to assess the role of odor by measuring intake and observing the reactions of flies that had been rendered anosmic by removal of the antennae, labellum, and labial palpi. Anosmic flies were tested in all of the situations already described. In no case did their behavior differ from that of the normal flies.

Contact chemoreceptors

It was clear from observing the behavior of flies in the ring tests that they were able to differentiate between protein and sucrose by means of the tarsi and the

labellum. Accordingly, tests were made on the chemosensory hairs of these appendages. Small drops of either protein or 0.1 *M* sucrose were applied to tarsal and labellar hairs of protein-hungry females and the presence or absence of proboscis extension noted. In each case care was taken first to satiate the fly with water and to test each hair first with water. About 40 different labellar and 20 different tarsal hairs were tested. The tests showed that some hairs were sensitive both to protein and to sugar; others, to sugar only. At this time no hairs were found which were sensitive to protein but insensitive to sugar.

Preliminary electrophysiological findings are in accord with these behavioral results, but the matter requires more extensive investigation before the activity of

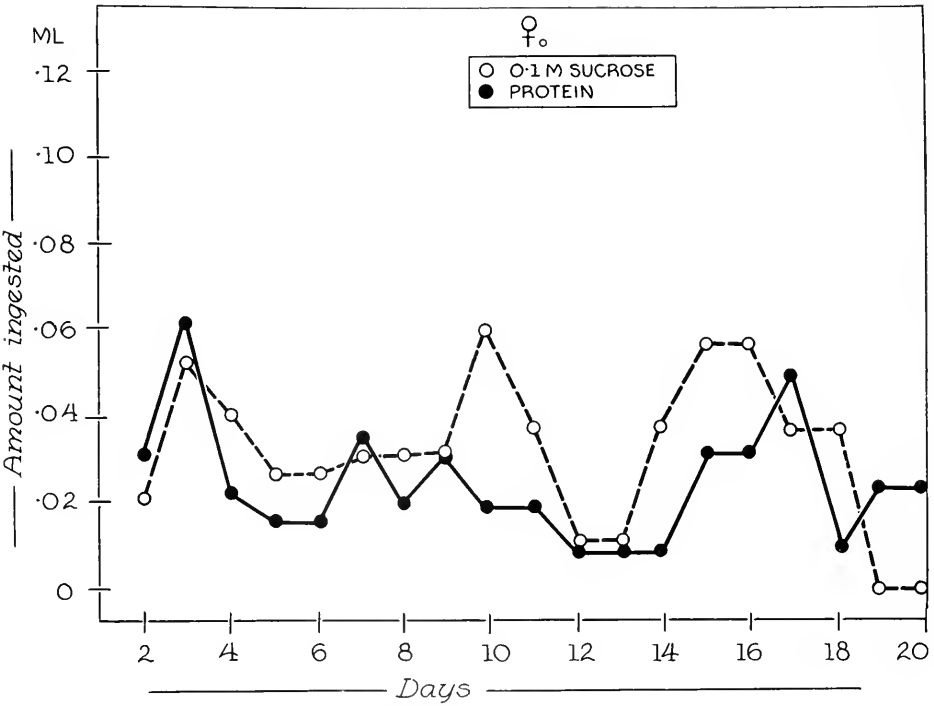

FIGURE 8. Daily intake of 0.1 *M* sucrose and brain-heart extract by a virgin female blowfly from which the ovaries have been removed.

each of the several neurons in the hair is understood. One of the long (ca. 300 μ) marginal labellar hairs tested was sensitive to water, fructose, NaCl, brain-heart extract, and crystalline hemoglobin. Activity was detected in three fibers (Fig. 10). One fiber, as Mellon and Evans (1961) have shown for these hairs, responded to water, one fiber to sugar, and one to sodium chloride. When sugar was applied, both the water fiber and the sugar fiber responded (Fig. 10B). When 1 *M* sodium chloride was applied, the water fiber was suppressed and only the salt fiber responded (Fig. 10D). When a mixture of fructose and sodium chloride was applied, three fibers (water, sugar, and salt) responded (Fig. 10G). When brain-

TABLE I

Total volumes (ml.) of protein and of sucrose taken in the first eight and first 22 days of adult life

Sex and condition	Protein		Sucrose		Total fluid
	8 days	22 days	8 days	22 days	22 days
Virgin female	0.25	0.27	1.08	1.33	1.60
Gravid female	0.26	0.33	1.03	1.37	1.70
Ovariectomized female	0.12	0.19	0.82	1.01	1.20
Female with sham operation	0.17	0.26	0.98	1.10	1.36
Male	0.07	0.09	1.05	1.14	1.23
*Allatectomized female	.12	.29	.25	.84	1.13
*Normal female	.16	.20	.19	.81	1.00
*Female with sham operation	.18	.27	.22	.70	0.97

* Flies in these categories were held for six days before testing. Tests on all other flies began on the day after emergence.

heart extract was added response could be detected in two fibers only (Fig. 10C), but it is not clear at this time which two fibers are responding. In an attempt to clarify this point a mixture of brain-heart extract and fructose was tested and also a mixture of brain-heart extract and sodium chloride. In each case activity could

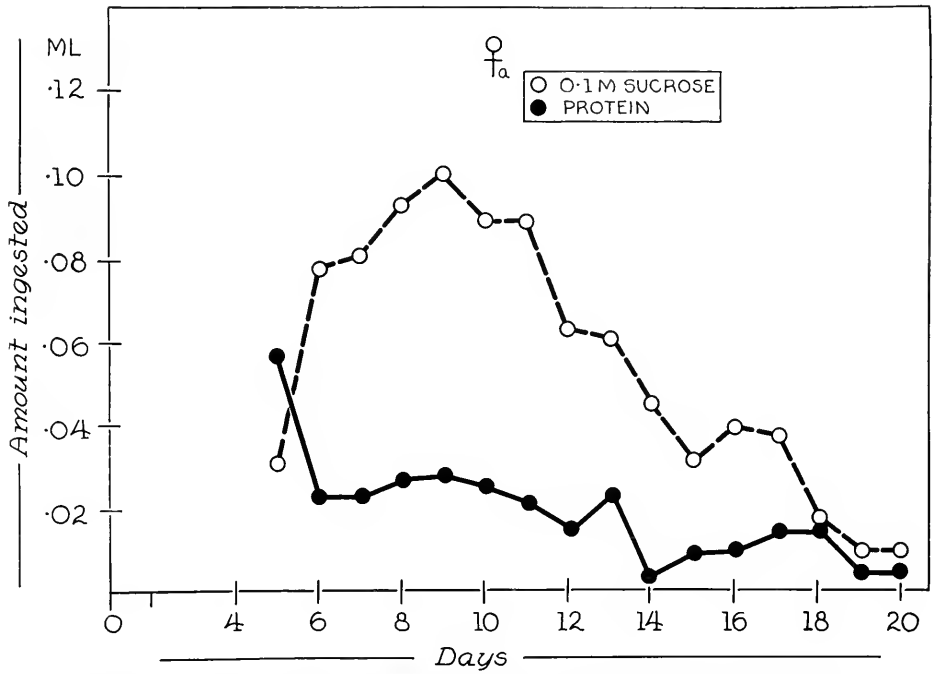

FIGURE 9. Daily intake of 0.1 *M* sucrose and brain-heart extract by a virgin female blowfly from which the corpus allatum has been removed.

be detected in two fibers only (Fig. 10E and F). Crystalline hemoglobin appeared to stimulate only one fiber.

Tests with a medium-sized hair (ca. 100 μ) presented a different picture. This hair responded to water, sugar, and salt; however, when either brain-heart extract or crystalline hemoglobin was applied, all electrical activity was reversibly blocked.

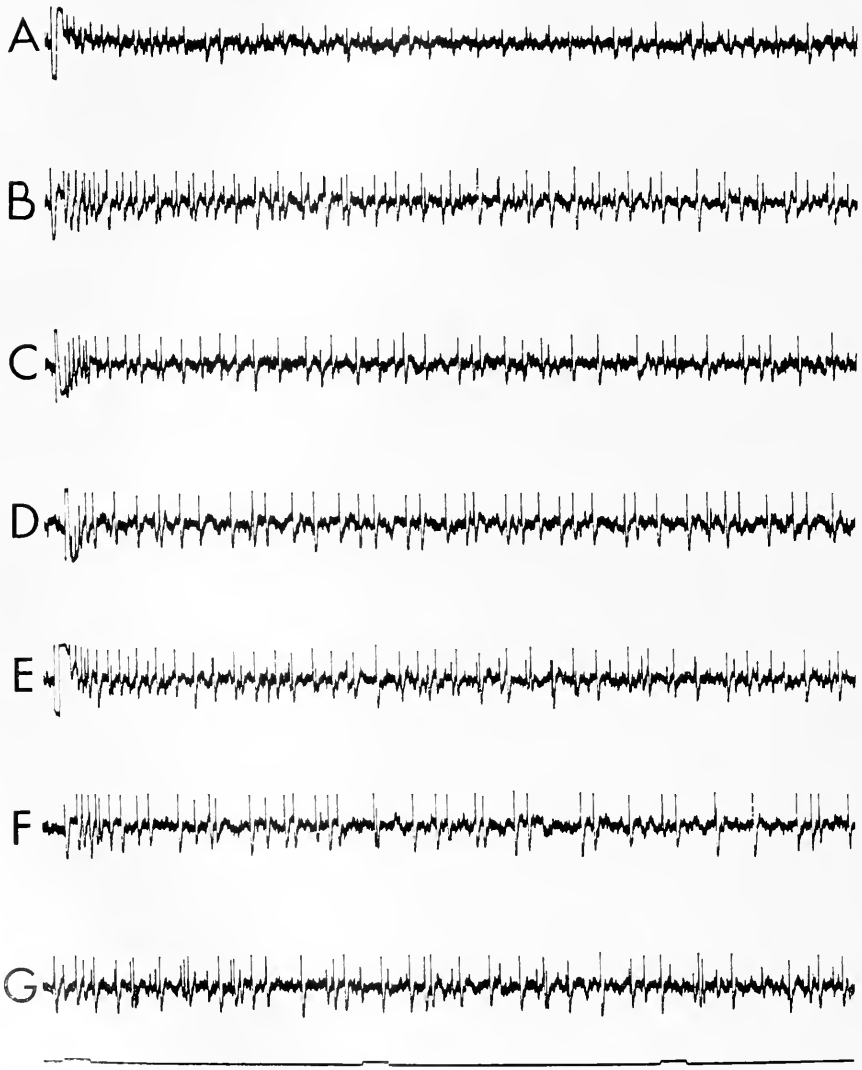

FIGURE 10. Receptor activity recorded through the side wall of one of the large marginal hairs of the labellum of a protein-deprived female *Phormia regina*. A, Response to water. B, Response to 1 *M* fructose. C, Response to brain-heart extract. D, Response to 1 *M* NaCl. E, Response to brain-heart extract plus fructose. Mixture is 0.5 *M* with respect to fructose. F, Response to a mixture of brain-heart extract and NaCl. Mixture is 0.5 *M* with respect to NaCl. G, Response to an equimolar (0.5) mixture of NaCl and fructose. Time, 0.2 second.

Internal factors affecting protein intake

A complicated and still only partially understood hormonal relation exists between the ovaries, the corpus allatum, the corpora cardiaca, and the median neurosecretory cells of the brain. Since preference tests revealed that the pattern of protein intake differs in the male and female and is correlated with ovarian changes, the ovaries and various endocrine organs were removed surgically and protein intake measured.

Removal of the ovaries did not alter the pattern of protein and carbohydrate intake (Fig. 8). Although the total volume of protein taken was less than that of the normals (but not of controls that had had a sham operation), it still showed the typical peak in the first six-day period and still exceeded that of males. There was a slight but not significant decrease in total carbohydrate intake (Table I).

When the corpus allatum was removed, eggs failed to develop; but there was no marked change in the pattern of protein and carbohydrate intake (Fig. 9). The reduction that did occur was matched by that of animals receiving sham operations (Table I).

Flies from which the median neurosecretory cells had been removed failed to survive longer than two weeks. During that period, however, the females exhibited the usual protein peak. Furthermore, in the ring tests they behaved like normal flies in their reactions to protein and carbohydrate.

Discussion

As Strangways-Dixon (1959, 1961) had already demonstrated with *Calliphora,* the volume of protein ingested by female blowflies is greater than that ingested by males and varies with phases in the reproductive cycle. It is clear from studies with *Phormia* that variations in ingestion reflect differences in feeding preferences. Thus, when a fly is given equal opportunity to ingest protein and carbohydrate, it refuses or ingests only a small amount of carbohydrate immediately after emergence and following each period of oviposition. At these times a large volume of protein is taken. The preference is accentuated if the fly is temporarily denied protein during these critical periods.

Observing the behavior of the females deprived of protein one gets the impression that there is a distinct "hunger" for protein. This reaction is particularly interesting because protein by itself is inadequate for survival. Thus, during these periods the fly eschews a nutritionally adequate diet (carbohydrate) for one which meets its reproductive requirements. From an evolutionary point of view, reproduction of the species takes precedence over survival of the individual.

The relationship between protein and carbohydrate ingestion is, however, not absolute. There is a strict dependence on the stimulating effect of the two substances on the sense organs. If, for example, the choice is between a highly stimulating carbohydrate (*e.g.,* 1.0 *M* sucrose) and protein, ingestion is biased in favor of the carbohydrate. Conversely, if the carbohydrate is only weakly stimulating (*e.g.,* 0.001 *M* sucrose), the ingestion of protein is tremendously augmented. Changing the kind of protein also alters the volumes taken. Homogenized liver is preferred to brain-heart infusion and both are preferred to hemoglobin; yet each suffices for egg production (although hemoglobin is the least satisfactory).

At first thought it would appear that the characteristic odors of these materials might be a deciding factor in choice and ingestion. Anosmic flies, however, are no different in their reactions toward protein and carbohydrate than normal flies. This finding is in agreement with data reported by Dethier and Chadwick (1947) and Evans and Barton Browne (1960). The fact that flies can detect the difference between protein and carbohydrate before ingesting them is inescapable. Observations of the behavior toward rings of solutions drawn on paper support the conclusion that discrimination is accomplished through the agency of the contact chemoreceptors on the legs and mouthparts.

Recent behavioral, electrophysiological, and histological work has shown that the innervation of the labellar hairs of *Phormia* is neither so simple nor uniform as originally believed (Dethier and Evans, 1961; Mellon and Evans, 1961; Larsen, personal communication). Some hairs have three neurons; others, four and possibly five. In addition to a neuron responding to bending there is one sensitive to water, one to sugar, and one to monovalent salts. While no specific protein receptor has been found thus far, there are differences among hairs with respect to the protein tested. One type of hair responds to carbohydrate and protein while another type responds only to carbohydrate. Protein appears to block activity in this hair. Both types respond to water and to sodium chloride. Although additional studies will be required before it is possible to assign protein sensitivity to a particular neuron, the point of importance now is that the fly has a peripheral mechanism for protein and carbohydrate discrimination.

Since it is true that the fly's response to protein and to carbohydrate varies concurrrently with events in the reproductive cycle, it must be inferred that the sensory contribution to behavior varies. The two most likely alternatives as to the level where changes occur are: the sense organs themselves; some intermediate level in the central nervous system. Either the relative sensory thresholds to protein and carbohydrate change periodically or some change occurs in the central nervous system where the sensory information is processed. There is as yet no direct information relating to sensory thresholds although by analogy with other sensory modalities in the fly it is unlikely that changes occur here. The alternative is that changes associated with protein metabolism alter integration by the central nervous system of sensory information coming to it. There are a number of ways in which changes occurring in the reproductive cycle might be linked with sensory input.

There are complicated and still only partially understood relations between the endocrine system, the reproductive system, and protein metabolism (*cf.* Wigglesworth, 1954; Strangways-Dixon, 1959). In *Phormia*, as in certain other blowflies, females fed carbohydrate alone are unable to bring the eggs to full development. If protein is provided, eggs develop fully, but they will not be laid unless copulation occurs. If the corpus allatum or medial neurosecretory cells are removed, egg development will progress only as far as in carbohydrate-fed flies.

It is conceivable, therefore, that differences in the fly's behavior toward protein and carbohydrate might be determined by one or more hormones or by changes in protein titer. The two possibilities for endocrine control are that the hormones increase sensitivity to protein or decrease it. If the first alternative is true, removal of the corpus allatum or medial neurosecretory cells should cause changes

in behavior toward protein. Neither changes in behavioral threshold nor difference in intake between operated and normal flies occurred. It might be argued that there was enough residual hormone to prevent a change; however, this appears unlikely since there was obviously not enough to permit egg development. Furthermore, allatectomized flies could be held for eight days on carbohydrate and still show a preference when presented with protein. These experiments rule out any hypothesis that hormones are *directly* concerned with nervous activity leading to protein ingestion.

In no case did removal of any of the endocine glands or of the ovaries prevent the fly from showing a protein peak shortly after emergence. In all cases subsequent protein peaks were absent. Removal of the ovaries alters protein levels simply because there are no developing eggs to create protein demands. Removal of the corpus allatum or medial neurosecretory cells alters protein levels because the absence of hormones also prevents egg development. Additionally, removal of the medial neurosecretory cells interferes with protein metabolism (Thomsen and Møller, 1959). Insofar as protein is concerned Strangways-Dixon (1959, 1961) obtained similar results with *Calliphora*. He did report, however, that allatectomy depressed carbohydrate intake, and this did not occur with *Phormia*.

The occurrence of an initial peak in protein ingestion unaffected by allatectomy, removal of the ovaries, or removal of the medial neurosecretory cells might be explained by assuming that all flies emerge from pupation with a protein deficit and that it affects behavioral threshold. This would explain the occurrence of initial protein ingestion in both sexes. In males the quantity is small and soon approaches zero. In the newly emerged female the initiation of egg development causes withdrawal of protein from the fat body (Strangways-Dixon, 1959) thus increasing the deficit. Accordingly, if females are denied protein for the first six days, the deficit becomes acute and sensitivity should increase (as indeed it does). In the males there is no such increase. Greenberg (1959) stated that there was no difference in the protein consumption of male and virgin female houseflies; however, his conclusion was based upon a comparison of mean daily intake, a measurement which tends to minimize the differences occurring shortly after emergence. After this time there are no pronounced differences. If a female is mated, a protein deficit develops, and the cycle is repeated.

The point at which protein deficit influences sensory input is at present unknown. It is not merely a matter of body pressure or osmotic relations in the body cavity because injection of carbohydrate, water, or hypotonic salt into the hemocoel fails to alter the pattern of protein ingestion. It is unlikely that it is any aspect of gut physiology because transection of the recurrent nerve, which innervates the gut, also fails to alter protein ingestion. When protein-deficient females are placed in a choice situation after recurrent nerve transection, they become hyperphagic, but do so by ingesting carbohydrate rather than protein.

The assistance of Mr. D. Mellon is gratefully acknowledged.

Conclusions

There is a difference in the pattern of protein feeding by the two sexes of the blowfly *Phormia regina* Meigen. Males, whether mated or not, gradually increase

their intake from the time of emergence until the fourth to eighth day. Thereafter little protein is taken. The pattern is similar for virgin females, but the volume ingested is greater. Mated females increase their protein intake after each batch of eggs is laid. If females are denied protein at times when the intake would normally be great, they show a decided preference for protein over carbohydrate in a choice situation. Choice is mediated by the contact chemoreceptors; odor is not a factor. The peak in protein ingestion that occurs after emergence is not altered by removal of the ovaries, corpus allatum, or medial neurosecretory cells. Subsequent peaks are abolished by any procedure that prevents egg development. Changes in feeding behavior are correlated with changes in protein levels which in turn are related to hormonal and reproductive cycles. It appears unlikely that hormones are directly concerned with nervous activity leading to protein ingestion.

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A HISTOPHYSIOLOGICAL STUDY OF THE RAT DIAPHRAGM

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Though some extensive studies have been made on the general morphology and physiology of the mammalian diaphragm as a whole, sufficient attention has not been paid to the regional and cellular organization of this organ. Morphologically, three distinct regions, namely vertebral (dorsal), costal (lateral) and sternal (ventral) have been described in man (Johnston and Whillis, 1949). But little information is available on the cellular components which constitute these regions, with regard to their histological features and metabolic activities. In a recent study Frunder (1954) observed that the oxygen uptake in the posterior and anterior parts of the diaphragm differed with regard to their glycogen content, and pointed out possible errors in respiration studies with selected parts of the diaphragm.

Recent studies conducted in our laboratories on the structure and physiology of mixed muscles, such as the pigeon breast muscle, which have been reviewed by Drummond and Black (1960), have shown the existence of two types of fibers: one broad, white and glycogen-loaded, adapted for an anaerobic metabolism in which glycogen is the chief fuel for energy; and a narrow, red fat-loaded variety for aerobic metabolism in which fat forms the chief fuel. The diaphragm also being a mixed type of muscle, it was thought desirable to carry out similar studies on its different regions with a view to correlating structure with function. In our present investigation the three regions of the rat diaphragm have been studied with regard to the nature, number and distribution pattern, diameter, metabolite load and concentrations of enzymes (lipase and succinic dehydrogenase) of the constituent fibers.

Materials and Methods

Freshly collected wild rats (*Rattus norvegicus*) were used as the material in the present investigation. The rats were decapitated and the whole diaphragm was quickly removed and spread on a clean filter paper. The three regions were demarcated and cut out separately. For histochemical observations thin strips of the tissue were then cut in line with the orientation of the fibers. For the quantitative estimations, each separate region was pooled from a number of individuals.

Fiber count and fiber diameter

Thin, frozen, hand sections of all the three regions, were separately cut out, spread on clean, dry slides and mounted in glycerine jelly. The respective numbers of red and white fibers within a certain lens field were counted. With the aid of an ocular eyepiece and micrometer scale the diameter of the fibers was determined.

Fat: histochemical localization and quantitative estimation

The tissue was fixed in Baker's calcium formol (Baker, 1946) for 24 hours and washed in running tap water for the same time and embedded in 20% gelatin. Thirty- to forty-micron sections were cut on a freezing microtome and stained for fat, using the Sudan Black B stain.

As already mentioned the material from a single individual was found to be insufficient for quantitative estimations and so the material from a number of individuals, irrespective of sex, was pooled together. It was then dehydrated by drying in a hot air oven at 100° C. The total fat content was estimated by the Soxhlet extraction method, using a 1 : 1 alcohol-ether mixture.

Glycogen: histochemical localization and quantitative estimation

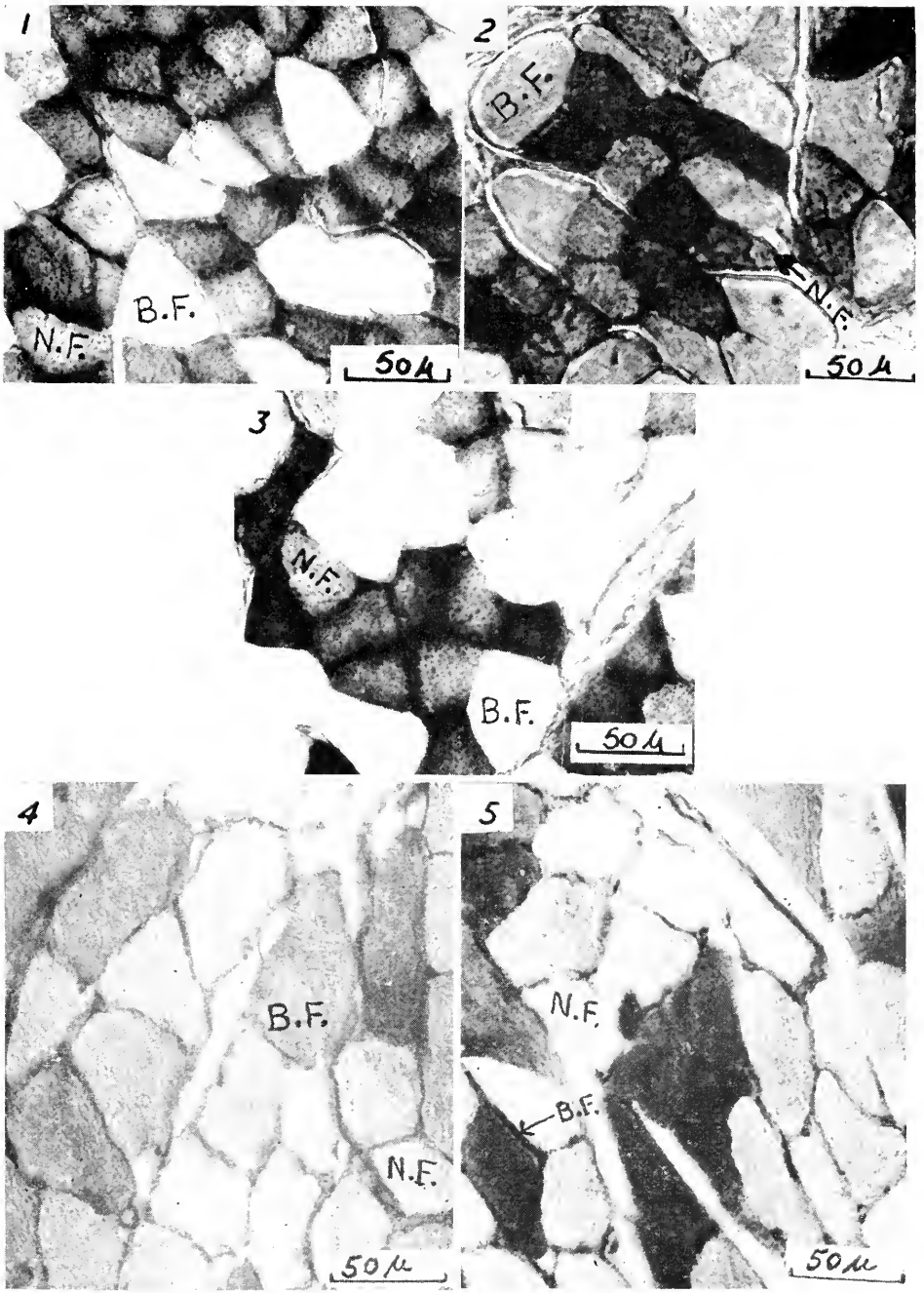
The tissue was fixed in cold alcoholic picroformol for 24 hours and embedded in paraffin wax of melting point 56 to 58° C. Sections 10 to 12 μ thick were cut and stained for glycogen by the periodic acid Schiff's reagent (Pearse, 1960).

Glycogen was estimated quantitatively by the micro method of Kemp *et al.* The material was pooled and treated with 80% methanol for the removal of glucose. The insoluble part was deproteinized and the clear supernatant hydrolyzed by concentrated sulphuric acid. The intensity of the pink color was measured on a Klett-Summerson photoelectric colorimeter, using a 520 mμ filter. The amount of glycogen present in the material was directly read from the standard graph. The results are expressed as μg. glycogen/100 mg. wet tissue.

Lipase: histochemical localization and quantitative estimation

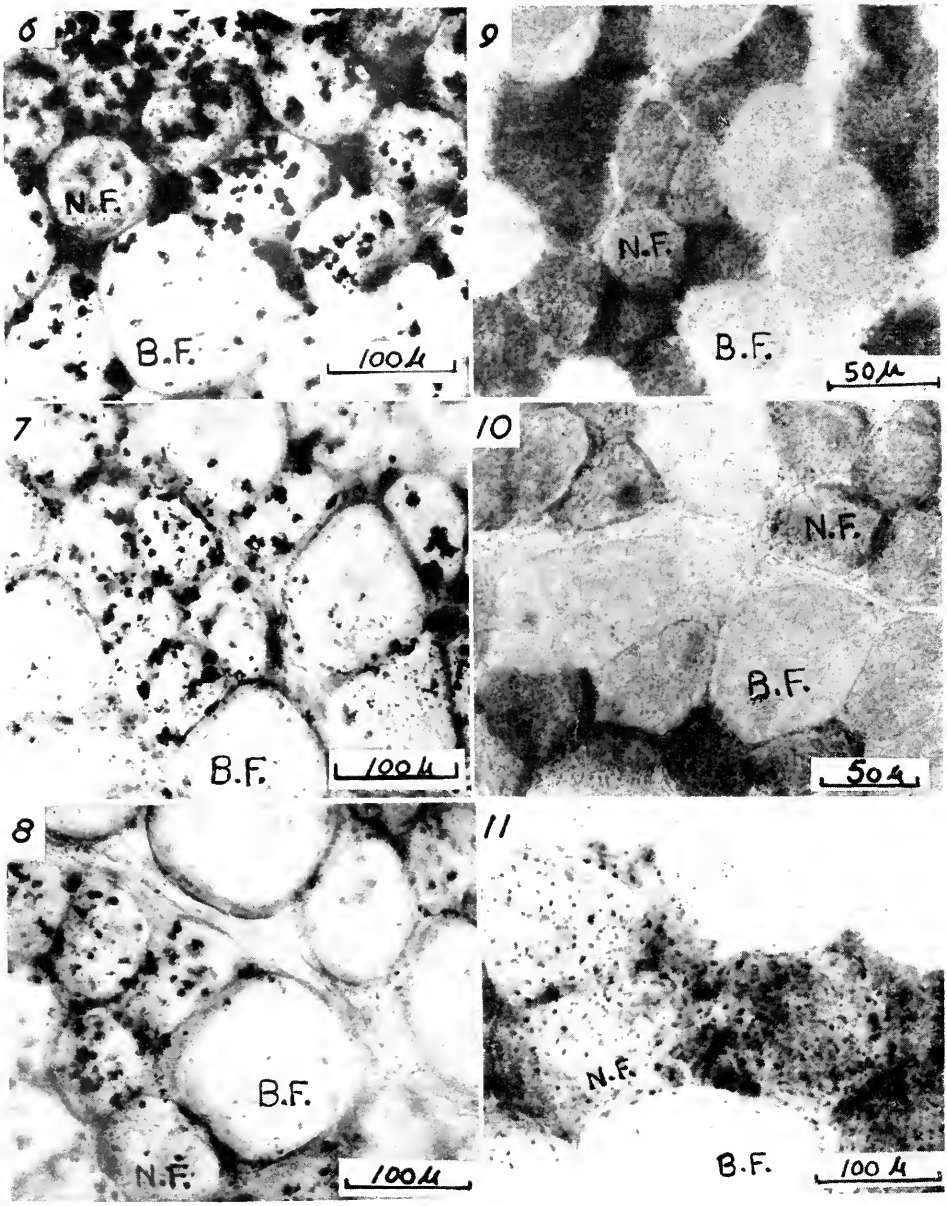
The lipase activity in the two types of fibers was studied histochemically using an improved technique (George and Iype, 1960). Thin frozen sections were cut according to the method of George and Scaria (1958) and dried on clean albumenized slides at room temperature. The sections were fixed in cold 6% neutral formalin for 3 to 4 hours, washed well in running tap water for about an hour and rinsed well with distilled water. A thin coating of gelatin (5%) was then applied on the sections and fixed in 6% neutral formalin for half an hour. It was washed in tap water for another half an hour. The sample sections were incubated for 12 to 16 hours in the incubation medium with "Tween 80" as substrate. The control sections were boiled for 10 minutes, coated with gelatin and incubated along with the sample sections. After incubation, the sections were washed well with distilled water and then with warm water (40° C.), so that the gelatin coating was completely removed. The sections were then treated with lead nitrate for half an hour and then with dilute yellow ammonium sulphide. According to the modifications made by George and Iype (1960), a final step of rinsing the sections in 1–2% acetic acid was also adopted for clearing the sections. The brownish black precipitates of lead sulphide formed as the result of the enzymic activity were seen very prominently.

The quantitative estimation of lipase activity was carried out according to the method of Martin and Peers (1953) using a Warburg manometric apparatus. The results obtained are presented as μl. CO_2 produced per mg. protein per hour.



FIGURES 1, 2, and 3. Transverse sections of the dorsal, ventral, and lateral regions, respectively, of the diaphragm stained with Sudan Black B for fat. The broad fibers (B.F.) are less sudanophilic than the narrow ones (N.F.).

FIGURES 4 and 5. Transverse sections of the dorsal and lateral regions, respectively, of the diaphragm stained with PAS for glycogen. The broad fibers contain more glycogen than the narrow ones.



FIGURES 6, 7 and 8. Histochemical localization of lipase activity in the fibers of the dorsal, ventral and lateral regions, respectively. The enzymic activity in the narrow fibers is more than in the broad ones.

FIGURES 9, 10 and 11. Histochemical demonstration of succinic dehydrogenase activity in the fibers of the dorsal, ventral and lateral regions. The deposition of the diformazan granules is more in the narrow fibers.

The protein content of the tissue was estimated by the micro-Kjeldahl steam distillation method (Hawk *et al.*, 1954).

Succinic dehydrogenase: histochemical localization and quantitative estimation

Thin frozen sections of the three regions of the diaphragm were separately cut out and the histochemical demonstration of succinic dehydrogenase was carried out by an improved method of George and Talesara (1961) using neotetrazolium chloride as the hydrogen acceptor. The sections were transferred to cold 0.1 *M* phosphate buffer (pH 7.4) for 10 minutes to destroy the endogenous substrates. They were then transferred to the incubation medium consisting of 0.1 *M* phosphate buffer (pH 7.4) 2.5 ml., 0.5 *M* sodium succinate 0.6 ml., 0.004 *M* aluminium chloride 0.5 ml., neotetrazolium chloride (3 mg. per ml.) 1 ml., methylene blue (1 mg. per ml.) 0.1 ml., 0.6 *M* sodium bicarbonate 0.3 ml. and 0.005 *M* magnesium sulphate one drop. The incubation was carried out under strictly anaerobic conditions by passing a mixture of nitrogen (95%) and carbon dioxide (5%) through the incubation medium at 37° C. for 5 to 10 minutes. The purple granules of the diformazan formed indicated the sites of the enzymic activity.

The enzyme activity was assessed quantitatively by the colorimetric method of Kun and Abood (1949). The results are expressed as μg. formazan formed per mg. dry weight per two hours.

Results

Histochemical

Fat. In all the three regions it was found that the broad fibers were less sudanophilic than the narrow ones (Figs. 1, 2, 3).

Glycogen. In the dorsal and lateral regions, the broad white fibers were found to contain more glycogen than the narrow ones (Figs. 4, 5). In the ventral region, however, the glycogen content was so low that satisfactory histochemical demonstration was not possible.

Lipase. The level of lipase activity in the three regions differed according to the nature and distribution of the fibers present, the broader fibers having less lipase activity (Figs. 6, 7, 8).

Table I

Giving the relative distribution, metabolite load and enzyme (lipase and succinic dehydrogenase) activity in the two types of fibers in the three regions of the rat diaphragm

Region of the diaphragm	Number of fibers in % per unit area		Ratio of the fibers B:N	Diameter of the fibers in μ		Diameter ratio B:N	Area covered per unit area in μ^2	
	Broad	Narrow		Broad	Narrow		Broad	Narrow
Dorsal (vertebral)	29.23	70.76	1:2.4	78.30	40.50	1:1.9	32,230	21,530
Lateral (costal)	32.29	67.70	1:2.1	90.72	43.20	1:2.1	40,100	19,060
Ventral (sternal)	32.24	67.78	1:2.1	49.40	37.80	1:1.3	18,890	23,020

TABLE I—(*Continued*)

Region of the diaphragm	Area ratio B:N	Glycogen μg./100 mg. wet tissue	Fat % dry weight	Lipase μl. CO_2/ mg. protein/ hour	SDH μg. formazan/ mg. dry weight/ 2 hours
		S.D.	S.D.	S.D.	S.D.
Dorsal (vertebral)	1:0.7	232.72 ± 24.95	16.19 ± 1.43	41.54 ± 5.52	10.97 ± 2.45
Lateral (costal)	1:0.5	297.42 ± 21.37	13.17 ± 1.55	24.55 ± 4.25	14.16 ± 3.22
Ventral (sternal)	1:1.2	127.42 ± 15.68	21.41 ± 0.80	29.79 ± 3.11	8.06 ± 2.81

Succinic dehydrogenase. The broad fibers showed considerably less enzyme activity than the narrow ones, in all the regions. In the lateral region, however, the narrow fibers, even though broader than the narrow fibers of the other two regions, showed high enzyme activity (Figs. 9, 10, 11).

Quantitative

The data obtained from the quantitative estimations are given in Table I. From Table I it is clear that each of the three regions has its own characteristics. The dorsal region has the highest number of narrow fibers and the least number of broad fibers, the diameter of the narrow fibers being less than that of the narrow fibers of the lateral region but higher than those of the ventral region. The diameter of the broad fibers is, however, less than that of the broad fibers of the lateral region but more than that of the broad fibers of the ventral. The area covered in a unit space by the broad fibers is less than that covered by the broad ones of the lateral and the area covered by the narrow fibers in the same space is less than that covered by the narrow ones of the ventral. The glycogen content is less than that in the lateral but more than that in the ventral. The fat content is higher than that in the lateral while it is less than that in the ventral. The dorsal region, again, has the highest lipase activity among all the three regions and succinic dehydrogenase activity less than that in the lateral but more than that in the ventral.

Of the three regions the ventral has the narrowest narrow fibers; narrowest broad fibers; the largest area covered by narrow fibers; has the least glycogen content; highest fat content; high lipase activity, much higher than the lateral but lower than in the dorsal, and the least succinic dehydrogenase activity.

The lateral region, on the other hand, possesses the broadest broad fibers; broadest narrow fibers; largest area covered by the broad fibers; highest glycogen load; least fat load; least lipase activity but highest succinic dehydrogenase activity.

DISCUSSION

That the diaphragm is a mixed type of muscle, having fibers of different sizes, is well known. Gunther (1953) studied the human, dog, and rat diaphragms and distinguished two types of muscle fibers, tetanic and tonic ones, the latter amounting to 10% in the human, 42% in the rat and 5% in the dog. He thus suggested a functional difference in the two types of fibers. Recently Nachmias and Padykula

(1958) in their histochemical study observed in the rat diaphragm two types of fibers, those with smaller average diameter having greater succinic dehydrogenase activity and others with larger diameter with lesser enzymic activity. In the cat diaphragm, however, they described three types of fibers, the third type being characterized by intense uptake of Sudan Black B in the subsarcolemmal position.

In the present study, two distinct types of fibers, namely a broad white and a narrow red, similar to the two types described in the pigeon breast muscle (George and Naik, 1957), existing side by side in all the three regions of the diaphragm, have been recognized. However, the distribution pattern of these two types of fibers, in contrast to that of the pigeon breast muscle (George and Naik, 1959), does not show any definite design and appears to be at random. It was also observed that the diameter of the fibers, unlike that of the pigeon breast muscle, showed a high degree of variation in each of the two types. Nevertheless, from our histochemical observations it is seen that there is a correlation between the diameter of the muscle fiber and its color, metabolite load, mitochondrial content and enzyme concentrations. Such a relationship has been shown in the pectoralis muscle of birds (George and Naik, 1957; George and Scaria, 1958a; George and Talesara, 1960, 1961), bats (George, Susheela and Scaria, 1958) and the flight muscles of insects (George and Bhakthan, 1960a, 1960b). However, in the case of the fibers of the diaphragm in which the narrowest white fiber and broadest red fiber have diameters of 49 μ and 43 μ, respectively, the above correlation was not so marked as in the case of the broad and narrow fibers of the pigeon breast muscle. In the bat pectoralis such variations in diameter were observed (George, Susheela and Scaria, 1958). The fibers of the pigeon breast muscle are therefore to be regarded as an instance of extreme specialization achieved in the differentiation of the two types of fibers.

In the diaphragm the broadest white fibers are distinctly loaded with glycogen and contain less mitochondria (as evinced by the staining for lipid) and less lipase and succinic dehydrogenase activity. In the case of the narrower white fibers and broader red fibers, however, it was not possible to arrive at definite conclusions based on histochemical observations. It also became clear that instead of the high degree of specialization resulting in the two distinct fiber types as in the pigeon breast muscle, in the diaphragm, the differentiation of the component fibers achieved a greater extent of variability in structure and perhaps function too. On the other hand, the diaphragm achieved a specialization in having three distinct regions, which is revealed from our histochemical and quantitative observations.

The fact that the dorsal region possesses the highest number of narrow fibers, high fat content, highest lipase activity and high succinic dehydrogenase activity suggests a predominance of fat metabolism involving the use of fat as fuel for muscular contraction. And it should be mentioned here that the utilization of fat by the diaphragm has been demonstrated by Wertheimer and Ben-Tor (1952). It has also been observed (George and Susheela, 1961) that during starvation there is a six-times actual reduction of fat in the dorsal as well as in the ventral regions over the lateral region, of the rat diaphragm.

The predominance of fat metabolism also appears evident in the ventral region where there is the highest fat content and high lipase activity. But this region comparatively has the lowest level of oxidative capacity in terms of succinic de-

hydrogenase activity. This indicates that in this region there is more of building up of fat from fatty acids for storage than fat metabolized for energy. Some indirect evidence for the possibility of fat being built up as energy store at sites rich in lipase activity, but poor in oxidative enzyme activity, has been recently presented by George and Iype (1960). They have demonstrated in the sheep heart high lipase activity and very low succinic dehydrogenase activity in the Bundle of His, and suggested the possibility of this site in the heart being a center for lipogenesis. They also demonstrated high concentrations of both the enzymes in the myocardium which is known to utilize fat as the major fuel for the activity of the heart. Further, the works of Cogan and Kuwabara (1957) and Kuwabara and Cogan (1960) have shown that the Purkinje fibers, the structural units of the Bundle of His, have the capacity to synthesize sudanophilic fat.

From the characteristics of the lateral region mentioned earlier, it is obvious that this region is specialized for carbohydrate metabolism in which glycogen forms the fuel for muscular contraction. The very high succinic dehydrogenase activity, even higher than what is seen in the fat-loaded dorsal and ventral regions, is certainly indicative of high oxidative metabolism involving the rapid oxidation through the Krebs cycle of the products of the Emden-Meyerhof cycle. Another possibility is that, under conditions of prolonged rapid respiratory activity, this region might well be capable of oxidizing fatty acids transported from the other regions of the diaphragm and/or from other sites in the body where fat is stored. In this context it may be mentioned that a recent study of the blood supply of the rat diaphragm (Beck and Baxter, 1960) has revealed the existence of an elaborate and copious system of circulation in this organ. Of the three regions, the lateral region has the major blood supply while the ventral has the least. Moreover, the blood supplies to the lateral and dorsal regions are more directly from the aorta through the phrenic arteries, than the ventral which receives blood from a small branch of the internal mammary artery. Their study has also shown the remarkable consistency in the main features of the venous drainage and the presence of an anastomatic system of veins in the diaphragm, connecting the inferior vena cava and azygous veins.

The present investigation on the diaphragm has revealed more of the complex nature of this organ than it has actually contributed to the elucidation of our knowledge of its structure and physiology. To that extent we feel amply rewarded. However, more extensive histophysiological and biochemical studies on the different regions are called for and are in progress.

One of us (A.K.S.) is indebted to the Council of Scientific and Industrial Research (Govt. of India), for the award of a Junior Research Fellowship.

Summary

1. The nature and distribution of two types of fibers in the rat diaphragm, with respect to its dorsal (vertebral), lateral (costal), and the ventral (sternal) regions, have been studied.

2. Histochemical as well as quantitative studies on the fat and glycogen contents and the enzymic activity (lipase and succinic dehydrogenase) have been made in the above three regions.

3. It is concluded that the narrow red type of fibers is well adapted for aerobic metabolism, involving mainly the oxidation of fat for energy, and the other, the broad white variety, for anaerobic metabolism where glycogen is the chief fuel. The significance of the regional differences in the two types of fibers in the physiology of the diaphragm is discussed.

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THE EFFECT OF THIOUREA, ADMINISTERED BY IMMERSION OF THE MATERNAL ORGANISM, ON THE EMBRYOS OF LEBISTES RETICULATUS, WITH NOTES ON THE ADULT GONADAL CHANGES[1]

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The terms oviparous, viviparous and ovoviviparous are commonly found in the literature discussing embryology and physiology. Oviparous and viviparous may be defined with relative ease and the interpretations of most authorities are in agreement. Briefly, oviparous refers to animals that deposit eggs outside of the body, while viviparous applies to animals that retain the ova and have their development within the soma. The latter term is applied to those forms having highly specialized structures, such as placentae, which attach the developing young to some part of the reproductive system of the adult in order to provide an avenue of material exchange, including nutritive materials for the growth of the embryo during the greater part of its development within the parent. The third term, ovoviviparous, has been variously defined and is less definite. It is agreed that in those forms described as being ovoviviparous the young are retained within the maternal reproductive system and leave the mother's body fully formed, viable and without any surrounding membranes. There does not appear to have been a permanent attachment, however, and the degree of dependence of the young on the parent is a moot question. The eggs of many species referred to as ovoviviparous are macrolecithal, and many authors believe the produced egg contains enough yolk to meet the nutritive needs of the developing young until it leaves the parent. The young is materially independent of the adult and uses the latter only as a harbor while developing. The distinction between viviparity and ovoviviparity is therefore classically based on anatomical and physiological differences.

A true anatomical uterus and placenta, which permits a firm attachment of the embryo to the parent, is wanting in teleosts. Structures and modifications of parts of the reproductive tracts (of both male and female) which permit retention and internal hatching of the young have been reported to occur in diverse orders of the class (Turner, 1942, 1947). In some of the species studied it has been shown that both parent and young may possess structures whose histological and cytological make-up, spatial arrangement and time of appearance suggest a material exchange between parent and young (Blake, 1867, 1868; Eigenmann, 1892; Fraser and Renton, 1940; Lane, 1903; Mendoza, 1937, 1938, 1939, 1940, 1941, 1943; Purser, 1938; Turner, 1933, 1936, 1937a, 1938a, 1938b, 1940a, 1940b, 1940c,

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1940d; Wyman, 1854). Some physiological studies (Bailey, 1933; Scrimshaw, 1944, 1945) comparing egg and embryo weights indicate the supply of materials to the young by the parent in certain species of teleost.

This paper reports the results of a study designed to reinvestigate the question of exchange between parent and young in one teleost, the guppy, *Lebistes reticulatus* (Peters), often referred to as ovoviviparous, by using the anti-thyroid chemical, thiourea, which is known to have the ability to cause the alteration of the morphology and physiology of the thyroid gland. It was thought that the chemical, when supplied to the adult, would produce discernible changes in the young if a material transfer does exist. *Lebistes reticulatus* was used as the experimental animal because of its hardiness, macrolecithal egg, and the presence of structures (Purser, 1938) that could possibly aid in the transfer of substances.

The author expresses his appreciation to Dr. Harry A. Charipper for the kind advice and guidance given during the course of this project. Thanks are extended to Dr. Charles M. Breder, Jr. and to Dr. Ross F. Nigrelli for their valuable suggestions. The major part of this work was performed in the Laboratories of the Department of Fishes and Aquatic Biology of the American Museum of Natural History, New York.

Materials and Methods

Seventy-five brood-bearing *Lebistes reticulatus* were utilized in this work. They were selected from a larger group of females exposed continuously to males soon after birth up to and one day after the delivery of their third brood. The fish were all of approximately equal size and found to present young at 28–31-day intervals. They were divided into 5 groups of 15 each as follows:

Group C: Received no treatment, served as controls.

Group 1D: Placed in a 0.04% thiourea solution one day after the delivery of their third brood. This timing exposed the adults to the solution at the time a group of ova were maturing, during fertilization and the entire ontogeny of the young.

Group 1W: Placed in a 0.04% thiourea solution one week after the delivery of their third brood. This timing exposed the adults to the solution at approximately the time of fertilization and during the entire ontogeny of the young.

Group 2W: Placed in a 0.04% thiourea solution two weeks after the delivery of their third brood. This timing exposed the adults to the solution during the latter two-thirds of the ontogeny of the young.

Group 3W: Placed in a 0.04% thiourea solution three weeks after the delivery of their third brood. This timing exposed the adults to the solution during the last one-third of the ontogeny of the young.

The young of the groups were labeled with a "Y" after the parents' group, giving the symbols CY, 1DY, 1WY, 2WY, 3WY.

Homotypic conditioned water was used in the preparation of the thiourea solutions and in the tanks containing control fish. Each animal was kept separate in a one-gallon container without plants, "scavengers" or aeration. Solutions were

changed semi-weekly. All fish, except as noted below, were allowed to present their broods. To insure against a long exposure of the newborn young to the thiourea solution all tanks were checked four times a day and any young found were fixed immediately. In only two cases, were broods presented during the night. These young may have been in the solution for a maximum of 10 hours. Seventeen non-control fish were observed to be delivering young and were sacrificed during the birth process. All other adults were killed when young were found in the tanks. Water temperatures ranged from 72 to 78° F. throughout the experiment. A portion of the controls and of each of the treated groups coexisted at all times. This arrangement served as a check on possible seasonal effects. Tanks containing control fish were always adjacent to the tanks containing treated fish in order to assure an equal distribution of light to all. Daylight was supplemented with artificial light during the late fall and winter months.

The fish were killed and fixed in Bouin's solution. As soon as movement ceased the adults were removed from the liquid, their abdominal wall was slit and they were reimmersed in the agent. This procedure allowed a more rapid and direct penetration of the fixing fluid into the body cavity. Adult fish were decalcified with nitric acid plus phloroglucin; young were not decalcified. Xylol was used as the clearing agent in preparation for embedding in 56–58° C. Histowax. Sagittal serial sections of the entire fish were cut at five micra and stained either with Harris' hematoxylin and eosin or a modified Masson procedure.

Thyroid cell height was measured with a calibrated ocular micrometer. A minimum of 100 cells was observed for each fish, involving as many different follicles as possible. The data obtained were subjected to statistical analysis and their significance determined.

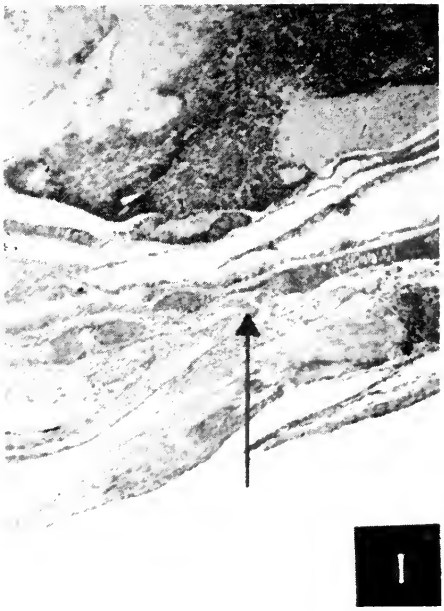
Brood sizes and intervals of all individuals of all groups were recorded. The ovaries of all adult fish were examined macroscopically and microscopically and the contained ova were grouped according to size.

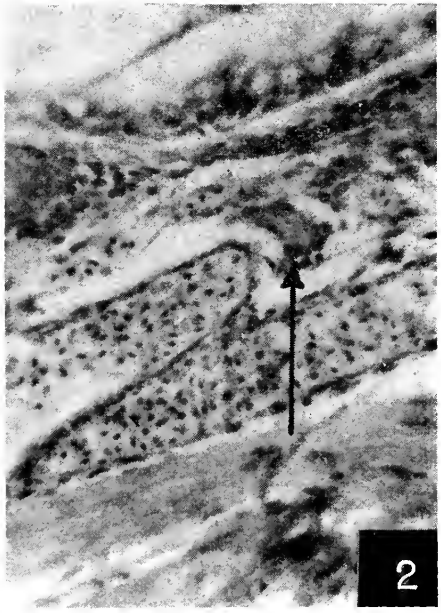
Results

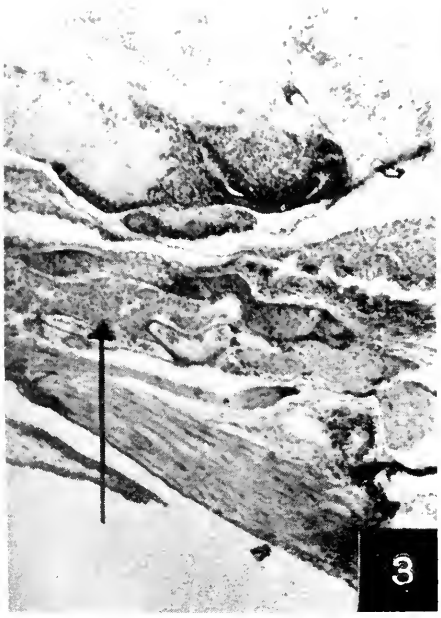
I. *Thyroid*

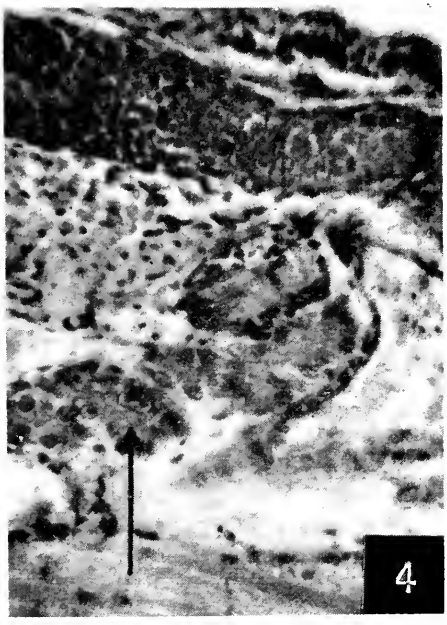
A. *Normal.* The thyroid gland of the normal *Lebistes reticulatus* was found to be a diffuse and unencapsulated organ consisting of many separate follicles in the connective tissue of the floor of the mouth. They are arranged alongside the ventral aorta and other blood vessels and extend from the anterior part of the mandible to the gill region; see Figures 1 and 2. Thyroid epithelia were not found in any other part of the body. Without exception the follicular cells are of a squamous or a very low cuboidal nature, as seen in Figure 5. The contained colloid is dense, homogeneous and fills the interior of all the follicles. The picture is essentially the same for the adult and the newborn.

B. *Treated Adults.* After immersion in the thiourea solution, hyperplastic changes were noted in all fish. Follicles increased greatly in size with very great variation. Without exception, follicles increased in number and extent. Some were found posteriorly to the level of the heart, throughout the connective tissue of the entire lower jaw, crowded around the blood vessels, and in the gill region. Members of group 1D showed the greatest degree of metastasis. In this group, thyroid cells were found in the myocardium in 12 cases, in the kidneys in 9 cases, in







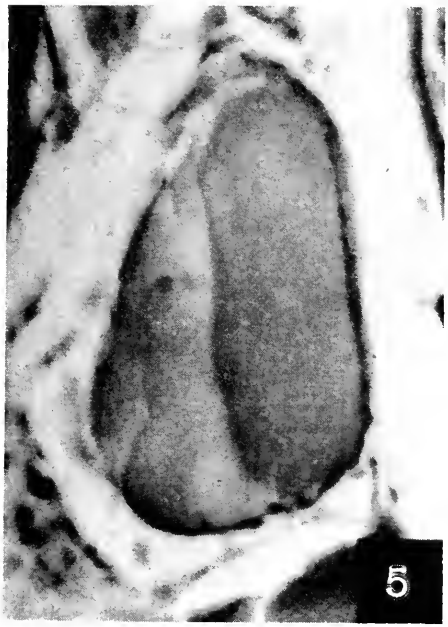


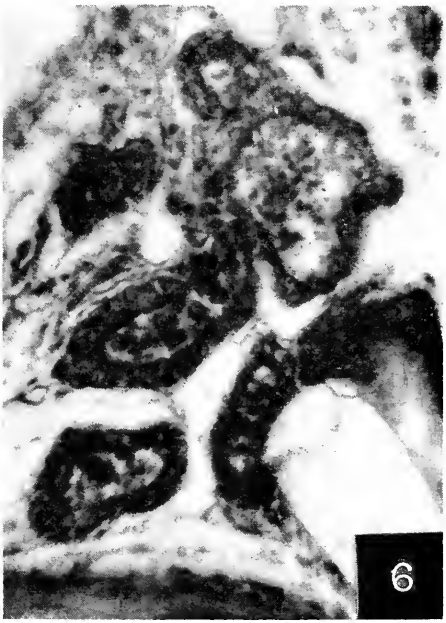
Tissues represented by Figures 1–7 were stained with Harris' hematoxylin and eosin, Figure 8 represents an unfixed, unstained young.

Plate I

FIGURES 1 and 2. Section of the floor of the mouth of a normal young showing few thyroid follicles. Figure 1, × 132; Figure 2, × 525.

FIGURES 3 and 4. Section of the floor of the mouth of a young from a thiourea-treated fish showing massing of cells and afollicular arrangement of cells near the blood vessels. Figure 3, × 132; Figure 4, × 525.





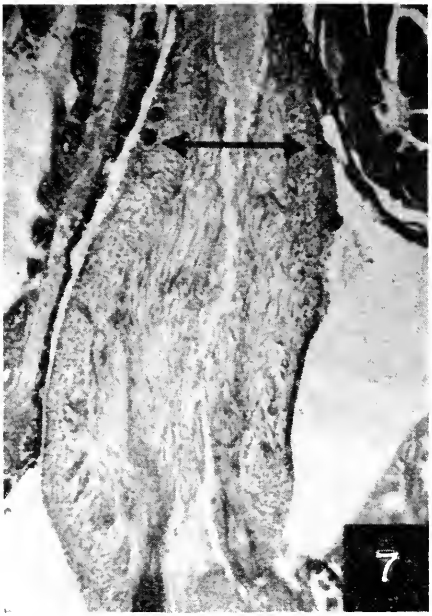


Plate II

FIGURE 5. Thyroid follicle from an untreated fish. × 525.
FIGURE 6. Thyroid follicle from a thiourea-treated fish. × 525.
FIGURE 7. Heart of a thiourea-treated fish showing follicles in the myocardium. × 132.
FIGURE 8. Aborted young from a thiourea-treated fish. × 15.

the intestines and spleen in 8 cases. Scattering of thyroid tissue took place to a lesser extent in fish of groups 1W and 2W. Group 3W did not show any atypical location of thyroid cells. The invading tissue in the new areas was arranged in small follicles or was massed in layers approaching an afollicular disposition. Colloid was reduced in amount. It was of an acidophilic nature and appeared foamy and vacuolated.

Thyroid cell height was markedly increased, the amount increasing with the time of exposure (Fig. 6). In all cases, the difference between the experimental and control animal data proved to be statistically significant (see Table I). Cytoplasmic staining was more intense. The nuclei increased in size, became more vesicular and basal in position. Nucleoli became more prominent.

TABLE I

Experimental condition of the fish and thyroid cell height

State of immersion	Mean thyroid cell heights (μ)	S.E.*
Control—adults	2.71	0.010
Young male	2.22	0.008
Young female	2.33	0.009
1 day—adults	7.21	0.031
Young male	9.66	0.021
Young female	9.81	0.027
1 week—adults	6.70	0.032
Young male	9.55	0.022
Young female	9.69	0.021
2 weeks—adults	6.52	0.019
Young male	9.04	0.017
Young female	9.22	0.019
3 weeks—adults	6.17	0.014
Young male	8.60	0.020
Young female	8.84	0.015

* Standard error (standard deviation of the mean): S.E. $= \sqrt{\frac{Ed^2}{n(n-1)}}$.

C. *Young from treated adults.* All the young from all groups showed extensive invasion of new areas by thyroid cells. The gill regions, heart, kidneys, spleen and intestines contained thyroid tissue. Myocardium invasion is seen in Figure 7. No one specimen showed metastasis to all sites, however. Follicular arrangement became the exception rather than the rule. Cells were massed in large groups without follicular arrangment, as seen in Figures 3 and 4. Colloid was scant or, in many cases, absent. Thyroid cell height was increased and in all cases proved to be significantly different from the controls.

II *Gonads*

A. *Normal adult.* It can be seen macroscopically as well as with a dissecting microscope that after the presentation of a brood the ovary contains a large number

of ova. Study and measurements showed that they could be arranged in groups. The oldest and most mature group consists of eggs measuring 0.8 to 1.5 mm. These are orange in color and rather transparent. In numbers they approximate the size of the last brood or may be slightly more numerous. A second group consists of ova measuring 0.3 to 0.5 mm. These immature eggs, in addition to being smaller, are white, dense and opaque. Their number is considerably larger than either the first group or the brood last presented. Eggs in a still more immature stage of development are also present. They are by far the most numerous. Schematic grouping of the latter is not feasible because of too great a variation in size, almost continuous gradation existing within this group. Definite intervals existed between the three major groups, however.

B. *Normal young.* Sexual distinction of newborn *Lebistes* cannot be accomplished macroscopically. The anal fin modification (the gonopodium) and the typical coloration is not obtained by the male for nearly two months. Histological study of the immature gonad does reveal the sex of the individual, however, and may be used to sex specimens.

TABLE II

Experimental condition of the fish and brood size, brood interval and ova development

	Brood size		Brood interval in days		0.8–1.5 mm. ova		0.3–0.5 mm. ova	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
Control	14.6	0.25	29.5	0.08	15.3	0.23	20.3	0.33
1 day	8.5	0.25	35.0	0.23	4.6	0.12	1.8	0.07
1 week	9.5	0.23	33.0	0.07	6.1	0.18	2.2	0.08
2 weeks	10.6	0.20	32.0	0.06	6.8	0.13	3.2	0.08
3 weeks	14.2	0.26	30.8	0.07	12.6	0.25	12.5	0.26

C. *Treated adult.* There is a very apparent decrease in gonadal activity. Without exception, all members of groups 1D, 1W and 2W presented a brood smaller in number than either of their previous broods or the average of their three previous broods. This is most evident in group 1D. Members of group 3W were not so affected, as shown in Table II. Three of the 15 members of group 1D and one member of the 15 in group 1W presented their young earlier than the expected date, that is, the date calculated from their previous performance and the average for the group. The aborted young were not viable. Their bodies were blackened and were undergoing resorption (see Figure 8). A statistically significant increase in the interval between the presentation of broods in a normal manner took place after exposure to thiourea (see Table II). The ovaries of members of groups 1D, 1W and 2W contained fewer than the anticipated number of large, orange, transparent ova. Their number was smaller than the number of young in the broods presented by the fish since the start of treatment with the anti-thyroid drug and smaller than either of its pre-thiourea broods. In addition, the number of ova of the 0.3-0.5 mm. group was markedly reduced. Ovaries of all controls contained more small ova than either the number of the young born in a brood or the eggs composing the more mature group. These data are included in Table II.

D. *Young from treated adults.* The gonads of all young from treated adults were developed sufficiently to permit sexing of the individual by means of histological examination.

Discussion

It has been known for a long time that some teleost fishes retain their young within their body. This live-bearing habit is found in widely distributed families. According to Turner (1947), there seems to be no relation to ecological conditions, geographic distribution or taxonomical relationship. Live-bearing species are found in cold or warm water, lagoons, swift running streams, lakes, caves, at muddy bottoms, at water surfaces and among rocks in fresh or salt waters throughout the world. Although the group of teleosts is old and has had time for evolutionary change, none of the species has evolved a true placental attachment, erosion of maternal tissue by the embryo or an anatomical uterus to harbor the young. The length of time of retention of the young is variable. Some species (for example, the scorpaenids) present their broods in a very immature state. Turner (1942) referred to this condition as "incipient viviparity." Other species present their young in a more developed state. Males of *Cymatogaster aggregatus* are sexually mature when born. The number of broods developing at any one time depends upon the species in question. Superfetation is found in a number of groups. In the extreme case of *Heterandria formosa*, as many as nine broods in different stages of development may be contained within the ovary. All of the live-bearers, of course, show internal fertilization. The sperm traverse the gonaduct to reach the ovary. Egg activation and fertilization may take place immediately or the sperm may be immobilized and stored for a period of months in the ovary and used to fertilize several succeeding broods, according to van Oordt (1928) and Purser (1938). In the latter case, part of the ovarian epithelium acts as a nurse cell. No rule can be stated to describe the site of fertilization, time of ovulation, or area utilized to contain the developing young. If fertilization is intrafollicular, the development may proceed either within the follicle, or the contents of the follicle may be evacuated into the ovarian cavity, or the zygote may be extruded immediately. Turner (1947) referred to the first type as "follicular gestation," the second as "ovarian cavity gestation." Both of these necessarily mean that ovulation follows the fusion of the egg and sperm. Superfetation is found to be superimposed upon follicular gestation; in this case all follicles are not emptied at the same time. Hatching may take place in the follicle, ovarian cavity or after expulsion from the adult's body.

There is no correlation between site of harboring and the amount of yolk contained in the egg. The deutoplasm may be abundant or relatively scarce in either follicular gestation or in ovarian cavity gestation. Neither has the amount of yolk been found to be correlated with gestation time. The yolk may be relatively abundant during a short gestation period or it may be at a minimum while the gestation period is lengthy. The latter finding, coupled with the existence of temporary structures (both maternal and embryonic) present only during the gravid condition, as well as changes in permanently established organs, suggests *a priori* a material exchange between adult and embryo may proceed.

Structural developments and organ changes of some kind and degree have been found to exist in all live-bearing fish so far studied, regardless of egg yolk contents.

In forms showing a macrolecithal condition and displaying follicular gestation, maternal changes are mainly a thinning of the follicle wall and greatly increased vascularity of that area. The embryonic vascular pericardial sac usually becomes enlarged and drawn over the head region to form a hood-like covering which definitely increases the embryonic vascular surface (Purser, 1938; Turner, 1940c). On the other hand, species whose embryos are equipped with a small yolk supply show a greater number and degree of modifications in both parent and young. The follicular lining, in addition to becoming highly vascular, develops a large number of vascular extensions very much like villi. These may make contact with and be closely applied to the surface of the embryo or be suspended in the fluid contained in the follicle. The pericardial sac is more highly expanded than in the previous case.

Species utilizing the ovarian cavity as the embryo-harboring site display a number of more complex structures. Following fertilization and while still within the follicle, the embryos possess for a short period of time the distended pericardial sac described for the follicular gestation embryos. When in the ovarian cavity the structure is lost and is replaced by extensions of the anal area called trophotaeniae (Mendoza, 1937; Turner 1933, 1937a). In the developing embiotocid fishes, the soft tissues between the ray ends of the vertical fins become vascular and extended (Blake, 1867, 1868). At this time the adult embiotocid cavity wall develops a number of vascular flattened processes which may become applied to the surface of the embryo gill or mouth area. The epithelial lining has been described as high columnar and secretory at this time (Mendoza, 1939, 1940). The spatial arrangement of these structures, the time of their appearance, and their structure certainly support the hypothesis of material exchange in the possessor. Turner (1940) has employed the term "pseudo-placentae" to describe them. The fluid within the cavity which bathes the embryos is said to contain degenerating cells and non-surviving embryos. Both the fluid and these cells may serve as a source of food materials (Turner, 1937a, 1947).

Scrimshaw attempted to provide more definite proof for an interchange of substances. He determined the dry and wet weights of embryos at various stages of development. In 1944, he found that the dry weight of the *Heterandria formosa* embryo increases from 0.017 mg. at the time of fertilization to 6.8 mg. at the time of birth. Wet weight measurements were not sufficiently accurate, for no way was found accurately to eliminate the excess water. Scrimshaw in 1945 applied this method of attack to many genera of Poeciliidae. He found that the dry weight of the intact follicle, determined at various stages of development, showed no significant weight change in 18 species. This is in marked contrast to the situation in oviparous fish where approximately one-third of the initial weight of the egg is used for maintenance during development. Accordingly, Scrimshaw concluded that in the species studied, the adult contributes positively to the embryo and that they receive as much nourishment quantitatively as they require for maintaining their metabolic rate.

In the present study, *Lebistes reticulatus*, a live-bearing Poeciliid was utilized. This species displays follicular gestation and the embryos are supplied with much yolk. *Lebistes* is often referred to as ovoviviparous and it is thought by some workers that the material exchange is nil or at most is restricted to gases. Purser (1938) studied *Lebistes* and found an extremely vascular area covering the yolk sac

and extending between the eyes and opercula and making its way over the head. This strap of tissue is almost completely covered with a vascular network. Purser referred to this development as a "specialized respiratory mechanism." The present writer confirms the head covering and records ridges containing blood vessels on the internal surface of the follicular wall.

Anti-thyroid compounds such as thiourea and related substances have been shown to affect the thyroid in all classes of vertebrates. The usual finding is an increase in the size of the gland and its cells and the production of a hypofunctioning organ. Studies have included many animals, among which are the following: rat (Astwood *et al.*, 1943; Mackenzie and Mackenzie, 1943; Hughes, 1944), monkey (McGinty and Wilson, 1949; Mixner *et al.*, 1944; Astwood *et al.*, 1944), reptiles (Ratzersdorfer *et al.*, 1949; Adams and Craig, 1949, 1950, 1951), amphibians (Gordon *et al.*, 1943, 1945b; Hughes and Astwood, 1944; Joel *et al.*, 1949; Adams and Craig, 1949), fishes (Goldsmith *et al.*, 1944; Nigrelli *et al.*, 1946; Lever *et al.*, 1949; Rasquin, 1949; Osborn, 1951; Chambers, 1951; Scott, 1953).

Goldsmith *et al.* (1944), reported hyperplasia of the thyroid in a hybrid strain of *Xiphophorus helleri* and *X. maculatus* after immersion of the fish in a thiourea solution. Their fish were inhibited in growth and failed to develop secondary sex characters. The studies of Rasquin (1949) on *Astynax mexicanus* and Scott (1953) on *Brachydanio rerio* substantiate the hyperplasia previously reported. Lever *et al.* (1949) investigated the effect of a short thiourea treatment upon the thyroid of *Lebistes reticulatus* and *Callionymus lyra*. They reported an increase in thyroid cell height as early as the first week. Fish in the present study showed extreme hyperplasia within eight days, but checks at shorter intervals were not made. In addition to the localized changes, metastasis to many areas of the body was induced.

In many animals, a hypofunctional state of the thyroid may be produced surgically or chemically by a number of goitrogenic compounds. The relationship between oxygen consumption and the metabolic rate is understood and known to be affected by the thyroid status in homoiothermal animals. Hypofunction leads to decreased oxygen consumption while hyperfunction results in the increase of oxygen consumption. Attempts to alter the oxygen uptake in fish by methods which have proven to be effective and reliable in other animals have yielded results that are contradictory. Drexler and Issekutz (1934) found that thyroxine in a 1:500 000 concentration failed to increase the metabolic rate of *Lebistes*. The work of Etkin *et al.* (1940) failed to reveal any increased metabolism after the feeding of desiccated thyroid tablets to goldfish for 9 weeks; the body weights were not changed even after treatment for 16 weeks. They interpreted the results as an absence of a metabolic effect of the thyroid in fish. Grobstein and Bellamy (1939), working with immature *Xiphophorus* (=*Platypoecilus*), produced an exaggerated exophthalmos, a change in body proportions and precocious sexual development. Smith and Everett in 1943 administered thyroxine and desiccated thyroid to new-born guppies for 50 to 90 days. They found no change in growth rate and the fish differentiated at the same time as the controls. Further, oxygen utilization was not different after a one-week exposure.

The administration of anti-thyroid drugs to fish has produced more consistent data. Goldsmith *et al.* (1944) considered the inhibition of growth and retarded

sexual development as (p. 133) "presumptive evidence that the thyroid gland is concerned with growth and development of sex characters of fishes." They concluded, "it appears that thiourea produces these effects by interfering with thyroid hormone production in much the same manner as reported for the mammal" (p. 133). Scott (1953) ascribed the retardation of growth in *Brachydanio* after thiourea administration to a hypofunctional thyroid. He stated (p. 59), "The evidence obtained from the observation of the glands indicated that such treatment produced a hypofunctional thyroid in the zebra fish." Nigrelli *et al.* (1946), working with *Lebistes reticulatus,* treated simultaneously with mammalian thyroid powder and thiourea, noted an increase in growth of these fish similar to that of their untreated controls and greater than that observed in their thiourea-treated fish. Osborn (1951) studied oxygen consumption by stonerollers (*Campostoma anomalum*) and bigmouth shiners (*Notropis dorsalis dorsalis*) immersed in thiouracil. Oxygen consumption was reduced about 20% with dosages of thiouracil that caused no mortality. The decrease was slight on the first day of treatment, but reached the 20% figure on the second day. A pronounced increase in oxygen consumption took place soon after replacing the fish in plain water. The increase was accomplished more slowly in fish treated for 22 days than in those treated for 9 to 11 days. The BMR is lowered in higher animals treated with goitrogenic compounds (Barker, 1949; Mackenzies, 1943; Leathem, 1945; Gordon *et al.,* 1946). Generally the decrease parallels that seen in surgically thyroidectomized animals. In view of the controversies concerning the fish thyroid, further study of the metabolic maintaining mechanism of these animals should be made.

The ovary of *Lebistes reticulatus* normally contains, after the presentation of young, a number of nearly mature ova (0.8–1.5 mm.) approximately equal to the number of young in previous broods. The number of the smaller and less developed ova (0.3–0.5 mm) is consistently larger than this. This finding indicates that a number of eggs undergo atresia. The thiourea-treated animals of groups 1D, 1W and 2W suffered a decrease in number of young in their fourth brood as well as a smaller than normal number of ova of the sizes mentioned above. Group 3W individuals, although presenting a fourth brood of normal number, possessed ovaries containing fewer than normal eggs of the 0.3–0.5 mm. group. These findings are evidence of a decreased ovarian function. The difference in the severity of reaction to thiourea between the groups is no doubt a reflection of the difference in time of exposure. Chambers (1951), Barrington and Matty (1952) and Scott (1953) also noted a gonad inhibition in fish after thiourea administration. It may be theorized that the decreased ovarian function is the result of a lowered production of a gonad-stimulating hormone elaborated by the pituitary, rather than being a direct action of the administered thiourea. Matthews (1939) has shown that gonadal function in the teleost *Fundulus* is dependent upon the presence of the pituitary. He recorded in 1939 that hypophysectomy results in lowered gonadal activity in that genus. It may well be that with the suppression of thyroid gland activity caused by thiourea, there is a great need for a rise in the titer of a pituitary thyroid-stimulating factor. The increased thyroid-stimulating factor production is accomplished by a sacrificing of the gonad-stimulating hormone to the level where the latter cannot maintain a normal functioning gonad. The present results are comparable to those reported to occur in rats. Barker (1949) noted a markedly

reduced fertility with altered estrous cycles after thiourea treatment. Decreases in size and weights of mice ovaries and uteri have been observed by Dalton *et al.* (1945). Jones, Delfs and Foote (1946) induced fetal resorption by thiouracil administration. They also stated that a decrease in the titer of gonadotropin occurred after prolonged treatment with the drug. The small size of the guppy makes hormone analysis of the blood a difficult, if not impossible, procedure at the present time.

It is well known that the secretory activity of the thyroid is regulated by an interaction and balance between that gland and the anterior lobe of the pituitary. A decrease in thyroid hormone output reflects itself in an increased production of the thyroid-stimulating hormone by the pituitary (Gordon *et al.*, 1945a; Grasso, 1946). A hypofunctioning thyroid may be produced by the administration of thiourea and related compounds. It has been found that the ability of the thyroid to take up iodine is depressed within one hour after the injection of anti-thyroid compounds into rats (Larson *et al.*, 1945). As a consequence of the decreased amount of circulating thyroid hormone and an increase in the stimulating factor, the thyroid is changed morphologically and physiologically. The thyroid tissue is a very labile one and soon demonstrates morphological alterations in an attempt to adjust its hormone output. Hyperemia and heightened epithelium are seen within 24 to 48 hours. Hyperplasia becomes pronounced in most forms within a week.

The scheme outlined in the preceding paragraphs, *viz.*, a decreased thyroid function followed by an increased circulating titer of thyroid-stimulating hormone and a decreased amount of a gonad-stimulating hormone, may well explain the interference with reproductive activities seen after thiourea immersion.

The thyroid tissue of all young of all treated groups gave evidence of great stimulation. Heightened cells, afollicular cell arrangement, masses of thyroid tissue without colloid, and metastasis to abnormal sites were found. Two main hypotheses may be offered to explain the stimulation. It may be argued that thiourea itself passed from the blood vessels of the maternal ovarian follicle wall to the embryo by way of the structures previously described. This compound, circulating within the embryo, suppressed the activity of its thyroid, resulting in an increased embryonic thyroid-stimulating hormone titer. The high level of the stimulating factor then caused morphological changes in the target organ. This hypothesis assumes fetal thyroid and pituitary function. The second possible explanation depends upon an increase in adult thyroid-stimulating factor (which must be granted in order to explain the adult thyroid changes) and the transfollicular transmission of this agent from the adult to the embryo. The maternally derived factor then stimulated the embryo thyroid. Valuable information on this question might be obtained if gravid females could be hypophysectomized in the latter part of the gestation period and subjected to thiourea starting at that time. If thiourea and an embryonic pituitary factor were responsible for the hyperplastic thyroid of the young noted in the present work, these same changes would follow this procedure. On the other hand, if the adult pituitary factor was the sole agent causing the hyperplasia the thyroid would not be changed. This method of attack assumes that the females could maintain and deliver their young after being deprived of the pituitary. Considering molecular size alone, other factors being equal, it would be easier for thiourea to pass from the adult to the embryo. The molecular weight of thiourea is 76.12. The

thyroid-stimulating hormone in a rather purified form obtained from sheep and beef pituitary glands possesses a molecular weight of approximately 10,000. Because of species differences, this finding cannot be carried over directly to the fish pituitary factor, but it does allow some speculation as to the size of the molecule. Direct placental transmission of anti-thyroid compounds has been shown to occur in the rat (Hughes, 1944; Williams, 1944; Goldsmith *et al.,* 1945) and in the mouse (Kaufman *et al.,* 1948).

Accepting the fact that the *Lebistes reticulatus* embryo is contained within an ovarian follicle and has no connection with the external environment except by way of the adult, one must concede that a transfollicular movement of material is possible and did occur in the present work. The use of the term ovoviviparous, in its strict sense, to describe the guppy is therefore open to question.

Summary

1. The adult thyroid of *Lebistes reticulatus* after exposure, by immersion, to a 0.04% thiourea solution displayed an extreme hyperplasia. An increase in follicle size and number and their establishment in abnormal areas took place. The heart, kidney, spleen, and intestines were invaded by the thyroid epithelial cells. The mean height of the follicular cells of the fish receiving the shortest thiourea treatment increased approximately 130%. With longer periods of exposure to thiourea, greater heights were produced.

2. The thyroid gland of the newborn of thiourea-treated females had undergone hyperplasia and metastasis during ontogeny. The thyroid became afollicular, lost colloid and possessed tall epithelial cells. Hearts, kidneys, spleens and intestines harbored the invading cells. A transfollicular transmission of an agent causing these changes took place.

3. *Lebistes reticulatus,* while not having an anatomical uterus, placenta or other means of attaching the developing embryo firmly to a part of the adult female reproductive system, does have maternal and embryonic modifications which permit an exchange of material. The species is therefore not a true ovoviviparous form.

4. The gametogenic function of the adult ovary was suppressed by thiourea administration as evidenced by abortions, decrease in brood size, longer brood intervals and smaller than normal number of ova in the gonad. This finding may be a reflection of a decreased gonad-stimulating hormone titer. The latter may be due to a shift to a greater thyroid-stimulating hormone production at the expense of a gonad-stimulating hormone by the pituitary.

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OSMOTIC STUDIES OF AMPHIBIAN EGGS. III. OVULATED EGGS

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In the two previous papers in this series (de Luque and Hunter, 1959; Hunter and de Luque, 1959) a study was made of the movement of water and cations into and out of the eggs of *Bufo marinus* and *Hyla labialis* with the emphasis on ovarian eggs. It was hoped that by making a similar study with fertilized and unfertilized eggs laid in water, it might be possible to find a basis to explain the apparent decrease in osmotic pressure of these eggs after they have left the ovary. These observations are included in the present paper.

Materials and Methods

As in the previous experiments, *Hyla labialis* were obtained locally and *Bufo marinus* were shipped to Bogotá by air. *Bufo* pituitaries suspended in 0.66% NaCl were injected to obtain ovulation. Since with these two species, sperm suspensions, obtained by removing the testes from a male, cannot be used to fertilize the eggs, the males also had to be injected. It has now been established (A. S. Hunter, unpublished data) that one can find *Hyla* with mature ova twice a year, associated with the rainy seasons. Pituitary injections at these two seasons usually result in fertilized eggs. However, in the case of *Bufo* nothing is known about their normal season in Colombia for ovulation, and consequently fertilized eggs were obtained from this species only occasionally.

As previously described, the jelly layer was removed from the eggs with watch-maker's forceps and the eggs were suspended in various NaCl and KCl solutions. Since volume changes have previously been reported, only a few of these measurements were made. Water and Na and K content were measured as before. A new attempt was made to measure non-solvent water using a distribution technique (*cf.* Parpart and Shull, 1935). The quantity of glycerol was determined by the method of Bailey (1959).

Results

Bufo ovarian eggs. In the first paper of this series it was suggested that *Bufo* ovarian eggs separated from their follicular layers swelled less than these eggs plus their follicular layers. In those experiments volume changes of eggs plus layers were measured after a short exposure and after a longer exposure to solutions with different osmotic pressures. Similar measurements using eggs without layers were made only after short exposures to the solutions since such eggs rapidly become aspherical. Since the apparent difference in volume changes

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between eggs with and without their follicular layers could have resulted simply from a difference in time of exposure, these experiments were repeated with observations at the end of an hour and at the end of 24 hours. At the end of an hour's exposure to solutions of NaCl (0.75–0.55%) the same small volume changes were observed with both types of eggs. This is similar to the changes previously reported (Fig. 2, p. 462 and the open symbols in Fig. 1, p. 461). At the end of 24 hours, the eggs without follicular layers swelled appreciably in all of the solutions (*cf.* closed symbols in Fig. 1, p. 461) with larger volume changes occurring in more hypotonic solutions. Measurements of Na and K content of these eggs after 24 hours in these solutions also indicated that Na and K could move across the cell membrane. It can be concluded, then, that water and salts can move slowly into ovarian eggs and also into the cells forming their follicular layers.

TABLE I

The amount of Na or K present in jelly following suspension in solutions of NaCl or KCl

Number of experiments	Na—meq./mm.^{3} $\times 10^5$					
	0.60% NaCl		0.38% NaCl		0.20% NaCl	
	Observed	Calculated	Observed	Calculated	Observed	Calculated
8	9.9	10.2	8.0	6.5	5.6	3.4
	K—meq./mm.^{3} $\times 10^5$					
	0.60% KCl		0.38% KCl		0.20% KCl	
7	7.6	8.0	5.1	5.1	2.9	2.7

Ovulated eggs. In all of the experiments made using eggs laid in water, whether fertilized or not, interpretation of the data becomes difficult because of the presence of a layer of jelly of varying thickness surrounding the eggs. The majority of the jelly was removed but it is almost impossible to obtain a completely clean egg on which to experiment. Moreover, if all of the jelly is removed the eggs do not develop normally (a higher percentage of exogastrulae results). For this reason, studies had to be made of isolated jelly in order to determine its contribution to the results obtained from eggs with a small jelly layer.

Since it was difficult to obtain sexually active males and females of *Bufo* simultaneously, only a few samples of *Bufo* fertilized eggs were studied. Because of the theoretical importance of a possible change in the amount of non-solvent water in the ovulated eggs (as compared to ovarian eggs) all of the samples of fertilized *Bufo* eggs were used for these studies, which unfortunately did not give repeatable results. The data on water, Na and K content of isolated jelly and of laid (unfertilized) *Bufo* eggs and of fertilized *Hyla* eggs follow.

Jelly. The jelly will be considered first, since it is involved in all of the other determinations. The difference between wet and dry weight showed that at least

TABLE II

Changes in water content of unfertilized eggs of Bufo marinus placed in solutions of NaCl of different osmotic pressures

Bufo no.	0.60% NaCl		0.38% NaCl		0.20% NaCl		Water	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	87	4.96	90	6.02	87	4.97	95	13.39
2	88	6.16	86	5.09	89	6.69		
3	95	5.37	96	9.50	95	6.47		
Av.	90	5.50	91	6.87	90	6.04		

TABLE III

Changes in salt content (meq./mg. $\times 10^5$) of unfertilized eggs of Bufo marinus placed in solutions of NaCl of different osmotic pressures

Bufo no.	0.60% NaCl		0.38% NaCl		0.20% NaCl		Water	
	Na	K	Na	K	Na	K	Na	K
1	24.8	14.0	21.8	14.8	19.0	14.0	7.3	15.0
2	33.4	16.0	27.6	16.4	18.6	14.0		
3	25.8	4.8	27.7	4.5	13.0	3.9		
Av.	28.0	11.6	25.7	11.9	16.9	10.6		

TABLE IV

Changes in water content of unfertilized eggs of Bufo marinus placed in solutions of KCl of different osmotic pressures

Bufo no.	0.60% KCl		0.38% KCl		0.20% KCl	
	%	mg./egg	%	mg./egg	%	mg./egg
1	89	5.42	88	5.96	90	6.91
2	82	4.44	88	6.68	87	6.24
3	87	3.09	88	3.77	93	6.42
Av.	86	4.32	88	5.47	70	6.52

TABLE V

Changes in salt content (meq./mg. $\times 10^5$) of unfertilized eggs of Bufo marinus placed in solutions of KCl of different osmotic pressures

Bufo no.	0.60% KCl		0.38% KCl		0.20% KCl	
	Na	K	Na	K	Na	K
1	13.5	45.0	6.8	29.2	7.7	39.2
2	7.4	35.5	8.2	23.2	5.8	20.2
3	8.0	59.9	3.1	23.3	4.3	28.7
Av.	9.6	46.8	6.0	25.2	5.9	29.4

TABLE VI

Changes in water content of fertilized eggs of Hyla labialis placed in solutions of NaCl of different osmotic pressures

Hyla no.	0.60% NaCl		0.50% NaCl		0.38% NaCl		0.20% NaCl		Water	
	%	mg./egg	%	mg/.egg	%	mg./egg	%	mg./egg	%	mg./egg
1	75	1.93	78	2.01	79	2.30	81	3.11	87	4.24
2	65	1.43	77	1.82	72	1.45	75	1.60	90	5.93
3	80	1.92			82	2.96	83	2.40		
4	71	1.52					82	3.06		
Av.	76	1.70	78	1.92	78	2.24	80	2.54	89	5.09

99.5% of the jelly of both types of eggs is water. Solvent water measurements, which in the case of jelly are probably valid, showed that 100% of the water in the jelly is solvent. In 6 determinations on *Hyla* jelly, values between 98 and 144% were obtained.

Hyla jelly was placed in NaCl and KCl solutions of different concentrations. After one hour the Na and K content of the jelly was determined. In Table I a comparison is made between the amount of Na or K present in the jelly after one hour in the various solutions and the theoretical amount expected with diffusion equilibrium. In solutions of NaCl, no K was present in the jelly and vice versa. From this it can be concluded that normally there is little, if any, Na or K in jelly.

It can readily be seen that in all of the six solutions there is good agreement between the calculated and observed values. It can be concluded that in solutions of NaCl and KCl the jelly reaches diffusion equilibrium rather rapidly.

To calculate the volume of jelly surrounding each egg, measurements were made in water of the diameter of the egg and of the egg plus jelly. In one series of experiments the average volume of an egg was found to be 1.98 mm.^{3} and the volume of the egg plus jelly, 5.60 mm.^{3}. Even when extreme care was used to remove as much jelly as possible without injuring the eggs these volumes were 1.65 mm.^{3} for an egg and 2.86 mm.^{3} for egg plus jelly. Similar measurements of eggs in 0.38% NaCl gave an average value of 1.03 mm.^{3} for the volume of an egg and 2.11 mm.^{3} for the volume of egg plus jelly. The difference between these

TABLE VII

Changes in salt content (meq./mg. $\times$ 10^5) of fertilized eggs of Hyla labialis placed in solutions of NaCl of different osmotic pressures

Hyla no.	0.60% NaCl		0.50% NaCl		0.38% NaCl		0.20% NaCl		Water	
	Na	K	Na	K	Na	K	Na	K	Na	K
1	15.8	18.0	10.6	13.1	8.1	13.2	10.0	13.5	4.1	11.9
2	17.2	14.0	10.8	11.2	11.4	11.8	4.5	8.5	4.8	12.9
3	19.6	10.5			16.2	14.0	11.7	8.7		
4	13.7	10.1					16.0	14.7		
Av.	16.6	13.2	10.7	12.2	11.9	13.0	10.7	11.4	4.5	12.4

TABLE VIII

Changes in water content of fertilized eggs of Hyla labialis placed in solutions of KCl of different osomotic pressures

Hyla no.	0.60% KCl		0.38% KCl		0.20% KCl		Water	
	%	mg./egg	%	mg./egg	%	mg./egg	%	mg./egg
1	81	2.42	84	2.93	80	2.53	83	2.86
2	61	1.73	72	1.91	63	1.45	84	4.08
3	75	2.17	77	2.46	73	2.20	88	5.64
4	64	1.26	67	1.44	72	2.05	85	4.09
5	72	2.05	77	2.83	60	1.22	83	4.60
Av.	71	1.93	75	2.31	70	1.89	85	4.25

values obtained in water and in 0.38% NaCl suggests that in water both the egg and the jelly swell but much more water is taken up by the jelly. It can be seen that in all of the subsequent experiments to be reported the volume of the jelly is larger than the volume of the eggs.

Bufo, unfertilized eggs. Tables II–V show the changes in water and salt content of unfertilized *Bufo* eggs after having been in various NaCl and KCl solutions during an hour. Since these eggs are always very irregular in form, volume measurements were not made.

Hyla, fertilized eggs. Similar data for *Hyla* fertilized eggs are presented in Tables VI–IX.

ANALYSIS OF RESULTS

Because of the nature of the above experiments, various assumptions have to be made in trying to interpret the data. It is quite possible to change one's assumption and arrive at a completely different conclusion. The analysis which follows, then, represents one possible interpretation of the data. The only point the authors want to make is that the data are not inconsistent with these suggestions.

In general, a comparison of the above data with those previously reported shows that all of the changes are less with ovarian eggs. From the data in Table X it can be seen that the volume of unfertilized eggs (plus jelly) of *Bufo* is much

TABLE IX

Changes in salt content (meq./mg. $\times$ 10^5) of fertilized eggs of Hyla labialis placed in solutions of KCl of different osmotic pressures

Hyla no.	0.60% KCl		0.38% KCl		0.20% KCl		Water	
	Na	K	Na	K	Na	K	Na	K
1	7.9	28.6	8.8	18.9	7.5	15.5	7.8	15.0
2	5.9	15.8	3.7	10.5	5.1	28.0	4.6	7.2
3	5.6	14.2	5.5	12.8	2.9	25.2	5.5	8.0
4	6.0	12.8	6.0	12.6	4.7	25.1	5.1	8.4
5	5.0	13.8	4.7	13.1	3.2	25.6	4.7	8.2
Av.	6.1	17.0	5.7	13.6	4.7	23.9	5.5	9.4

larger than that of ovarian eggs. The unfertilized eggs also have more Na and K. Fertilized *Hyla* eggs (plus jelly) are much larger than ovarian eggs and in NaCl solutions they have more Na and K. The presence of the jelly obviously could contribute to this difference.

One interesting point of comparison is that in spite of the differences in quantity and percentage of water in the eggs in the different solutions (Tables II, IV, VI, and VIII) the dry weight per egg, as calculated from these data, is always essentially the same, as would be expected. The dry weight of a *Bufo* ovarian egg as previously published was 0.59 mg. The unfertilized eggs in NaCl and KCl solutions give values of 0.61–0.75 mg./egg. The published figure for *Hyla* ovarian eggs is 0.65 mg./egg. In the present experiments with fertilized eggs the values are 0.54–0.81 mg./egg. Considering the difference in size of eggs from different females it seems reasonable to say that the eggs neither gain nor lose considerable quantities of substances included in the dry weight when they leave the ovary.

It is obvious from the measurements mentioned earlier of the volume of an egg and the volume of its jelly that a large part of the volume changes in Tables II,

TABLE X

A comparison of water (mg./egg) and salt content (meq./mg. × 10^5) of ovarian and laid eggs in isotonic solutions (0.66% and 0.38%, respectively)

Type of egg	NaCl			KCl		
	Water	Na	K	Water	Na	K
Bufo, ovarian	1.17	12.2	7.8	0.93	2.6	12.9
Bufo, non-fertilized	6.87	25.7	11.9	5.47	6.0	25.5
Hyla, ovarian	0.64	7.2	3.1	1.47	5.8	15.7
Hyla, fertilized	2.24	11.9	13.0	2.31	5.7	13.7

IV, VI, and VIII may be due to changes in the amount of water in the jelly. To try to determine whether or not water enters or leaves the egg let us consider that 0.38% salt solution has one unit of osmotic pressure and that the volume of the water in the egg plus jelly is 1. The expected volume of water in any other solution can be calculated using the formula PV = constant. Table XI compares these calculated values with the actual changes in the volumes of the eggs.

With the exception of the first value, the changes in the volume of water are always less than would be expected. That is, in hypertonic solutions the eggs shrank less and in hypotonic solutions they swelled less than the calculated values. An average of the per cent deviation of the last 6 values gives a figure of 28%. Using the figures previously given (egg plus jelly, 2.11 mm.^{3}; egg, 1.03 mm.^{3}) 28% of 2.11 mm.^{3} gave a value very similar to the quantity of water in an egg with a volume of 1.03 mm.^{3}. Using the other measurements (egg plus jelly, 5.60 mm.^{3}; egg, 1.98 mm.^{3}) a similar calculation leads to the same conclusion. This would suggest that at least the major portion of the change in water content occurred in the jelly. Tentatively, then, let us suggest that these studies of changes in water content might be interpreted as indicating a decreased rate of movement of water across the membrane of an egg after it has left the ovary.

TABLE XI

A comparison of the theoretical and observed volume changes in water content

Egg	Solution	Water content		
		Calculated volume	Observed volume	% difference
Bufo	0.60% NaCl	0.63	0.62	2
	0.60% KCl	0.63	0.79	25
	0.20% KCl	1.90	1.20	37
Hyla	0.60% NaCl	0.63	0.76	21
	0.50% NaCl	0.76	0.85	12
	0.20% NaCl	1.90	1.13	41
	0.60% KCl	0.63	0.84	33

In Table XII an attempt was made to determine how much of the increase in salt in the egg could be attributed to salt dissolved in the jelly. Two methods were used to calculate the *Hyla* data. The volume of the egg was considered to be 1.03 mm.^{3} (obtained from the measurements previously given) or 1.44 mm.^{3} (obtained by adding the dry weight to the amount of water in an ovarian egg). Only the latter method (volume = 1.23 mm.^{3}) was used in calculating the *Bufo* data. It was assumed that this volume did not change in the different solutions. (Probably this assumption is not completely correct but the error introduced would not change the argument.) This value was subtracted from the total volume of water actually measured in the various solutions (Tables II, IV, VI, and VIII) to give a minimum value for the amount of water in the jelly. The volume of jelly water was then multiplied by the calculated milliequivalents of Na or K in the different solutions to give a theoretical value for the amount of Na or K in the water in the jelly, assuming diffusion equilibrium. These figures can then be compared with the total amount of Na or K per egg calculated on the basis of 0.65 mg. per egg as a dry weight of *Hyla* eggs and 0.59 mg. for *Bufo* eggs.

In the case of *Bufo* eggs, comparing the last two columns, with the exception of 0.20% KCl (these data are inconsistent in every respect), the increase in measured amounts of Na and K could have been due completely to the solution

TABLE XII

A comparison between calculated values of Na and K in the jelly layer and observed values in eggs plus jelly

Solution	Ion	Calculated quantity in *Hyla* jelly		Obs. *Hyla*	Calculated quantity in *Bufo* jelly (1.23 mm.^{3})	Obs. *Bufo*
		(1.03 mm.^{3})	(1.44 mm.^{3})			
0.60% NaCl	Na	6.9	2.7	10.8	44.0	16.5
0.38% NaCl	Na	7.9	5.2	7.7	36.7	15.2
0.20% NaCl	Na	5.1	3.7	7.0	16.3	10.0
0.60% KCl	K	7.2	3.9	11.0	24.8	27.6
0.38% KCl	K	6.5	4.2	8.8	21.6	14.9
0.20% KCl	K	2.3	1.2	15.5	14.2	17.3

of these ions in the jelly. In the case of *Hyla* eggs, the same conclusion could be reached. This suggestion could also explain the observation that in the present experiments there is more salt in the eggs plus jelly in more concentrated salt solutions, while with the ovarian eggs the amount of salt in the eggs was essentially the same regardless of the concentration of the surrounding solution.

Another method to analyze the data to test the same point follows. If we assume that the concentration of Na or K in the jelly is the same as in the surrounding solution, and if we further assume that the amount of Na or K in the egg is constant, three simultaneous equations can be solved for each series of experiments to determine the amount of Na or K in the eggs. If we let a = volume of jelly in mm.^{3} and b = milliequivalents of Na (or K) in the egg, then for *Bufo* eggs in NaCl solutions:

$$10.3 \times 10^{-5}a + b = 19.1 \times 10^{-5}$$
$$6.4 \times 10^{-5}a + b = 17.5 \times 10^{-5}$$
$$3.4 \times 10^{-5}a + b = 11.5 \times 10^{-5}$$

With these three equations an average value of 1.17 mm.^{3} for the volume of the jelly was obtained. Using this value, the amounts of Na in the *Bufo* eggs in the three NaCl solutions were 7.0, 10.1 and 7.5×10^{-5} meq./mg. These values are quite similar to those previously obtained with ovarian eggs. Making similar calculations for *Bufo* eggs in KCl solutions and for *Hyla* eggs in NaCl and in KCl solutions, the values for Na or K in the eggs in these experiments are similar to, or less than, the amount previously reported in ovarian eggs.

The above calculations would suggest that salts as well as water enter and leave ovarian eggs more rapidly than they enter and leave eggs that have left the ovary.

Another calculation which is of interest is that previously made with ovarian eggs (Hunter and de Luque, last line, p. 476). From the data in Table II, the dry weights of the eggs in each of the three solutions of NaCl can be calculated. Multiplying these dry weights by the values of Na and K in Table III, the total milliequivalents of cations per cell can be calculated. Dividing this by the amount of water per cell, the concentration (in milliequivalents of Na plus K per liter) can be calculated. Values of 44, 37 and 31 were obtained with these data. These values are to be compared with 140 previously calculated for ovarian eggs. Similar calculations with *Bufo* eggs in KCl solutions and *Hyla* eggs in NaCl and KCl solutions yielded values considerably lower than for ovarian eggs. Although it is difficult to assess the effect of the jelly in these calculations, one might conclude that the osmotic pressure of eggs that have left the ovary is less than the osmotic pressure of ovarian eggs.

Discussion

The various measurements of volume changes of ovarian eggs, unfertilized and fertilized eggs in solutions with different osmotic pressures have shown that water can move into or out of the three different types of eggs. The studies of Na and K content also suggest that these cations can move across the membranes of the three types of eggs. One analysis of the present data leads us to the conclusion that a decrease in permeability to water and salts in eggs that have left the ovaries is not inconsistent with the data. As was mentioned in the review of the literature

in the first paper of this series, similar suggestions have previously been made, based on studies of various eggs.

If the above suggestion is correct, it could explain how an egg laid in water could survive without losing all of its salts and without swelling indefinitely due to the entrance of water. However, it does not explain the apparent decrease in osmotic pressure in the eggs when they are laid.

The present data do not support the idea that the decrease in osmotic pressure of the eggs after they have left the ovary results from the loss of salts or from the entrance of a large quantity of water. On the contrary, the data in Table XIII suggest that there is an increase in the amount of K in the eggs that have left the ovary. The values of t (calculated by the Method of Fischer) indicate that there is no difference in the amount of Na in *Hyla* ovarian and fertilized eggs but there is a difference in K in these eggs significant at the 2% level. There is no significant difference in the amount of Na in *Bufo* ovarian and unfertilized eggs but a highly significant difference in the amount of K in these eggs.

In making the salt analyses of the egg homogenates, the proteins are precipitated with trichloracetic acid. This means that whatever quantities of Na and/or

TABLE XIII

A comparison of the Na and K content of ovarian and non-ovarian eggs of Hyla labialis and of Bufo marinus

	meq./mg. $\times 10^6$		t
	Ovarian	Ovulated	
Hyla, Na content	4.4 ± 0.20	5.5 ± 1.22	0.32
Hyla, K content	5.4 ± 0.03	12.4 ± 0.95	4.10
Bufo, Na content	6.8 ± 0.32	7.2 ± 0.29	3.12
Bufo, K content	8.3 ± 0.25	12.3 ± 0.31	72.0

K are bound to the proteins of the egg are not included in the Na and K measurements. If one postulates: (1) that in the ovarian eggs some K is bound to proteins, and (2) that on leaving the ovary there is a change in the proteins which decreases their capacity to bind K, the observed increase in K could be explained. If this is true, one might also postulate that the amount of water bound to proteins decreases. Unfortunately, with the methods available in this laboratory, satisfactory values of non-solvent water could not be obtained. Using the glycerol-distribution technique with fertilized eggs of *Hyla,* the amount of glycerol that disappeared from the solution increased more or less indefinitely with time. The possibility that these eggs are metabolizing glycerol is being investigated. Because of the importance of determining whether or not there is a change in solvent water when the eggs leave the ovary, one of the authors (O. de L.) will continue these studies in another laboratory using isotopic techniques.

SUMMARY

1. Studies of eggs of *Bufo marinus* and of *Hyla labialis* that have left the ovary indicate that there is no large change in the total amount of water nor of Na and K.

2. A possible interpretation of the data is that after the eggs have left the ovary there is a decrease in their permeability to water and to Na and K.

3. A statistically significantly higher quantity of K in these eggs might suggest a change in the binding-capacity of the egg proteins.

4. If such a change does occur, there might be a decrease in the amount of water bound to the proteins which might explain the lower osmotic pressure of eggs that have left the ovary. Attempts to obtain experimental data to support this hypothesis failed for technical reasons.

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PREPARATION AND USES OF INSECT HEMOCYTE MONOLAYERS IN VITRO[1]

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Repeated subculturing of rapidly proliferating insect cells has not as yet been achieved, and to date no insect cell strain is available (Day and Grace, 1959; Martignoni, 1960). On one occasion a series of tissue subcultures from *Callosamia promethea* (Drury) suggested adaptation to a culture medium (Grace, 1958), but a cell strain was not established. This lack of a continuous cell strain may be responsible for the relatively slow progress in insect virology as compared to the rapid advances in vertebrate virology.

Explantation techniques have been adopted frequently by insect morphologists. However, for biochemical and virological purposes the use of cell suspensions is the most appropriate, and a number of investigations in this direction have been undertaken (St. Amand and Tipton, 1954; Martignoni, Zitcer and Wagner, 1958; Liu, Hsia and Kao, 1958; Aizawa and Vago, 1959). Recently we have developed a method for the preparation of monolayer cultures using insect hemocytes. Although these monolayers have the few disadvantages of primary cultures, they can be prepared easily and the cells are not subjected to treatment with enzymes or chelating agents. Insect blood is a good source of cells. Between 700,000 and 800,000 cells per larva are routinely obtained from the variegated cutworm, *Peridroma saucia* (Hübner) [= *P. margaritosa* (Haworth)], using the technique described in this paper. In comparison, the embryo of the green-striped grasshopper, *Chortophaga viridifasciata* (De Geer), produced about 13,000 cells after hyaluronidase and trypsin digestion (St. Amand and Tipton, 1954), and approximately 160,000 cells can be obtained from the three thoracic segments of a mature larva of *P. saucia* after treatment with snail hepatopancreas extract (Martignoni, Zitcer and Wagner, 1958).

As vertebrate leukocytes have been used successfully for the propagation of viruses (Dunne, Luedke and Hokanson, 1958), and since some insect viruses are known to multiply in hemocytes, *in vivo*, an attempt to prepare hemocyte monolayers for the propagation of insect viruses seemed justified.

Methods

Hemocyte source

Hemocytes were obtained from 2- to 4-day-old sixth instar larvae (last larval instar) of *Peridroma saucia* (Hübner) [= *P. margaritosa* (Haworth)] mass-

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produced according to methods described by Finney (1958). The mean weight of these larvae was 923 mg.

Balanced salt solution (BSS)

Composition. See Table I.

Preparation. This balanced salt solution (BSS) was prepared with extremely high-resistance water obtained by passage of distilled water through a $30 \times 1\frac{3}{4}$-inch column of mixed-bed analytical grade ion exchange resin (AG 501-X8, D, 50–100 mesh; Bio Rad Laboratories; Richmond, California) and storage in polyethylene bottles. One hundred-ml. amounts of saline were prepared as follows: the salts, orthophosphorous acid, and l-ascorbic acid were dissolved in 90 ml. water, and the pH adjusted to 6.45 with 1 *M* NaOH. Distilled, de-ionized water was added to complete the volume of 100 ml., and the solution was sterilized by filtration (Millipore filter, type HA). This saline was preserved in the refrigerator, in rubber-stoppered 100-ml. bottles, for about one week; after

TABLE I

Composition of the balanced salt solution (BSS)

Compound	Grams/liter	Molarity
KCl	6.262	0.084
$CaCl_2$	2.553	0.023
$MgCl_2 \cdot 6H_2O$	3.660	0.018
$H_2(HPO_3)$	1.149	0.014
NaOH	1.068	0.027
l-ascobic acid	1.000	0.006

mole ratio Na/K	0.3
mole ratio K/Ca	3.7
pH	6.45
osmotic pressure (moles NaCl)	0.160

this period much of the ascorbic acid was oxidized and the solution was discarded. For a large-scale operation, it is advisable to have on hand large volumes of NaOH-neutralized solution of salts and orthophosphorous acid, without ascorbic acid. Whenever small volumes of fresh solution are needed (50 to 100 ml.) l-ascorbic acid is added to this stock solution, then neutralized (to pH 6.45), and the saline filtered through a Millipore membrane. The ascorbic acid-free stock solution can be used for the preparation of the culture medium, too (see below).

Although phosphate buffers have been used repeatedly in salines and culture media for insects (*e.g.* Trager, 1935; Ludwig, Tefft and Suchyta, 1957), their use limits the Ca and Mg levels in the solutions. The solution described here is buffered with sodium hydrogen phosphite, which has one considerable advantage when compared to phosphate buffers: the concentration of calcium and magnesium ions which can exist with phosphite in solution, without precipitation, is much greater than that with phosphate (Robertson and Boyer, 1956). This advantage is particularly important in insect tissue culture, as the salines often must contain large amounts of the two cations. A detailed comparison of the solubility of phosphite and phosphate salts has been presented by Robertson and Boyer.

Furthermore, the buffering capacity of phosphite is highest in the vicinity of pH 6.5, the blood pH of sixth-instar *P. saucia*. The approximate pK_2 of phosphite is 6.5. Orthophosphite is known to be nontoxic to cultured mammalian cells.

The blood pH of mature sixth-instar larvae of *P. saucia* was measured in humidified gas mixtures with various proportions of carbon dioxide. Equilibration with CO_2 was necessary, as there is a rapid loss of the gas from insect blood in air (Buck and Friedman, 1958). The pH measurements were made by using a one-drop electrode assembly with a silver chloride reference electrode (Fig. 1).

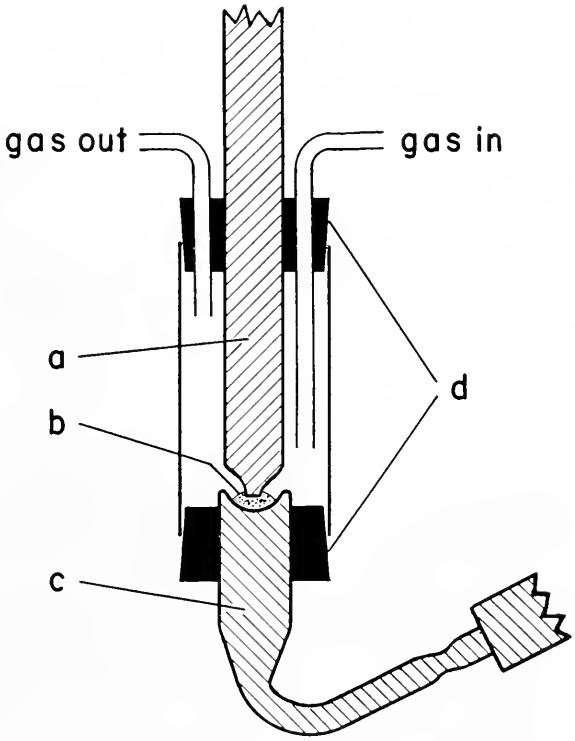


FIGURE 1. Electrode assembly for the measurement of the blood pH in an equilibration chamber: a, silver chloride reference electrode; b, blood; c, one-drop glass electrode; d, rubber stoppers used as closure of glass cylinder.

To obtain blood, an incision was made on one proleg of a larva, and bleeding followed immediately. With sufficient practice, two operators were making the first pH measurement within 10 seconds. Each determination was made on the blood obtained from a single larva, and each curve involves 9 to 15 larvae. The results are reported graphically in Figure 2. The least shift in pH was observed with 5% CO_2, with only a slight drift towards lower values. The 45 values obtained at the first measurement (5–15 seconds after blood extraction) formed a statistically homogeneous population: the mean was pH 6.47, with a standard error of 0.012.

As a basis for establishing the ion ratios in the BSS, the ionic content of four samples of pooled blood plasma (10 to 20 individuals) from sixth-instar larvae of *P. saucia* was determined by flame photometry. The mean contents were the following: Na, 10.3 meq. per liter; K, 33.5 meq. per liter; and Ca, 18.6 meq. per liter. The mean mole ratios were 0.3 (Na/K) and 3.7 (K/Ca). These mole ratios are well in agreement with those of other related phytophagous species

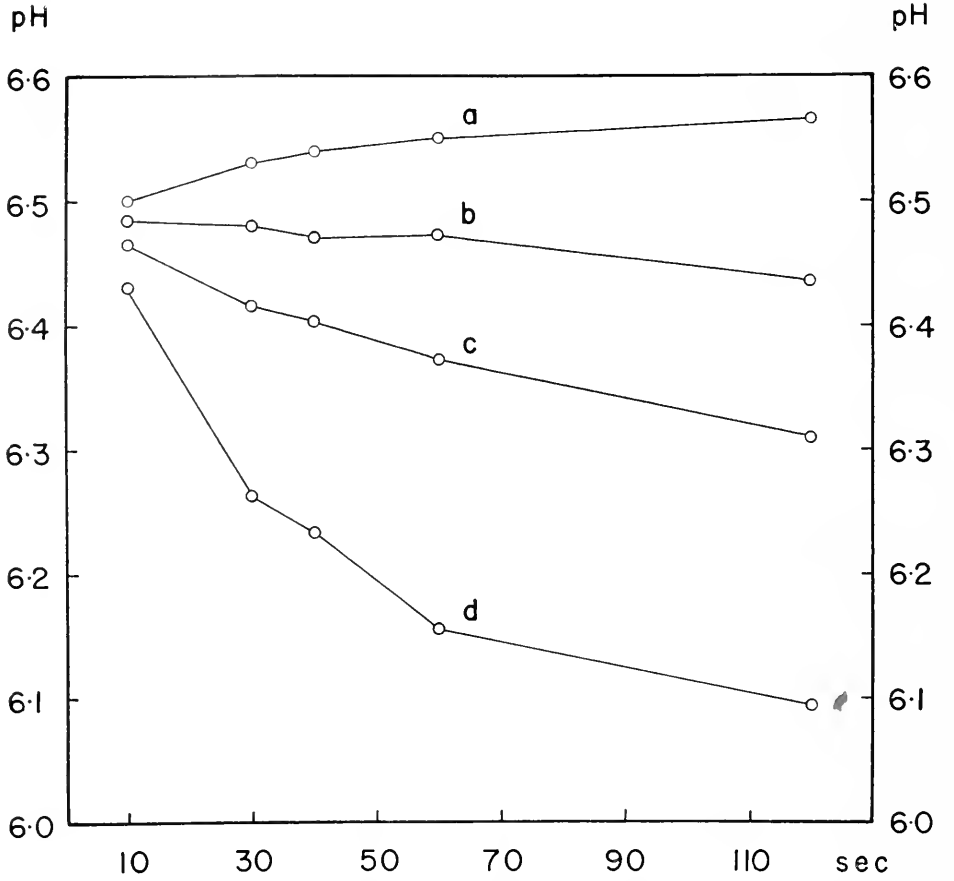


FIGURE 2. Results of pH measurements on whole blood of 2–4-day-old sixth-instar larvae of *Peridroma saucia* (Hübner), in an equilibration chamber, under a flow of: a, air; b, 5% CO_2 and 95% N_2; c, 20% CO_2 and 80% N_2; d, 100% CO_2. Time: seconds from start of bleeding.

(see the comprehensive compilation of Buck, 1953). It is now well established that these ionic ratios (particularly K/Ca) are very important in the formulation of physiologic solutions for insects (Ludwig, Tracey and Burns, 1957).

The BSS is isotonic with the blood of sixth-instar larvae of *P. saucia*. The blood osmotic pressure was determined by the method of Gross (1954). This method permits determinations on samples of less than 0.01 ml. volume to an

accuracy of 0.02° C. Twenty-one sixth-instar larvae were used, and the determinations were replicated two or three times for each larva. The mean osmotic pressure computed from this sample and expressed in m*M* NaCl was 155 (range: 138 to 176), with a standard error of 2.6 m*M*.

One of the important characteristics of the saline described here is that it is poised at a low oxidation-reduction potential (E_h around + 80 mv), with the addition of l-ascorbic acid (molarity: 0.006). Phenylthiourea has been recommended as a tyrosinase inhibitor in insect tissue culture (Schmidt and Williams, 1953), but we found this substance to affect cell cultures adversely. This confirms previous observations by Wyatt (1956), who reported toxic effects of this substance, or an impurity in it, in tissue cultures of *Bombyx mori* (Linnaeus). The mechanisms of the adverse effects of phenylthiourea are not well known, but it is possible that this substance may act also on other vital oxidative reactions: DuBois and Erway (1946) demonstrated that rat lung succinic dehydrogenase was considerably inhibited by thiourea (100% inhibition) and by phenylthiourea (94% inhibition). Jones and Wilson (1959) found that phenylthiourea had a pronounced depressant action on the rate of oxygen consumption of final instar larvae of *Samia cynthia* (Drury). This depressant action may be exerted on parts of the respiratory chain, according to a number of investigators. Ascorbic acid does not specifically inhibit tyrosinase, but maintains a low oxidation-reduction potential in the saline (approaching the situation in the larval hemocoel), sufficiently so to avoid immediate melanosis in cell cultures. The useful concentration of ascorbic acid (6 m*M*) was determined by recording the reactions of the cells and blood plasma to varying amounts of the substance, from 0.23 m*M* to 0.23 *M* per liter. Since l-ascorbic acid undergoes oxidation, the solution must be used fresh (from one to five days old), and stored at 4° C. While we used ascorbic acid, it is possible that other oxidation-reduction systems may work as well.

Maintenance medium (26C)

Composition. See Table II.

Preparation. Medium 26C was prepared with stock solutions: 100 × concentrates of the L-amino acids and of the vitamins are available commercially, and were routinely used in our laboratory. A 100-ml. volume of medium 26C was prepared as follows: (1) place in a beaker 90 ml. of ascorbic acid-free, buffered (pH 6.45) BSS; (2) add 1 ml. each of 100 × concentrates of the amino acids and vitamins; usually L-glutamine is stored as a separate 100 × concentrate; (3) add 1 ml. of 100 × concentrate of penicillin and streptomycin; (4) add and let dissolve 70 mg. glucose, 40 mg. fructose, and 40 mg. trehalose; (5) add and let dissolve 100 mg. l-ascorbic acid and carefully neutralize, under constant stirring, using molar NaOH solution, to pH 6.45; about 1 ml. will be required; (6) bring volume up to 100 ml. with BSS; (7) sterilize by filtration (Millipore filter, type HA); store at 4° C.; do not use if older than 5 days.

Fetal bovine serum gave better results than human, lamb, and horse sera. However, we have not completed yet a systematic investigation of a number of serous fluids. In this connection, it is worth mentioning a report by Gessard (1901), who found that calf and rabbit sera have tyrosinase-inhibiting properties.

TABLE II

Composition of the maintenance medium (26C)

Components	Concentration
Inorganic compounds and l-ascorbic acid	As in BSS described above
13 L-amino acids HeLa	As in Eagle (1955)
9 vitamins	As in Eagle (1959)
3 carbohydrates	As in Wyatt (1956), with d(+) trehalose instead of sucrose
Antibiotics	
Penicillin G	200 units/ml.
Streptomycin sulfate	100 μg./ml.

This medium was supplemented with 15% homologous insect blood plasma and 15% fetal bovine serum.

Gas mixture

Cultures prepared with medium described above were held in a gas mixture of 96% (by volume) nitrogen (minimum purity 99.996%), 3% carbon dioxide (minimum purity 99.95%), and 1% oxygen (minimum purity 99.5%). The three gases were mixed after measuring the rate of flow of each in Matheson Universal Flowmeters. The cultures were kept in a specially adapted jar (capacity: 9 liters), provided with a double phase manometer: after evacuation to 15 inches of mercury the jar was refilled with nitrogen (to lower the oxygen partial pressure), then the flow of the N_2-CO_2-O_2 mixture was maintained at the rate of 600 ml. per minute for 2–3 hours. The low level of oxygen was selected in order to inhibit phenol oxidase activity. A series of tests performed with blood of sixth-instar larvae of *P. saucia* showed that the period from blood extraction to onset of melanosis was prolonged by a factor of 2 or more only when the oxygen content of the gas mixture bathing the fluid was decreased from normal atmospheric level to less than 3%. No melanosis occurred in pure nitrogen. Tyrosinase in insect plasma can be inactivated by heat treatment (Wyatt, 1956); however, satisfactory culture of hemocytes of *P. saucia* is not possible even in the presence of inactivated homologous plasma, unless the oxidation-reduction potential of the culture medium is maintained at a low level by the addition of reducing agents and by exclusion of excess oxygen. We have observed cell melanosis in our cultures even in total absence of homologous plasma. As reported by Kawase (1960), in the case of mature silkworm larvae, hemocytes have a limited, but fairly constant tyrosinase content.

There is some indication in the literature that certain insect tissues may be considered microaerophilic: the hemocytes of *P. saucia* may be placed in this category. Schneiderman, Ketchel and Williams (1953) indicated that spermatogenesis in cultures of spermatocytal cysts of *Hyalophora cecropia* (Linnaeus), in presence of phenylthiourea, is not inhibited by reductions of the oxygen tension from normal atmospheric partial pressure to about 1% of atmosphere. Inhibition was observed below 1%. Similarly, Harvey and Williams (1958) observed that the heartbeat of diapausing pupae of the same species was independent of external oxygen tensions from normal to 0.5% of atmosphere. Oxygen tension seems to

have an effect also on the rate of growth of some mammalian cells. It is worth mentioning that Cooper, Burt and Wilson (1958) found that the liquid-phase oxygen levels for fastest growth of a rabbit kidney cell line were considerably less than air equilibrium values, and as low as 14% oxygen by volume.

Temperature

All cultures were held at 25° C., ± 0.5° C., in darkness.

Preparation and maintenance of cultures

The larvae of *P. saucia* were anesthetized with ethyl ether for about ten minutes, surface sterilized with 0.2–0.4% aqueous solutions of Hyamine 10-X for 5 minutes, with agitation (Martignoni and Milstead, 1960), and then allowed to dry between folded sterile paper towels. The larvae were placed in the germicide solution in the general laboratory area, but the transfer from germicide to the sterile towels was best performed in the culture room. Fifteen to twenty larvae could be handled as one group. It is not advisable to use more than this number at one time, since the effects of the ether anesthesia might end before the last larva is bled, and regurgitation, a common aftereffect of anesthesia in these larvae, may occur. About two minutes were allowed for drainage of the germicide. During this time, 3 to 4 ml. of culture medium were placed in a 12-ml. conical graduated tube, covered with an inverted glass beaker: the exact amount was noted. Each larva was lifted with forceps and carefully placed on the forefinger of the left hand (the operator was right-handed), taking care that the ventral integument of the larva did not come in contact with the skin of the operator. Thumb and middle finger delicately held the larva in place (Fig. 3). One proleg was amputated with a very sharp, blunt-pointed scissor, while the larva was held over the tube (Fig. 4). Between 0.10 and 0.14 ml. blood would flow from the cut and fall in the medium; pressure was not applied on the larval body, as the gut might rupture quite easily. After all larvae were bled, the total volume of blood collected was readily determined by a reading on the tube, graduated in 0.1-ml. units. From a dilution table available to the operator in the culture room the final amounts of the three components were read, and the proper additions made. The composition of the final culture was: 15% insect blood, 15% fetal bovine serum, and 70% maintenance medium.

After gentle mixing by pipette, the cell suspension was distributed in Petri dishes, each containing one Corning 22 × 22 mm. No. 1 coverglass. The volumes were 3.5 ml. for 60-mm. glass Petri dishes and 2 ml. for 35-mm. wettable polystyrene dishes (Falcon Plastics, Los Angeles). The cover glasses could be used for permanent preparations, after the culture was discarded. The Petri dishes were placed without delay in the incubator, in the gas mixture described in this paper. The medium was replaced every third day. We obtained good results without insect blood plasma, by using a replacement medium consisting of 30% fetal bovine serum and 70% medium 26C.

Culturing was performed with strictly aseptic techniques. The routine culture methods, the preparations of materials, etc., are not discussed here, since their description is readily available in most tissue culture textbooks.

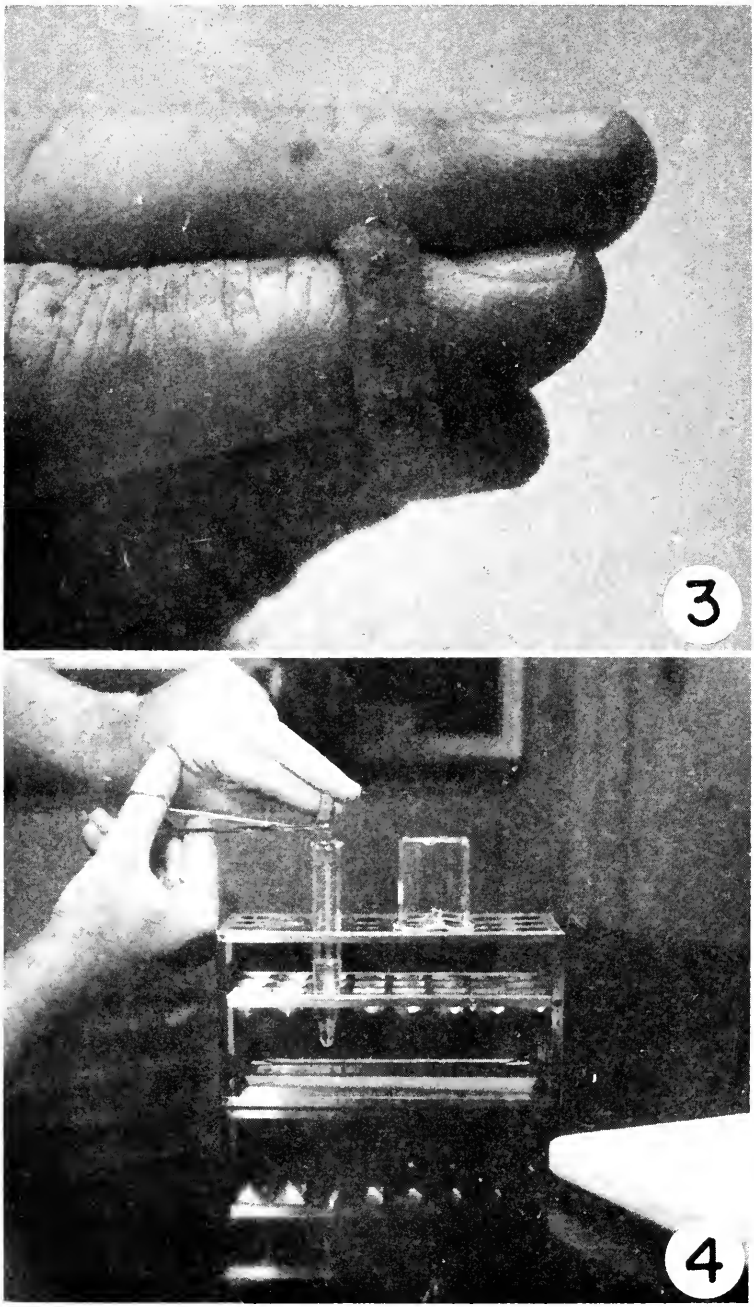


FIGURE 3. The handling technique for positioning of anesthetized larva of *Peridroma saucia* (Hübner). The prolegs are visible on the ventral surface of the larva.

FIGURE 4. The aseptic collection of larval blood (usually performed in a sterile culture room).

Results and Discussion

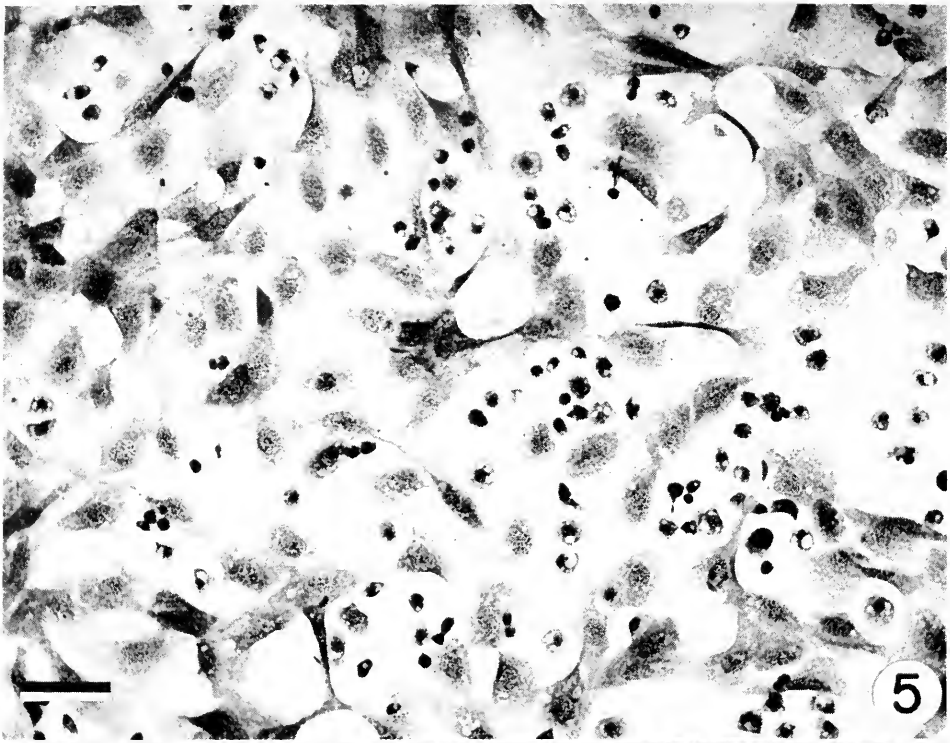
Cells cultured by the methods described here attached to glass readily and formed a sheet in less than 12 hours (Fig. 5). A number of cells remained floating in the culture vessel and did not settle on glass even after one to three days of incubation. These cells were eliminated when the medium was changed. The final cell sheet (Fig. 6) was composed mainly of plasmatocytes (amoebocytes of Wigglesworth, 1956), prohemocytes, and spheroidocytes (terminology according to Jones, 1959). Some degenerating cells were also present. These monolayers could be maintained in perfect condition for 10 to 15 days. Subcultures were not attempted.

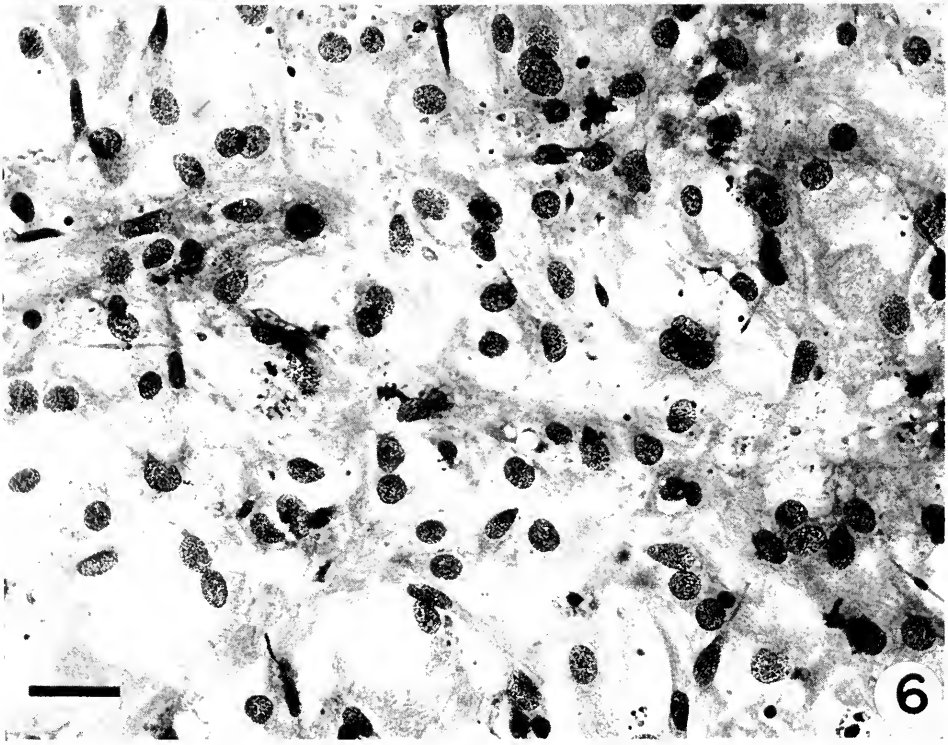
Wigglesworth (1959; and in Campbell, ed., 1959) suggested that the cells migrating from the ovariole of Lepidoptera maintained *in vitro* are hemocytes formerly adherent to the tissues. The striking similarity of cells in cultures from the ovariole sheath of *Samia advena* Packard (Jones and Cunningham, 1960) with the hemocytes in our cultures seems to support this view: unfortunately, the two types of cultures have not been compared experimentally, to our knowledge. While occasional mitoses may be observed in cultures of insect hemocytes, proliferation is limited (or absent in some cases: Rosenberger and Jones, 1958), in the culture conditions available at present. Proliferation has been scanty also in cultures from the ovarian tissue of insect immatures (Grace, 1958; Jones and Cunningham, 1960), as compared with growth curves normally obtained in primary cultures of vertebrate tissue. Growth in insect tissue cultures is probably limited by the amount of endogenous ecdyson in tissue explants (growth "momentum": Schneiderman, in Campbell, ed., 1959).

In the cultures we have observed occasional variations that did not derive from changes in the cultural techniques, but were probably to be attributed to variability of the insect, as cell agglutination and violent tyrosinase activity. The larvae of *P. saucia* were fed with fresh alfalfa (*Medicago sativa* Linnaeus) and seasonal changes are known to occur in the plant. Furthermore, a certain variability in the insectary stock of *P. saucia* was inevitable. The presence of occasional aberrations is, of course, one of the main disadvantages of primary cell cultures.

Two criteria were used, in absence of considerable growth, to judge the suitability of a particular culture medium, substance, or method. One criterion was necessarily subjective: a judgment of the general appearance of the culture and of the conditions of the cell nucleus and cytoplasm. This appraisal was supplemented by a differential cell count, using a Howard disc inserted in one of the microscope eyepieces. A division of the cells into three categories was sufficient for our purposes: (I) prohemocytes; (II) plasmatocytes; and (III) spheroidocytes and other cells (including degenerating cells). Differential counts made on a sample of 62 cultures showed that the prevalent cell was the plasmatocyte, with a mean frequency of 80.35% of the cell population ($\sigma_{\bar{x}} = 1.015\%$) on the coverglass.

The plasmatocyte count was used in a series of tests (Table III) as a measure of the activity of a number of substances. This method may prove to be useful for bioassay *in vitro*. One should be reminded, of course, that at this point no direct extrapolation from cell to intact organism can be made without additional





FIGURES 5 and 6.

assay *in vivo*. Kinetin (6-furfurylaminopurine; Mann Research Laboratories, New York) was used at the level of 2 μg./ml. Increased rates of cell division after addition of low doses of kinetin to the culture medium have been reported for the epithelial outgrowth of human skin of the breast and for fibroblasts from a carcinoma of the breast (Orr and McSwain, 1957), and for *Paramecium caudatum* Ehrbg. (Guttman and Back, 1960). In our cultures, the addition of kinetin caused a decrease in plasmatocyte numbers. There was a proportional increase in cells of group III, and no change in the number of prohemocytes. An increase in mitotic activity was not noticed.

The addition of DL-carnitine hydrochloride (California Corporation for Biochemical Research, Los Angeles) at 5 μg./ml. did not cause appreciable changes in our cultures, while this vitamin was reported to increase the rate of mitosis in cultured chick-embryo fibroblasts (Fraenkel and Friedman, 1957).

TABLE III

Effect of some additives on the plasmatocyte ratio in vitro. The control cultures and the test cultures are from the same cell pool.

Test	Number of cultures	Per cent plasmatocytes		Level of significance (*t* test)
		$\bar{x}$	$\sigma_{\bar{x}}$	
Kinetin, 2 μg./ml.	25	80.34	1.39	8%
Controls	15	84.03	1.18	
Fly embryo extract, 10% EE_{50}	13	83.78	3.78	10%
Controls	4	67.48	11.66	
Carnitine, 5 μg./ml.	14	77.51	4.11	more than 10%
Controls	13	81.05	2.75	
Proteose-peptone 1 mg./ml.	18	81.62	1.25	more than 10%
Controls	9	82.93	1.61	

Proteose-peptone (Difco) at the level of 0.1% (Harris, 1959) did not alter significantly the ratios of the three cell groups when added to the cultures in presence of nondialyzed fetal bovine serum and homologous insect plasma. This suggests that peptides or other protein split products were present in sufficient amounts, in the serous fluids added to our medium. Tests with dialyzed serous fluids were not conducted, and this may be a promising avenue for future exploration.

The addition of an extract from house-fly embryos (*Musca domestica* Linnaeus) produced cultures with a high ratio of plasmatocytes. The extract was prepared from 8- to 10-hour-old eggs (containing almost fully developed embryos), homogenized in an equal volume of BSS, following a method similar to the one routinely

FIGURES 5 and 6. Hemocyte monolayers from *Peridroma saucia* (Hübner). The culture in Figure 5 is 12 hours old, the one in Figure 6 is 4 days old. The dominant cell in the monolayers is the plasmatocyte. Fixative: Duboscq-Brasil. Staining: Giemsa. Photography: V. G. Duran. Scale: 50 microns.

used for bovine embryo extract (EE_{50}). The test cultures received 10% of the Millipore-filtered extract. Unfortunately, in the course of this particular experiment an unexplained wide variation in plasmatocyte rates was observed, as evidenced by the large standard errors. However, there is some indication that the addition of fly embryonic extract had stimulative effects in our cultures.

The need for a medium poised at a low oxidation-reduction potential for the culture of insect hemocytes and probably other insect cells was clearly demonstrated by the analysis of differential cell counts made on two groups of cultures, one in which the medium showed melanin formation, another in which no melanin was formed. Melanin, which is known to act as an oxidation-reduction indicator (Figge, 1939), was formed in the medium supplemented with insect blood plasma, after exposure to oxygen. The results, summarized in Table IV, show that an increase in oxidation-reduction potential was connected with a decrease in plasmatocytes: a proportional increase was observed in cells of group III, in this case mostly degenerating cells. In addition, the cultures in which oxidation occurred were qualitatively inferior to the control cultures.

One more use of the hemocyte cultures should be mentioned briefly. Propagation of nuclear polyhedrosis virus is now possible in the plasmatocytes cultured

TABLE IV

The effect of a shift in oxidation-reduction potential on the plasmatocyte ratio in vitro

Group	Number of cultures	Per cent plasmatocytes		Level of significance (*t* test)
		$\bar{x}$	$\sigma_{\bar{x}}$	
Melanin formed	15	53.57	4.35	less than 0.1%
Controls	25	76.84	1.66	

as described here (Martignoni and Scallion, 1961). Inasmuch as the preparation of these cell cultures is relatively simple, we expect a number of studies on the interaction between virus and host at the cellular level, in the controlled conditions of the culture vessel.

The cooperation of Mr. James E. Milstead, who made numerous osmotic pressure determinations, and was most helpful in other phases of this work, and of Mr. Glenn L. Finney and Mrs. Mildred Schutter, who reared the five thousand larvae used for this study, is gratefully acknowledged. We also wish to thank Dr. Ralph I. Smith, who introduced us to the art of semi-micro osmotic pressure measurement.

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STUDIES ON THE HEXAPOD NERVOUS SYSTEM. IV. A CYTOLOGICAL AND CYTOCHEMICAL STUDY OF NEURONS AND THEIR INCLUSIONS IN THE BRAIN OF A COCKROACH, PERIPLANETA AMERICANA (L.)

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Inclusions commonly regarded as products of secretory activity have been described in the nerve cells of numerous and diverse insects (see reviews by Scharrer and Scharrer, 1954; Gabe, 1954; Van der Kloot, 1960). Much of this information has been obtained by employing selective, but poorly understood, staining reactions such as the aldehyde-fuchsin and chrome alum-hematoxylin techniques. Few attempts have been made to compare inclusions in different nerve cells of a single insect species using a variety of the more rigorous cytochemical staining procedures currently available. It might be expected that such a study would provide data germane to a problem which perennially faces the insect neurocytologist and neuroendocrinologist, namely, that of neuron classification. The present study is also considered basic to investigations of the fine structure of the cockroach nervous system now in progress at this laboratory.

Methods

By dissecting away the cranial and cervical integuments of live *Periplaneta* adults and late instar nymphs, the brain and retrocerebral complex were exposed. After severing the circumesophageal connectives, major nerves, and foregut distal to the corpora allata, the foregut was removed and carried to the appropriate fixative together with the brain and its associated retrocerebral complex. With practice this operation can be completed within two or three minutes, and certainly before any appreciable drying of the exposed organs occurs. A comparison with results obtained after exposing the brain and fixing it *in situ*, or after bathing the brain in Yeager's cockroach saline during the dissection did not justify modifying this procedure. Indeed, it was considered less damaging to remove the brain prior to fixation than after.

The choice of fixative was dictated by both the cytochemical staining technique intended, and the quality of cytological preservation required. Alcoholic and aqueous Bouin's, Lillie's acetic-alcohol-formalin (AAF; Lillie, 1954), 10% neutral formalin, and Helly's, were among those used. Preservation of neuronal inclusions in paraffin sections was best after Helly's. Following fixation, each specimen was dehydrated in half-hour changes of 70%, 95%, and absolute ethyl alcohol, cleared in methyl benzoate, washed briefly in benzene, and vacuum infiltrated with Fisher's Tissuemat (60–62° C.). Serial sections were cut at 5–7 μ. Adjacent sections were commonly mounted on alternate slides, thus allowing the cytological and cytochemical staining characteristics of the same inclusions to be compared.

Cytological features were elucidated by staining with Gomori's aldehyde-fuchsin (AF) as modified by Halmi (1952), or Bargmann's modification of Gomori's chrome alum-hematoxylin-phloxine (CAHP; Pearse, 1960). Additional information was obtained by staining Helly-fixed sections with the periodic acid-Schiff (PAS) method and counterstaining with sudan black B. While observing a particular region with a water-immersed objective (N.A. 1.25) several drops of acetone can be applied to remove the sudan black. This leaves the PAS-positive components. In this way the staining of inclusions by the two methods within a single cell was readily compared.

The cytochemical procedures used included the PAS reaction for polysaccharides after diastase digestion (Pearse, 1960), after aniline-acetic acid blocking (Lillie and Glenner, 1957), and after omitting periodic acid oxidation. In an attempt to decide whether lipids were responsible for PAS-positive reactions, freshly dissected brains were extracted with equal proportions of chloroform:methanol in a closed container at 60° C. for 24 hours. The PAS reaction was applied to gelatin-imbedded frozen sections of such extracted brains, as well as to frozen sections of brains which had been fixed in aqueous Bouin's first, and subsequently extracted with 60° C. pyridine. Frozen sections of aqueous Bouin-fixed brains served as controls.

Lipids were stained with sudan black B (SBB) in Helly-fixed paraffin sections, as well as in frozen sections of material which had been fixed either in aqueous Bouin's, or in neutral 10% formalin followed by postchromation (Baker, 1946). Controls consisted of aqueous Bouin-fixed, or unfixed brains, which had been extracted 24 hours either with 60° C. pyridine in a closed container, or with boiling chloroform:methanol in a reflux condenser. Baker's acid hematein (AH) test for phospholipids (Baker, 1946) was applied to gelatin-imbedded frozen sections. Helly-fixed paraffin sections were also stained with the copper phthalocyanin method (CPM) of Klüver and Barrera (Pearse, 1960).

RNA was localized in paraffin sections of brains and thoracic ganglia which had been fixed in Lillie's AAF for one hour, rapidly dehydrated in 95% and absolute ethyl alcohol, cleared in methyl benzoate, and vacuum-infiltrated. Constituents which stained with Einarson's gallocyanin-chromalum method for nucleic acids (pH 1.64; Pearse, 1960) after incubation for 1–2 hours in distilled water (37° C.), but which were not stained in adjacent sections incubated for the same period in 0.73% ribonuclease (recrystallized 5 times, without protease; Mann Laboratories), were considered RNA. These constituents are also present in neurons which have been fixed in alcoholic Bouin's, and they bind methylene blue at a pH of 2.5. Their strongly acidic nature is in accord with the hypothesis that they do, indeed, represent RNA.

To estimate the extent CAHP was dyeing the RNA component of neurons, paraffin sections of brains which had been fixed either in 80% ethyl alcohol or Helly's were stained after ribonuclease digestion. Sections which had been incubated in distilled water for identical periods were also stained with CAHP and served as controls.

The performic acid-alcian blue (PAAB) method of Adams and Sloper for disulfide groups (Pearse, 1960) was applied to paraffin sections of Helly-fixed material. Controls for the PAAB technique consisted of adjacent sections which

had been treated in the same manner as test sections, save for the omission of performic acid oxidation. The tetrazolium mixture of Barrnett and Seligman (1954) was used in the alkaline tetrazolium reaction (Pearse, 1960). This test was also applied to paraffin sections of Helly-fixed material.

Neurosecretory Material Produced by A-Cells

Of the many nerve cell bodies in the cockroach brain, only a relatively small number in the pars intercerebralis contains inclusions which stain both purple with AF and intensely blue-black with CAHP (Fig. 1A). Using the scheme of classification proposed by Nayar (1955), these would be called "A-cells." Due to the presence of stainable granules within the axoplasm, processes emanating from these cells can be traced to the first cardiac nerves, and thence to the corpora cardiaca (Scharrer, 1952; Arnold, 1960). Scharrer (1952) has demonstrated that this stainable material accumulates proximal to a transection of the first cardiac nerves, and is markedly depleted distally, suggesting that it is normally transported along the axons to the corpora cardiaca, which serve as neurohemal organs.

Different A-cells in the same individual are not invariably filled to the same extent with neurosecretory material (NSM). In the pars intercerebralis of some roaches, for example, A-cells which are packed with NSM were found adjacent to cells which contain only small, separate clusters of fine ($< 0.5\ \mu$ in diameter) neurosecretory granules. It is not improbable that this appearance is correlated with different phases of secretory activity.

Granules of NSM stain identically in the A-cells, in their processes within the brain and first cardiac nerve, and in the corpora cardiaca (Fig. 1B, C). A-cell NSM is poorly preserved by alcoholic fixatives. Unlike the situation in certain vertebrates (Sloper, 1955), loss of NSM is not appreciably prevented by post-fixing in 10% formalin after initial fixation in 80% ethyl alcohol prior to paraffin embedding. The loss of cockroach A-cell NSM appears to be due to the solvent action of alcohol, not to subsequent extraction by water. This is also indicated by the following data which show that NSM contains a lipid component.

Careful examination of adjacent Helly-fixed paraffin sections, one series stained with SBB, the other with CAHP, revealed that A-cell NSM is moderately sudanophilic. SBB can be removed readily with acetone, and the section can be restained, which further indicates that the sudanophilia is specific (Lillie, 1954). The sudanophilia of this NSM is also demonstrable in frozen sections of formal-bichromate or aqueous Bouin-fixed material. It is not apparent in frozen sections after hot chloroform:methanol extraction. A-cell NSM stains blue after Klüver and Barrera's CPM, and blue-black in frozen sections stained with Baker's AH (Fig. 1C). The latter reaction is abolished by hot pyridine extraction. The cytochemical evidence, therefore, suggests the presence of a phospholipid.

A-cell NSM is moderately PAS-positive in diastase-digested sections of Helly-fixed brain and retrocerebral complex. A positive reaction is also manifested by controls which have not been oxidized with periodic acid, but it is not as intense as that obtained after oxidation. After blocking exposed aldehyde groups with aniline acetate the PAS reaction is negative. The significance of the positive reaction is questionable. It could be caused by lipids (Pearse, 1960), or it could signify the presence of a carbohydrate moiety containing 1:2 glycol groups, which

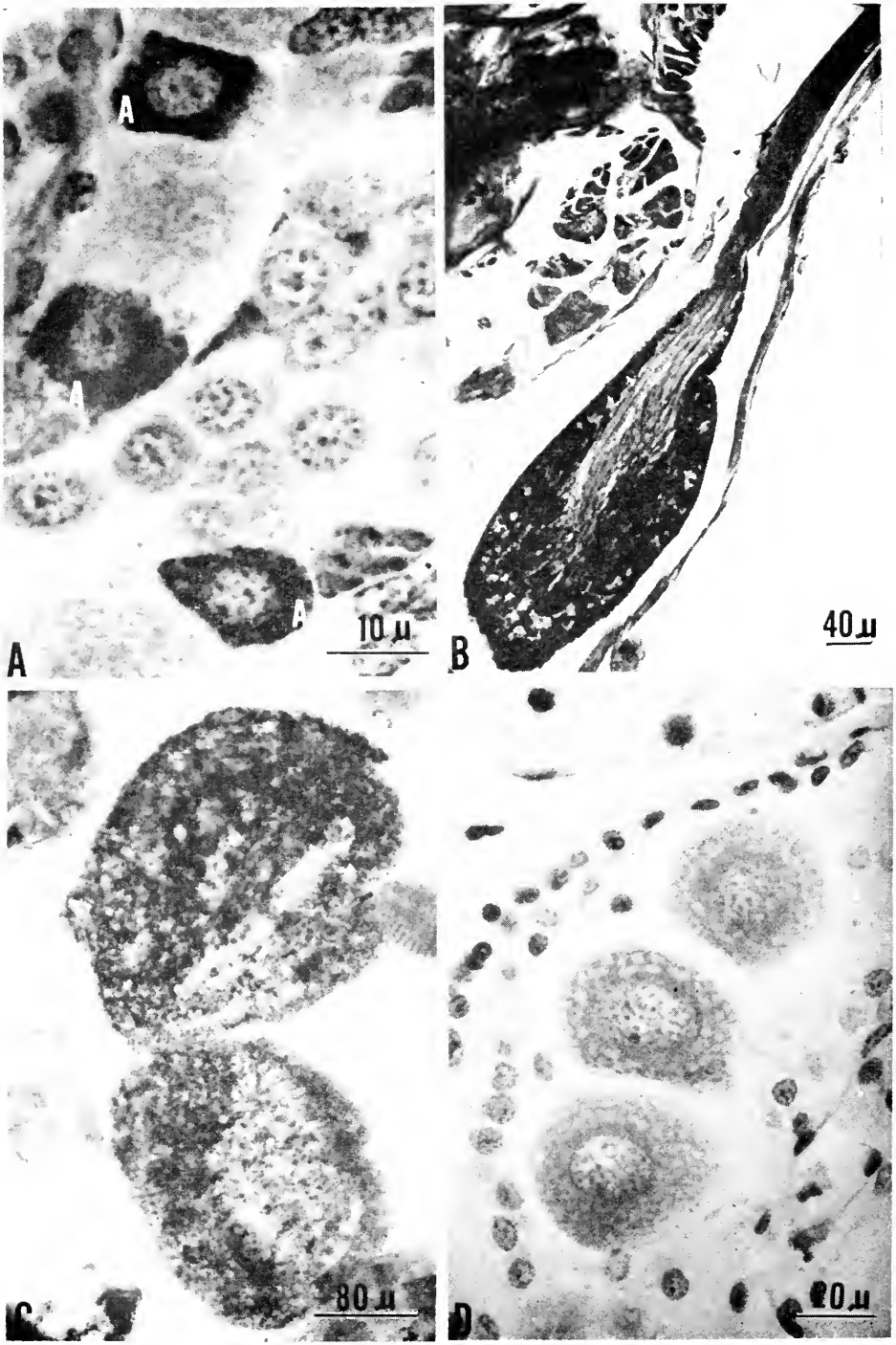

FIGURE 1.

occurs together with lipid. Since A-cell NSM is removed by lipid solvents, current cytochemical staining techniques do not permit a choice to be made between these possibilities.

A-cell NSM stains blue with the alkaline tetrazolium reaction, indicating the presence of protein reducing groups. Such an interpretation is further supported by the positive reaction noted with the PAAB method of Adams and Sloper for protein disulfide. The PAAB reaction is negative in controls which have not been oxidized with performic acid prior to staining with alcian blue. This is in agreement with the findings of Sloper (1957) in the cockroach *Leucophaea*. It should be emphasized, however, that this method stains other cytoplasmic inclusions in the nervous system besides A-cell NSM. Thus, cockroach gliosomes, first described by Scharrer (1939) and studied more recently by Pipa (1961), give a

TABLE I

Staining reactions of cytoplasmic inclusions in the brain of Periplaneta americana (L.)

Stain or test	A-cell NSM	Beta-granules	Delta-granules	RNA	Gliosomes
CAHP	Blue-black	Red	Pink or unstained	Blue-black	Blue-black
AF	Purple	Purple	Green	.....	Purple
Alkaline tetrazolium	Blue	Red	Gray	.....	Red
PAS (diastase)	++	++++	++++	.....	++++
PAS (unoxidized)	+	Faint, if at all	+	.....	+
PAS (blocked)	–	–	–	.....	–
SBB (frozen or Helly-fixed paraffin)	++	++++	++++	.....	Variable
SBB (lipid extracted)	–	–	–	.....	–
AH	++++	Faint, if at all	++++	.....	Variable
AH (hot pyridine extracted)	–	–	–	.....	–
Presence after alcohol fixation and paraffin method	Greatly reduced or absent	Greatly reduced or absent	Absent	Present	Greatly reduced or absent
CPM	++	++++	++++	.....	Variable
Gallocyanin-chromalum	–	–	–	++++	++++

positive reaction. Unlike neurosecretory granules, though, these inclusions stain red with the alkaline tetrazolium reaction. They also stain blue-black with Einarson's gallocyanin-chromalum, while A-cell NSM stains slightly, if at all (Table I).

RIBONUCLEIC ACID

A component cytochemically identifiable as RNA is present in the majority of neuronal perikarya. It is found distributed in patterns which vary in different

FIGURE 1. Photomicrographs of histological and cytological preparations; A, B, D fixed in Helly's; C fixed in 10% formalin followed by postchromation. (A) A-cells in pars intercerebralis stained with CAHP; (B) longitudinal section through corpus cardiacum and its nerve, stained with AF. Note intensely stained A-cell NSM in axons of nerve and in periphery of gland; (C) gelatin-imbedded frozen section of two corpora cardiaca; cross-section, stained with Baker's AH method for phospholipid; (D) three thoracic ganglion neurons stained with Einarson's gallocyanin-chromalum method for nucleic acids. A, A-cells.

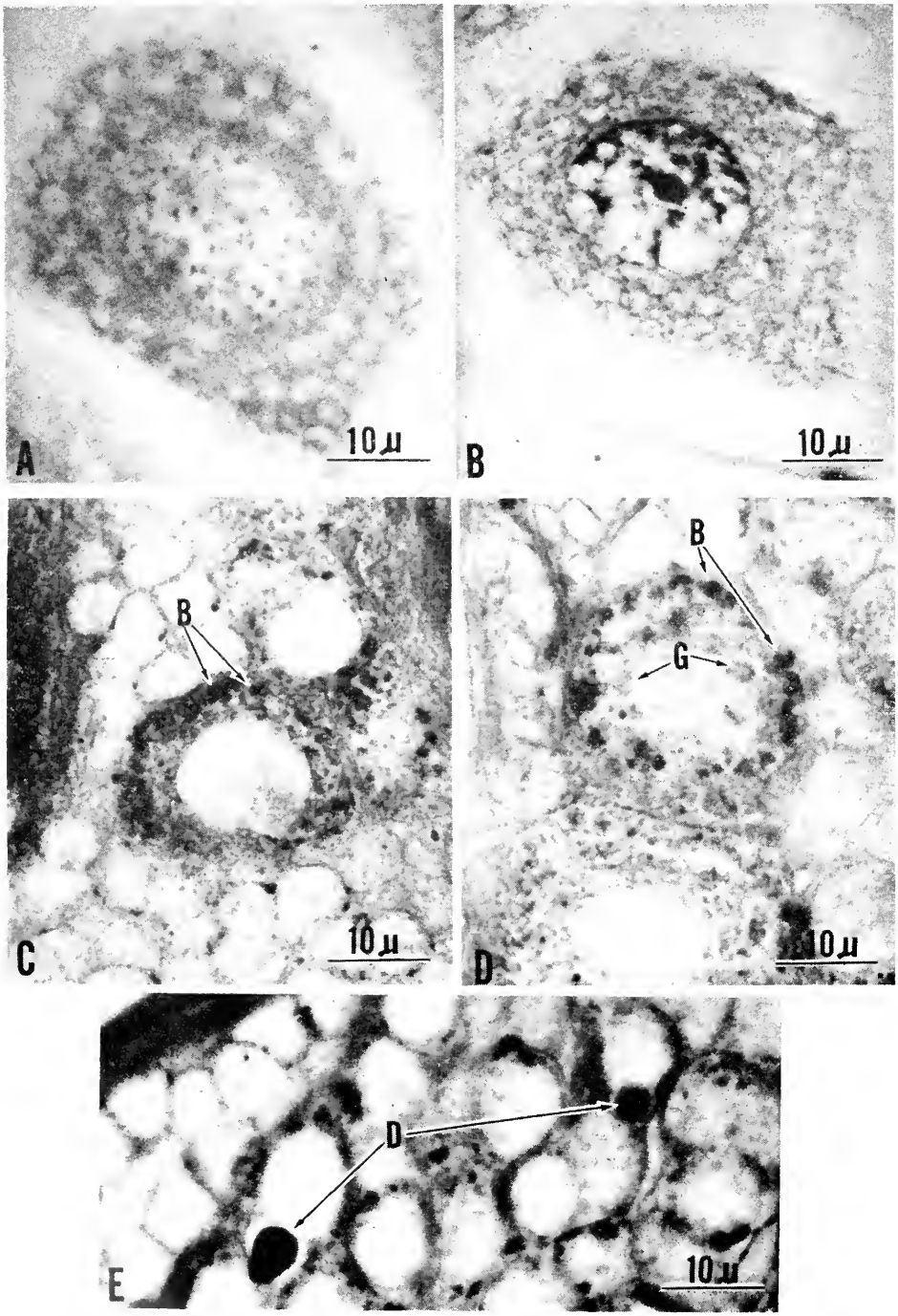

FIGURE 2.

neurons, commonly surrounding gallocyanin-chromalum-refractory spheroidal zones (Figs. 1D, 2A, B). Where these zones are reduced in diameter the distribution is more homogeneous. In agreement with the observations of Hess (1958), Arnold (1960), and Wigglesworth (1960), RNA was never found clumped to form well-defined Nissl bodies such as those described in vertebrate neurons. After alcohol fixation a vast majority of nerve cell perikarya in the brain stains blue-black with CAHP. That this staining is due to RNA is strongly suggested by the fact that it is abolished by ribonuclease digestion. Nerve cells of adjacent sections which were used as controls in this test stained after incubation in distilled water.

The perikarya of Helly-fixed neurons stain blue-black after CAHP, too. This is particularly noticeable when the phloxine counterstain is omitted. It is not surprising that this stainability remains essentially unaffected by ribonuclease, for this enzyme would hardly be expected to digest RNA in cells which have been fixed in a solution containing mercuric chloride. In all likelihood, though, the faint blue-black staining demonstrable in most Helly-fixed neurons after chrome alum-hematoxylin is also due to RNA. Fortunately, NSM within Helly-fixed A-cells stains more intensely than RNA after CAHP, and it is not difficult to distinguish these cells from other neurons found in the pars intercerebralis by this method.

Beta-Granules

A majority of the neurons contains inclusions which stain intensely with PAS, SBB, CPM, and AF, but which are not stained blue-black by CAHP (Fig. 2C, D). These inclusions range from 0.5 to 3.0 μ in diameter. They are especially well-preserved in frozen sections of formalin-bichromate-fixed material, and in Helly-fixed paraffin sections. They appear to vary in abundance in different neurons of the same brain, being most conspicuous in the axon hillock and periphery of each perikaryon, where they often occur in clusters. These inclusions were never found well-within the neuron process, and there was no evidence to support the contention that they represent neurosecretory products. Failure to find cytological evidence of axonal transport, however, does not eliminate the possibility that these inclusions represent a visible manifestation of products which leave the perikaryon via a different route, or in an invisible form. Although not all of these inclusions may be of the same type, they shall be designated beta-granules for the sake of discussion.

When counterstained sufficiently and not destained excessively, beta-granules demonstrate a slight phloxinophilia after CAHP, but this is less intense than that seen in nucleoli present in the same cell. They also stain red after the alkaline tetrazolium reaction, and are PAAB-negative.

The larger of these inclusions stained slightly, if at all, during the Baker AH procedure, whereas NSM of the A-cells, and inclusions believed to be mito-

Figure 2. Photomicrographs of cytological preparations; A, C, D, E fixed in Helly's; B in Lillie's AAF. (A) thoracic ganglion neuron stained with Einarson's gallocyanin-chromalum method for nucleic acids. Note distribution of RNA around unstained spheroids which are believed to represent loci of Golgi apparatus; (B) similar to (A), but after alcoholic fixation; (C) Beta-granules stained with PAS and SBB; (D) Golgi apparatus and beta-granules in same neuron perikaryon, stained with PAS and SBB; (E) Delta-granules stained with PAS and SBB. B, beta-granules; D, delta-granules; G, Golgi apparatus.

chondria, were intensely stained in the same sections. Beta-granules stain with SBB and PAS in gelatin-imbedded frozen sections after formalin-bichromate or aqueous Bouin fixation. They are seldom found in frozen sections of brains which have been both fixed and extracted with hot chloroform:methanol. The larger of these inclusions are still abundant and stainable with SBB and PAS in frozen sections of material which has been fixed in aqueous Bouin's and subsequently extracted with hot chloroform:methanol, or hot pyridine. These data suggest that beta-granules contain a lipid or glycolipid which is either not predominantly phospholipid, or from which phospholipid has been removed during fixation. They are rendered relatively resistant to the action of lipid solvents by formalin fixation, indicating chemical association with a protein.

Although beta-granules stain alike, it would be gratuitous to assume that they are chemically identical. The cytochemical tests used are generally considered specific for certain classes of compounds if appropriate controls are employed, but they do not identify compounds within each class. The presence of a common chemical group within two inclusions can hardly be considered proof that the two are chemically identical.

It might be argued that certain, if not all of these "beta-granules" represent mitochondria. Though this has not been dismissed as a possibility, it seems unlikely in view of the fact that inclusions believed to be mitochondria on the basis of electron microscope data generally stained yellowish green rather than purple with AF. Mitochondria also stain less intensely with PAS than do beta-granules. After Baker's AH test mitochondria stain blue-black, whereas the larger beta-granules within the same cells stain slightly, if at all. Preliminary electron microscope data which are currently at hand likewise indicate that beta-granules differ in fine structure from "typical" mitochondria found within the neuron.

Golgi Complex

When formalin-bichromate-fixed frozen sections, or Helly-fixed paraffin sections of neurons are stained with SBB, not only are the homogeneously-stained beta-granules mentioned above made evident, but sudanophilic crescent-shaped bodies and "hollow" spheroids frequently appear in the same cells. Each of these is comprised of an "externum" which stains intensely with SBB, and an "internum" which is variably sudanophilic (Figs. 2D, 3A). The "externa" are especially well-revealed in paraffin sections stained with Klüver and Barrera's CPM (Fig. 3B).

When Helly-fixed paraffin sections that have been stained with PAS and counterstained with SBB are flooded with acetone, SBB is removed, and the following observations can be made during the destaining process: (1) The beta-granules described above are both intensely PAS-positive and sudanophilic. Thus, when SBB has been removed they are still clearly evident. (2) The "hollow" spheroids and crescent-shaped structures which stained with SBB become indiscernible. Unlike beta-granules these structures stain slightly if at all with PAS. (3) Occasional beta-granules occur within the "hollow" spheroids or crescents. When SBB has been removed, red-staining inclusions of variable size can be seen within a zone which is either PAS-negative, or only very slightly PAS-positive.

The Golgi apparatus in the neurons, then, consists of sudanophilic, crescent-

shaped or spheroidal "externa" which are probably manifestations of lamellar membrane systems (Beams *et al.*, 1953; Palay and Palade, 1955; Bern *et al.*, 1961). These are frequently closely associated with variably sudanophilic "interna" which may contain beta-granules. The beta-granules are sudanophilic, intensely PAS-positive, and stain purple with aldehyde fuchsin. A summary of their staining characteristics is given in Table 1.

Nerve cells which have been stained by techniques used to demonstrate RNA (*i.e.*, gallocyanin, cresyl violet) commonly present a variably "mottled" appearance (Figs. 1D, 2A, B). As previously noted, this is due to the staining of RNA

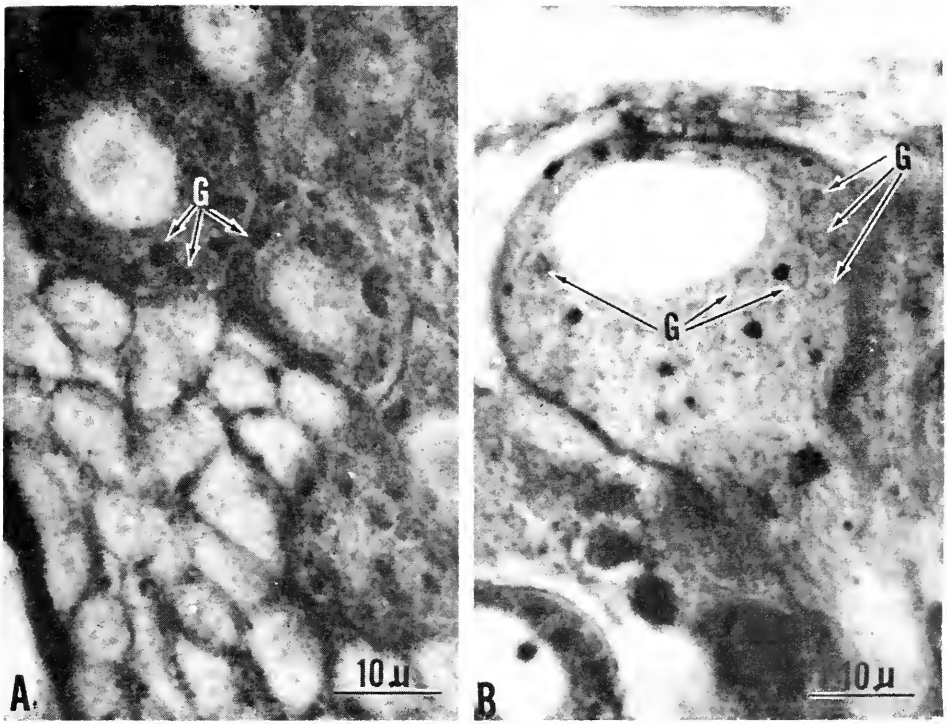

FIGURE 3. Photomicrographs of cytological preparations; fixed in Helly's. (A) Golgi apparatus stained with SBB. (B) Neuron stained with CPM; Golgi "externa" evident in cytoplasm. G, Golgi bodies.

which surrounds unstained spheroidal zones. An examination of adjacent Helly-fixed paraffin sections of the same neurons, one series stained with chrome alum-hematoxylin, the other with SBB, led to the conclusion that these spheroidal zones represent the loci of the Golgi apparatus which are devoid of ribosomes (*i.e.*, are present as "agranular reticula"; Palay and Palade, 1955; Palay, 1958; Bern *et al.*, 1961).

When frozen sections of aqueous Bouin-fixed, pyridine-extracted brains are stained with SBB and examined, the Golgi apparatus is no longer demonstrable. The cytoplasm now appears finely granular, save for the occurrence of the larger

beta-granules which continue to stain with SBB and which will also stain intensely with PAS. This may be taken as additional evidence that the Golgi apparatus and larger beta-granules are not one and the same structure.

Delta-Granules

Within the perikarya of certain of the smaller neurons (nuclear diameters of 10 to 15 μ) single, conspicuous, spherical inclusions are consistently encountered (Fig. 2E). These inclusions, here termed delta-granules, range from 2 to 5 μ in diameter. They occur within the brains of late instar nymphs, as well as in adult males and females. It is uncertain whether they are limited in occurrence to special cells, or if they occur sporadically in different neurons. Cells containing delta-granules may occur singly or in small groups. They were seen most frequently in the pars lateralis, less commonly in the pars intercerebralis.

Delta-granules do not stain homogeneously, for areas which fail to stain, or which stain unlike the remainder are noticeable at their centers. Delta-granules may stain faintly pink after CAHP, or they may fail to stain altogether. They are not fuchsinophil, but stain green with the counterstain employed in the AF technique. Their failure to stain purple, and the fact that they stain blue-black after Baker's AH distinguishes them from beta-granules (Table I). Since delta-granules no longer stain with AH after pyridine extraction it is likely that they contain appreciable phospholipid.

Discussion

The inherent limitations of staining reactions as criteria for categorizing cytoplasmic inclusions are painfully apparent. The fact that two structures stain alike does not necessarily mean they are chemically identical. When more rigorous cytochemical staining techniques are employed one may be led to conclude that two inclusions contain the same class of compounds, but it is seldom possible to tell precisely what the compounds are. It is common knowledge that structures which play disparate roles in the economy of the cell may contain identical classes of compounds, and may therefore stain identically. When two inclusions fail to stain alike, however, it is logical to consider them chemically or physically dissimilar.

In light of these precepts the advisability of using a diversity of staining techniques becomes obvious. In the present case such an approach has led to the recognition of three types of neuronal inclusions which are believed to exist in addition to mitochondria, ribosomes, and the agranular reticulum (*i.e.,* Golgi apparatus). To facilitate discussion these are called A-cell NSM, beta-granules, and delta-granules.

A-cell NSM was found in certain neurons of the pars intercerebralis, as well as in the corpora cardiaca. This is in agreement with the observations of Scharrer (1952) and Arnold (1960). The histochemical evidence indicates that this NSM contains a lipoprotein. This has also been noted in certain Lepidoptera (Rehm, 1955) and Hemiptera (Nayar, 1955). The fact that A-cell NSM stains intensely with Baker's AH before pyridine extraction, but not after, suggests the presence of phospholipid. The moderately PAS-positive reaction which this NSM exhibits is of unknown significance. That it is not due to glycogen is certain, but it

could be caused by a lipid component, or by a carbohydrate moiety associated with lipid. Since lipid solvents will remove A-cell NSM, current cytochemical staining procedures do not permit a choice to be made from among these possibilities.

As indicated by Sloper (1957) NSM produced by insect A-cells resembles that produced by certain neurons of the vertebrate hypothalamus, inasmuch as it shows evidence of containing a protein or polypeptide rich in cystine or cysteine. In other respects it differs significantly from vertebrate NSM, as reported by Sloper (1955), and Howe and Pearse (1956). These workers concluded that vertebrate NSM is mainly protein or polypeptide in nature, there being no indication of lipid. Their findings are in opposition to those of Schiebler (1952) who interpreted his histochemical data as indicating a glycolipoprotein complex.

Beta-granules are of variable size. Although they stain alike, it is possible that future study may reveal this to be a heterogeneous category. They occur in variable abundance in the majority of neuron perikarya, and are most conspicuous in the periphery, especially in the axon hillock region. A resumé of their staining characteristics is given in Table I. Beta-granules differ from mitochondria by staining intensely PAS-positive, and by staining purple with AF. More significantly, preliminary electron microscope data indicate that they differ from mitochondria in fine structure. Beta-granules were never found well-within the neuron process. If they do represent a type of neurosecretory material, they must be altered in size or stainability before passing out of the perikarya. Perhaps they are comparable to the chromophilic intraneuronal granules described by Thomas (1951).

Although it is not the intention of this study to enter into the "Golgi controversy" (Baker, 1957; Lacy and Challice, 1957; Palay, 1958), a comparison between locust "lipochondria" and structures noted here would seem appropriate. According to the interpretation of Shafiq (1953, 1954) and Shafiq and Casselman (1954) the classically described Golgi apparatus is an artifact resulting from the deposition of osmium or silver on the surfaces of homogeneous spheroids or "lipochondria" during impregnation. Malhotra (1956), however, reported seeing the binary spheroidal Golgi apparatus not only in osmium or silver impregnations, but also in living insect neurons, as well as in cytological preparations stained with SBB. He did not describe neuronal perikaryal inclusions other than mitochondria, Golgi binary spheroids (=lipochondria), and RNA particles.

The present account concurs with Malhotra's finding that in cytological preparations stained with SBB the Golgi apparatus is demonstrable as intensely stained crescentic "externa" associated with less intensely stained "interna." This appearance, then, is not entirely an artifact due to the deposition of osmium or silver on otherwise homogeneous spheroids. Electron microscope investigations have also revealed variably crescentic lamellar membrane systems ("agranular reticula") as part of the neuron Golgi apparatus (Beams *et al.*, 1953; Palay and Palade, 1955; Palay, 1958; Bern *et al.*, 1961), thus providing a probable fine-structure basis for the Golgi "externum" visible with the light microscope. The fact that no RNA could be demonstrated within the spheroidal zones ("externum" plus "internum") in the present work supports the contention that they may, indeed, represent the loci of the agranular (ribosome-free) reticula.

It is not clear whether by the term "lipochondria" Shafiq meant the spheroidal

zones (Figs. 1D, 2A, B) which represent the loci of the Golgi apparatus ("externum" plus "internum"), the larger beta-granules noted here, or both. It is quite certain, however, that the Golgi apparatus and the beta-granules are not one and the same structure. This interpretation is also supported by preliminary electron microscope data currently at hand.

The functional significance of the conspicuous delta-granules is likewise unknown. They were found solely in the perikarya of smaller neurons; never in the processes. A comparison between the staining affinities of these and certain other inclusions within the neurons is made in Table I.

The cytomorphology of neurons varies in different cells of the same brain. It also varies in the same group of cells in different brains. This variability is expressed as differences in the degree of Golgi apparatus elaboration, RNA distribution, and in number and size of inclusion granules. If we are willing to accept these static appearances as evidence for secretory activity, then it would seem that a majority of the neurons in the brain are secreting. Such cytomorphological features need not necessarily be correlated with neurosecretion, however, for they could conceivably reflect axoplasm replacement (Weiss and Hiscoe, 1948), or changes associated with ageing (*i.e.*, accumulation of lipofuscin).

On the basis of the cytological data presented here it would be premature to conclude that beta- and delta-granules represent neurosecretory products. It is also entirely possible that secretion may occur without the production of visible granules. Clearly, reciprocal physiological information is essential before this matter can be decided.

It is a pleasure to express my gratitude to Professor Berta Scharrer, Albert Einstein College of Medicine, New York, and to Professor Roderick Craig, University of California, Berkeley, for critically reading the manuscript, and for numerous suggestions. This does not imply endorsement or responsibility on their part for any conclusions or views presented.

Summary

1. A variety of cytological and cytochemical staining reactions has been applied in a study of neuronal inclusions in the brain of the American cockroach. Three types of cytoplasmic inclusions, which exist in addition to mitochondria, ribosomes, and the Golgi apparatus, are characterized. For discussion purposes these are called A-cell NSM, beta-, and delta-granules.

2. The cytochemical staining reactions used indicate that A-cell NSM is a cystine- or cysteine-rich phospholipoprotein.

3. Perikaryal inclusions which stain intensely with PAS, SBB, CPM, and AF, but which are not stained blue-black with CAHP, occur in the majority of neurons. These are called beta-granules. Despite similarities in stainability, there is a possibility that these inclusions are not all of one type. They clearly differ from the Golgi apparatus, and it is also highly unlikely that they are "typical" mitochondria. The cytochemical data suggest that beta-granules contain a lipid or glycolipid which is either not predominantly phospholipid, or from which phospholipid has been removed during fixation. The fact that they are rendered relatively

resistant to lipid solvents by formalin fixation indicates chemical association with a protein.

4. Delta-granules occur as single, conspicuous inclusions within the perikarya of certain of the smaller neurons. Unlike beta-granules, they stain green with AF. They appear to consist of a lipoprotein or glycolipoprotein which contains phospholipid.

5. The Golgi apparatus consists of sudanophilic crescent-shaped or spheroidal "externa" frequently closely associated with variably sudanophilic "interna" which may contain beta-granules. The "mottled" appearance of nerve cells which have been stained to demonstrate RNA appears to be due to the unstained Golgi spheroids.

6. The limitations of staining reactions as criteria for categorizing cytoplasmic inclusions, and the significance of cytomorphological features as indices of neurosecretory activity are discussed.

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UREASE ACTIVITY IN TRYPANORHYNCH CESTODES[1]

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The hydrolysis of urea has been reported as an activity of tissue preparations from a number of invertebrate animals. Urease activities were detected in a lamellibranch (*Mytilus edulis*), a gastropod (*Helix pomatia*), an oligochaete (*"Lumbricus agricola"*), and a decapod crustacean (*Astacus fluviatilis*) by Przylecki (1922); in five species of marine molluscs by Albrecht (1921); in the horseshoe crab (*Limulus polyphemus*) by Loeb and Bodansky (1926, 1927); in the eggs and developing embryos of a sea urchin (*Strongylocentrotus purpuratus*) by Brookbank and Whiteley (1954); in the larvae and larval secretions of a blowfly (*Lucilia sericata*) by Robinson and Baker (1939); in a sipunculid (*Sipunculus nudus*) by Florkin and Duchateau (1942). The latter authors also reported activities in tissues of a crayfish (*"Ecrevisse"*) and a lobster (*"Homard"*) (Florkin and Duchateau, 1943). Brunel (1938) also reported urease activity from *Mytilus edulis,* while Baldwin and Needham (1934) detected activity in the nephridium of *Helix pomatia.*

Several species of parasitic nematodes have been reported to possess a urease. Rogers (1952) demonstrated urease activity in *Nematodirus* spp., and stated that lesser activities were found in *Ascaridia galli* and *Haemonchus contortus.* Savel (1955) studied urease in several tissues of *Ascaris lumbricoides.*

Apparently there have been no previous observations of urease activity in flatworms. Van Grembergen and Pennoit-deCooman (1944) failed to detect activity in a trematode (*Fasciola hepatica*) and two cestode parasites of mammals (*Moniezia benedeni* and *Taenia pisiformis*).

The present paper will show that certain trypanorhynch tapeworms from elasmobranch hosts possess urease of quite remarkable levels of activity.

Materials and Methods

Infected elasmobranches were obtained through the courtesy of the Supply Department of the Marine Biological Laboratory. All the fishes came from a trap in Buzzard's Bay, Massachusetts, and were identified by reference to the paper of Bigelow and Schroeder (1953).

Fishes were killed by blows on the head, and cestodes were immediately collected from the spiral valves. A saline solution composed of 250 m*M* NaCl, 4.4 m*M* KCl, 5.1 m*M* $CaCl_2$, and 2.9 m*M* $MgCl_2$ was used for rinsing and incubating the worms. Following several saline rinses to remove adhering materials, the worms

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were incubated for 2–5 hours at 20° C. Such treatment was found to render the tetraphyllid cestode, *Calliobothrium verticillatum*, free of detectable endogenous urea (Read *et al.*, 1959). Subsequent to this incubation period, worms were blotted on hard filter paper and the desired amount of tissue weighed on a torsion balance.

Homogenates of worm tissue were prepared in TenBroeck tissue grinders of suitable size immersed in a cracked-ice bath. The homogenates were 1:9 (w/v) dilutions of wet tissue, the diluent being an ice-cold solution of 5 m*M* disodium ethylenediamine tetraäcetate (EDTA).

Urease activity of tissue homogenates was assayed by a modification of the method described by Summer (1955), which measures the ammonia produced from the hydrolysis of urea. A complete reaction mixture consisted of 200 microliters of a urea solution of appropriate concentration, 200 microliters of a 100 m*M* buffer

TABLE I

Urease activities in cestodes of elasmobranchs

Worm species	Host	No. determinations	Tissue activity (mean ±s.e.)
Tetraphyllidea			
Calliobothrium verticillatum	*Mustelus canis*	8	ND
Phyllobothrium lactuca	*Mustelus canis*	4	ND
Phyllobothrium foliatum	*Dasyatis centrura*	4	ND
Onchobothrium pseudo-uncinatum	*Dasyatis centrura*	4	ND
Inermiphyllidium pulvinatum	*Dasyatis centrura*	7	ND
Orygmatobothrium dohrnii	*Carcharias taurus*	4	ND
Disculiceps pileatum	*Carcharinus obscurus*	4	ND
Trypanorhyncha			
Lacistorhynchus tenuis	*Mustelus canis*	8	3,330± 40
Grillotia erinaceus	*Raja ocellata*	7	ND
Pterobothrium lintoni	*Dasyatis centrura*	8	33,290±610

Five-minute incubations at 20° C.
ND: not detected, less than 25 micromoles urea hydrolyzed per gram wet tissue per hour.

solution (phosphate buffer, pH 7.0, unless otherwise indicated), and 100 microliters of 10% tissue homogenate. The reaction was initiated by blowing in the homogenate and stopped by blowing in 200 microliters of 20% trichloroacetic acid (TCA). Control preparations for the assay included (1) untreated homogenate in a reaction mixture lacking urea, (2) heat-inactivated homogenate incubated in the presence of urea, (3) zero-time preparations with TCA added prior to the addition of homogenate, and (4) diluent alone (no tissue) in the presence of urea. The latter preparation never yielded detectable ammonia; the other control preparations gave low (about 25 micrograms of ammonia nitrogen per gram wet tissue), and practically identical, ammonia values. All assay values are reported as corrected for the maximum tissue blank value.

Ammonia produced in the reaction mixtures was liberated by the addition of 1.5 milliliters of a saturated solution of potassium carbonate and captured in 1 N

H_2SO_4 by the microdiffusion method of Seligson and Seligson (1951). Ammonia was determined by the procedure of Lang (1958).

Protein was estimated by a modification of the Lowry method (Lowry *et al.*, 1951) using crystalline bovine serum albumin (Armour and Company) for comparison.

Results are reported as tissue activities or as specific activities, here defined as micromoles urea hydrolyzed per gm. wet tissue weight per hour and micromoles urea hydrolyzed per mgm. protein per hour, respectively, assuming that two micromoles of ammonia recovered are equivalent to one micromole of urea hydrolyzed.

Additional experimental procedures will be described in context.

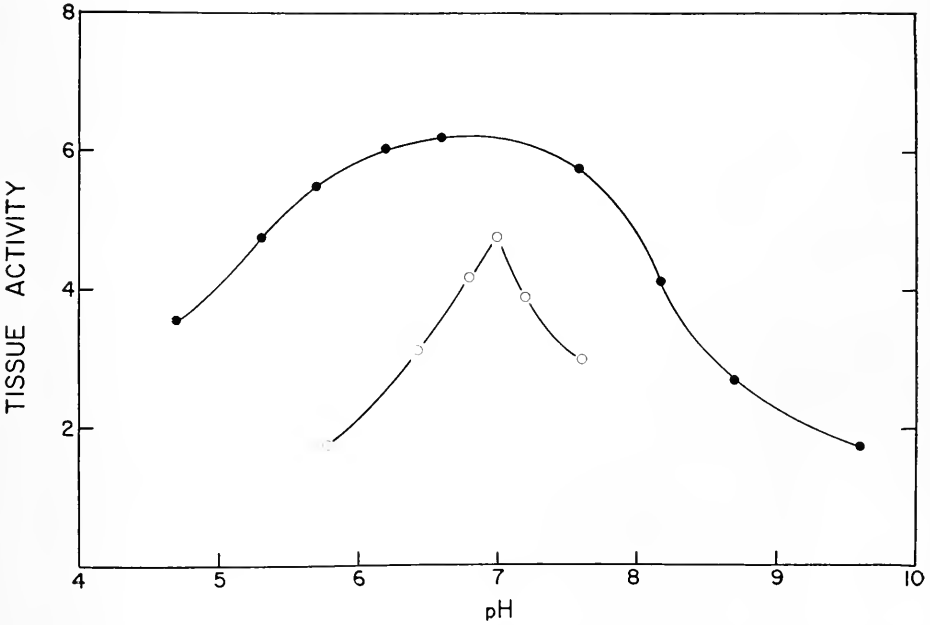

FIGURE 1. Effect of pH on *Lacistorhynchus* urease activity. Tissue activity as micromoles urea hydrolyzed per gram wet tissue per hour in thousands. Open circles with phosphate buffer; closed circles with Tris-maleate. One-minute incubations at 20° C. Each point represents the mean value of four determinations.

RESULTS

A. *Distribution of urease activity among cestodes of elasmobranch fishes*

Read *et al.* (1959) have shown that urea is an important osmotic constituent of certain Tetraphyllidea, a tapeworm group parasitizing elasmobranch fishes; data derived from studies with [^{14}C] urea indicate that in tetraphyllid cestodes urea is a metabolically inactive compound (Simmons *et al.*, 1960).

Ten species of tapeworm from elasmobranches were examined for urease activity by the procedures just described; results of the assays are given in Table I. Of seven tetraphyllids examined, none exhibited activity, while two of three trypanorhynch species examined were quite active in hyrolyzing urea.

Additional *Pterobothrium* material was not forthcoming, and further studies with tissue preparations were made with the more readily available *Lacistorhynchus*.

B. *Studies of Lacistorhynchus urease in tissue homogenates*

Effect of pH on activity

Figure 1 shows activity curves obtained when Sørensen's phospate buffer and tris(dimethyl)aminomethane (Tris)-maleic acid buffer (Gomori, 1955) are employed in the assay.

The very sharp optimum observed at pH 7.0 in phosphate buffer is characteristic also of jackbean urease (Sumner, 1951). Apparently there have been no studies with jackbean urease employing Tris-maleate although Wall and Laidler (1953) found that crystalline jackbean urease showed a marked optimum at pH 8.00 in Tris-sulfuric acid buffer. *Lacistorhynchus* homogenates show no such optimum in Tris-sulfuric acid when the pH is varied between 7.4 and 9.0 (Table II).

TABLE II

Urease activity of Lacistorhynchus homogenates with Tris-H_2SO_4 Buffer

pH	Tissue activity (mean ±s.e.)
7.4	3370 ± 40
7.8	3160 ± 50
8.0	2690 ± 42
8.2	2120 ± 50
8.6	2080 ± 43
9.0	1920 ± 20

One-minute incubations at 20° C.
Activities are mean values of four determinations.

Although the worm activities in Tris-maleate are considerably elevated above those obtained using phosphate buffer, especially on either side of pH 7.0, the latter buffer was employed in subsequent experiments since most studies of urease activities from other sources have employed phosphate buffer.

Activity as a function of enzyme concentration

Linearity of activity as a function of protein concentration was observed over the range examined, 0.068–1.09 mgm. protein (Fig. 2).

Effect of substrate concentration

Table III gives activities of *Lacistorhynchus* homogenates with increasing urea concentrations. The saturating concentration range, observed from about 250 m*M* to 350 m*M* is quite interesting in view of the fact that the urea concentration in the host blood is about 330 m*M* and that of the gut contents is similar (Read *et al.*, 1959).

The worm enzyme may be less susceptible to product inhibition than jackbean enzyme, since the latter exhibits decreasing activities in urea concentrations about

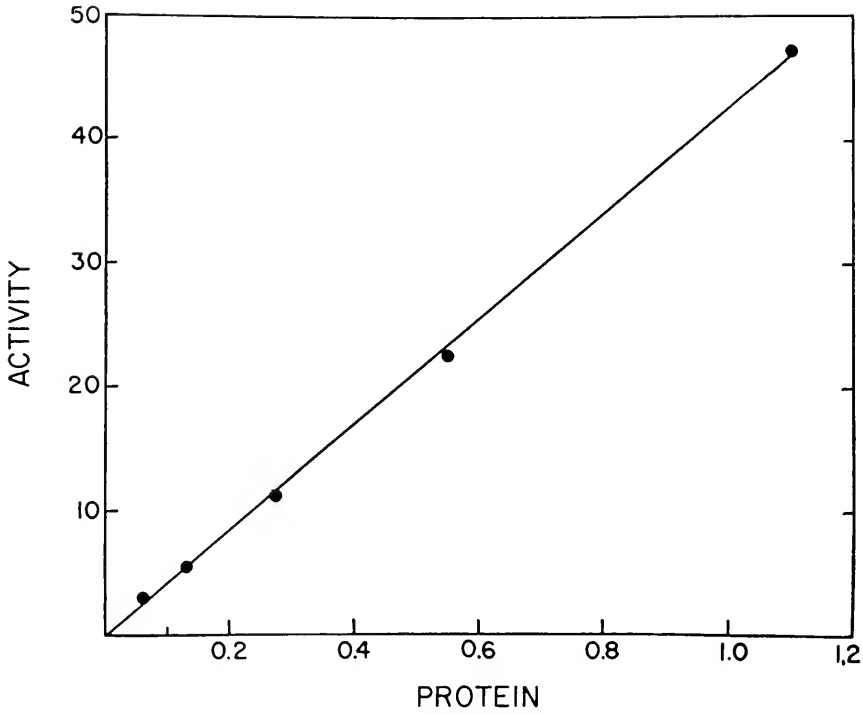

FIGURE 2. Urease activity of *Lacistorhynchus* as a function of protein concentration. One-minute incubations at 20° C. Activity as micromoles urea hydrolyzed per hour. Protein in milligrams. Each point represents the average of duplicate determinations.

250 m*M*–330 m*M*, presumably due to inhibition by the ammonium ions produced (Wall and Laidler, 1953).

Data obtained with the tapeworm urease give good agreement with Michaelis-Menten kinetics when plotted by the method of Lineweaver and Burk (1934), over

TABLE III

Urease activity of Lacistorhynchus homogenates with varying urea concentrations

Urea concentration m*M*	Tissue activity
6.25	835
12.5	1300
25.0	1780
50.0	2140
100.0	2490
200.0	2783
250.0	3108
300.0	3250
350.0	3150
400.0	2967

One-minute incubations at 20° C.
Activities are the average of duplicate determinations.

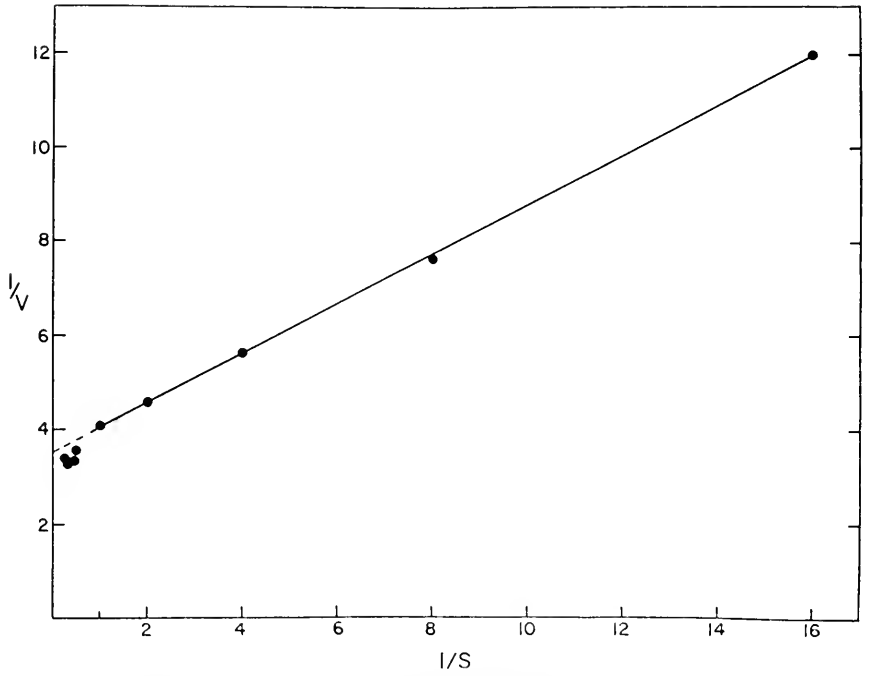

FIGURE 3. Lineweaver-Burk plot of *Lacistorhynchus* urease activity. Velocity reciprocal as (micromoles urea hydrolyzed per gram wet tissue per hour)$^{-1} \times 10^4$. Substrate reciprocal as (millimolar urea)$^{-1} \times 10^2$. One-minute incubations at 20° C. Each point represents the average of duplicate determinations.

the lower substrate concentration range (6.25 m*M* to 100 m*M* urea) (Fig. 3). From 200 m*M* to 400 m*M*, the velocity observed is in excess of that expected.

Activity in saturating substrate concentration

When incubated in the presence of 300 m*M* urea, *Lacistorhynchus* urease activity follows zero-order kinetics for at least four minutes (Fig. 4).

Enzyme stability

When held at 0° C. in the saline solution previously described, *Lacistorhynchus* remain viable for at least 110 hours, the total period of observation. Homogenates freshly prepared from such worms retain full activity (Table IV).

Homogenate preparations *per se* are, however, quite labile; about 93% of the activity is lost in 24 hours at 20° C., and the rate of decay is only little different at 0° C. (Table IV). The loss of specific activity with a concomitant loss of tissue activity is highly suggestive that the decreased activity is due to inactivation and not to general proteolysis.

Storing of whole worms at −20° C. followed by thawing at 0° C. resulted in a loss of activity in homogenates prepared from such material of about 90%; freezing, followed by lyophilization resulted in complete loss of activity.

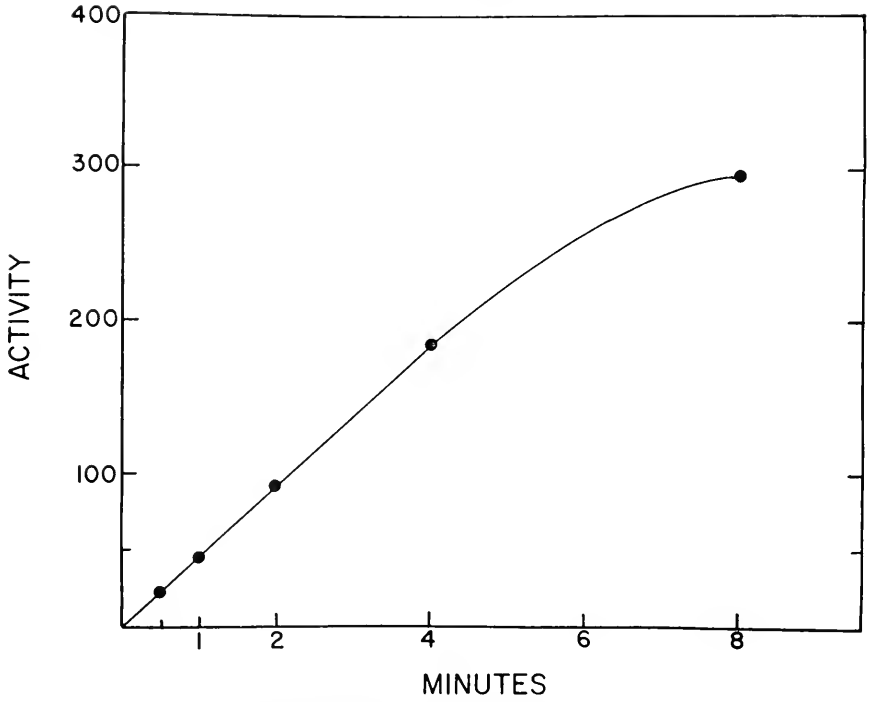

FIGURE 4. Activity of *Lacistorhynchus* urease with time. Incubations at 20° C. Urea concentration 300 m*M*. Activity as micromoles urea hydrolyzed per milligram protein. Each point represents the average of duplicate determinations.

TABLE IV

Stability of Lacistorhynchus urease activity

Material	Holding time (hours)	Holding temperature			
		0° C.		20° C.	
		Tissue activity	Specific activity	Tissue activity	Specific activity
Intact worms	Freshly collected	3450	45.6	—	—
	24	3558	45.6	—	—
	84	3568	45.1	—	—
	110	3336	41.7	—	—
Homogenates	0	3689	43.4	3689	43.4
	1	2329	27.4	1862	21.9
	2	1785	21.0	1360	16.0
	4	1292	15.2	799	9.4
	8	765	9.0	604	7.1
	16	587	6.9	408	4.8
	24	323	3.8	255	3.0

Activities are the average of duplicate determinations from one-minute incubations at 20° C.

C. *Activity exhibited by intact worms*

A single experiment was designed to demonstrate urease activity in intact *Lacistorhynchus*.

One hundred freshly collected worms were divided into four lots of 25 worms each and given a preliminary incubation of three hours at 20° C. in urea-free saline as previously described.

Experimental incubations were performed in respirometer vessels with two sidearms. The main compartment of each vessel contained 1.0 ml. saline with 300 m*M* [^{14}C] urea (California Foundation for Biochemical Research) having a specific activity of 6.67×10^{-3} microcuries per micromole. The center well of each vessel contained 200 microliters 30% KOH for CO_2 capture; sidearm 1 contained 400 microliters of a 1 : 1 (v/v) mixture of 20% TCA and 0.5 *M* lactic acid.

Sidearm apertures were closed with greased, ground-glass stoppers. A worm lot was introduced into the main compartment of each vessel and the mouth of the vessel was immediately closed with a greased rubber stopper, further secured with a rubber band.

TABLE V

Recovery of ammonia and $^{14}CO_2$ following incubation of intact Lacistorhynchus in [^{14}C] urea

Worm group	Wet weight (mgm.)	Acid tip-in (minutes)	Ammonia recovered (micromoles/gm. wet tissue)	$BaCO_3$	
				Mgm.	Radioactivity (counts/min./mgm./gm. wet tissue)
1	155.6	0	11.6	3.3	7,352
2	186.0	5	52.7	3.7	34,559
3	183.2	10	71.0	4.0	39,885
4	170.6	20	102.1	4.6	41,882

See text for experimental details.

The vessels were incubated in a water bath at 20° C. and the acid mixture was tipped in and thoroughly mixed with the contents of the main compartment at desired times. The vessels were allowed to stand 10 hours at room temperature for CO_2 capture, following which period they were opened and the KOH removed from the center well; the latter was rinsed with ten 200-microliter portions of water and these were added to the KOH solution.

For ammonia capture 200 microliters 1 N H_2SO_4 were placed in sidearm 2. One milliliter of a saturated solution of K_2CO_3 was added to the main compartment, the vessels were again sealed, and 24 hours were allowed for diffusion of the ammonia. The H_2SO_4 was removed together with five 200-microliter rinses of the sidearm, diluted to 10.0 ml. and portions were subjected to microdiffusion and ammonia determination as previously described.

Carbon dioxide was precipitated from the KOH solution by the addition of 3.0 ml. of 0.1 N (each) $BaCl_2$-NH_4Cl. The precipitated $BaCO_3$ was centrifuged down at low speed and twice washed with water, and finally plated as a slurry in 70% ethanol on tared stainless steel sample pans (Nuclear-Chicago, SC-12). Radioactivity was determined with a gas flow counter (Nuclear-Chicago, D-47, with "Micromil" window).

Examination of the data obtained, presented in Table V, clearly indicates the production of ammonia and $^{14}CO_2$ from [^{14}C] urea by intact *Lacistorhynchus.* Significant radioactivity of $BaCO_3$ from the sample in which the acid was tipped in at zero-time indicates that the acid mixture is not instantaneously lethal.

The ammonia captured following five minutes' incubation is equivalent to hydrolysis of 316 micromoles urea per gram wet tissue per hour, or about one-tenth the tissue activity of homogenate preparations (Table I).

Because of the high urea concentration employed in the experiment, urea disappearance could not be measured by the Archibald (1945) method; hence no attempt was made to relate urea disappearance and ammonia and $^{14}CO_2$ production.

Discussion

Considerable scepticism has attended the periodic reports of urease in lower animals. This has probably been in part due to the low activities recorded as well as to the inadequacy of experimental procedures used in some instances. Too, doubt regarding the validity of observations on animal urease has probably been strengthened by the fact that activities attributed on numerous occasions to gastrointestinal mucosae are, in fact, of bacterial origin (see Kornberg and Davies, 1955).

In any event, Sumner (1951) offered the opinion that the entire question of animal urease needs reinvestigation, an opinion reiterated by Cohen and Brown (1960) in a more recent review.

The quantity of activity exhibited by the tapeworm urease reported here is of considerable interest. In Table VI the present author has assembled data from a number of other reports of urease of invertebrate origin and compared these with jackbean activity. It is to be understood that because of the widely varying conditions under which these assays were performed, Table VI, of necessity, represents only approximate comparisons. Dry weight values were estimated to be 20% of wet tissue weights when only the latter were available.

It is easily seen that the tissue activity of the tapeworm urease herein reported compares favorably with that of the jackbean material.

The use of antibiotics or other procedures designed to insure bacteriostatic conditions was not resorted to in the present study. Since tapeworms lack a gut, microorganisms would only be present as surface contaminants. It is highly unlikely that the urease activities observed with *Lacistorhynchus* can possibly be attributed to microorganisms. If they were indeed due to surface contaminants, then one might reasonably expect activity to be exhibited in tissue preparations of *Calliobothrium,* a worm parasitizing the same host as *Lacistorhynchus* (Table I). Too, while living worms retain full activity for a considerable period of time, tissue preparations rapidly lose activity, which would be most improbable were the activity of microbial origin (Table IV).

Of great interest to students of parasitism is the fact that cestodes seem to have developed more than one method of adapting to the enormous physiological uremia of their elasmobranch hosts, a condition which Smith (1936) believes to be an archaic biochemical property of this vertebrate class. Among tetraphyllids, urea is apparently not metabolized, but rather is an important osmotic constituent of these worms. Urease activity does not seem to be ubiquitous among trypanorhynchs (Table I), and may not even be characteristic of smaller taxonomic groups within

the Trypanorhyncha, since Dollfus (1942) assigns *Lacistorhynchus* and *Grillotia* to the same family.

Although most previous evidence indicates that adult tapeworms are poikilosmotic animals, consideration of possible osmo-regulation must be given to those forms inhabiting elasmobranch hosts and metabolizing urea (see Read and Simmons, in press).

There are, as yet, no data clarifying the biochemical consequences of urea hydrolysis by *Lacistorhynchus*. Although the hydrolysis of urea is an exergonic reaction, it has never been demonstrated that the free energy available can be trapped for useful work (Cohen and Brown, 1960). The possibility that *Lacisto-*

TABLE VI

Estimated urease activities of various invertebrate species compared with jackbean

Source	Tissue tested	Activity (mgm. urea hydrolyzed per gm. dry weight per 5 mins.)	Temperature (degrees Centigrade)	pH	Approximate concentration of urea (molar)	Author
Jackbean	Meal (not defatted)	486–735	20	7.0	0.250	Damodaran and Sivaramakrishnan (1937)
Cestodes						
Lacistorhynchus tenuis	Whole	70	20	7.0	0.400	Present
Pterobothrium lintoni	Whole	700	20	7.0	0.400	Present
Nematodes						
Ascaris lumbricoides	Intestine	0.73	?	6.0	0.007	Savel (1955)
	Muscle	0.28	?	6.0	0.007	Savel (1955)
Nematodirus spp.	Whole	0.036	?	7.3	0.008	Rogers (1952)
Sipunculid						
Sipunculus nudus	Intestine	0.30	?	?	0.0013	Florkin and Duchateau (1942)
Annelid						
"*Lumbricus agricola*"	Whole	0.0007	?	?	0.167	Przylecki (1922)
Molluscs						
Mytilus edulis	Whole	0.009	?	?	0.167	Przylecki (1922)
Helix pomatia	Whole	0.014	?	?	0.167	Przylecki (1922)
Arthropods						
Lucilia sericata	Maggots reared aseptically	1.2	35–36	7.4	?	Robinson and Baker (1939)
Astacus fluviatilis	Whole	0.001	?	?	0.167	Przylecki (1922)

rhynchus might further utilize at least part of the ammonia and carbon dioxide produced by its urease must be considered. Campbell (1960) demonstrated the formation of α-[1-^{14}C] alanine and, probably, [^{14}C] succinic acid from the degradation of [2-^{14}C] uracil by the rat cestode, *Hymenolepis diminuta*, for which the most tenable explanation was a mechanism of carbon dioxide fixation; Daugherty (1954) has reported the synthesis of amino nitrogen from ammonium carbonate by the same species.

The author expresses appreciation to the Supply Department of the Marine Biological Laboratory for providing fishes. He is very grateful to Dr. Clark P. Read for encouraging the work, for supporting it, and for criticizing the manuscript, and to Dr. J. Wayne Campbell for providing certain facilities and for technical advice.

Summary

1. Strong urease activity was measured in tissue preparations of two species of trypanorhynch cestodes; activity was not detected in seven species of tetraphyllids, nor in a third trypanorhynch.

2. Tissue activity of *Lacistorhynchus tenuis* homogenates was in excess of 3000 micromoles urea hydrolyzed per gram wet tissue per hour; that of *Pterobothrium lintoni* was about ten times greater. Tissue activity of intact *Lacistorhynchus* was about one-tenth that of homogenate preparations.

3. The worm activity was partially characterized in homogenate preparations.

4. Living worms retain full activity for at least 110 hours, but homogenates rapidly decline in activity; less than 10% of the original activity is measured after 24 hours.

5. Production of ammonia and of $^{14}CO_2$ from [^{14}C] urea was demonstrated using intact *Lacistorhynchus*.

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TRYPTOPHAN PYRROLASE ACTIVITY IN THE LIVER OF ADULT RANA PIPIENS [1]

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It has been shown by Knox and Mehler (1950) that the activity of mammalian liver tryptophan pyrrolase (peroxidase-oxidase) was greatly increased after injection of tryptophan. The enzyme has been found in the livers of a variety of mammals but not in other tissues (Knox, 1955). Knox and Mehler (1951) further demonstrated that this induction of increased enzyme activity was not controlled by substrate alone. Increased liver enzyme levels could be produced by various stressing conditions, such as section of the spinal cord (Thompson and Mikuta, 1954), and by injections of such substances as histidine and tyrosine (Knox and Mehler, 1951) and histamine and adrenalin (Knox, 1951) in intact animals. In adrenalectomized animals liver enzyme activity could be increased by injections of adrenocorticotropic hormone (Geschwind and Li, 1953) and the glucocorticoid hormones (Thompson and Mikuta, 1954; Knox and Auerbach, 1955). Knox and Auerbach (1955) concluded that there were probably two types of inducing agents, the substrate and an adrenal hormone, and that the enzyme could be induced in adrenalectomized rats by the substrate independently of the hormone. Civen and Knox (1959) further demonstrated that the hormonal induction was independent of substrate concentration and not due to an increase in the level of tryptophan in the tissues produced by the glucocorticoid tissues. More recently Feigelson and Greengard (1961) have demonstrated that rat liver tryptophan pyrrolase is activated by an iron protoporphyrin located in the microsomes.

In the course of investigations on the synthesis of tryptophan pyrrolase during the embryonic development (to be reported elsewhere) of *Rana pipiens* it was found that the controlling mechanisms of tryptophan pyrrolase activity in the frog livers are quite different from those of the adult mammal. It is the results of this investigation on the liver of adult *Rana pipiens* that are reported in the present paper.

Materials and Methods

Large adult male and female *Rana pipiens* were obtained from Vermont and maintained in running tap water at 12–18° C. The procedure for assay of the enzyme was that described by Knox and Auerbach (1955) with two exceptions. The homogenate was prepared by grinding the liver in either a Teflon-Pyrex homogenizer [2] or a Virtis "23" homogenizer [3] for 2–3 minutes, and secondly the reactions were run in an air atmosphere. All other details were *exactly* as described by Knox and Auerbach. Under the conditions specified by these authors

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[2] Kontes Glass Co., Vineland, New Jersey.

[3] Virtis Company, Inc., Yonkers, New York.

enzyme activity was directly proportional to concentration of frog liver homogenate. The Knox and Auerbach procedure is based on the conversion of tryptophan to kynurenine which is the product actually measured during the assay. This conversion, however, is a two-step reaction, tryptophan pyrrolase catalyzing the first step, the conversion of tryptophan to formylkynurenine. The enzyme formylase, which catalyzes the conversion of formylkynurenine to kynurenine, the second step, is found in excess in mammalian liver homogenates and is not rate-limiting (Knox and Mehler, 1950). In all control and experimental frog livers, no reaction products absorbing at 321 mμ were detected. This indicated the absence of formylkynurenine, and formylase, therefore, is probably present in excess in frog livers and is not rate-limiting. L-tryptophan, L-histidine (free base), bovine adrenocorticotropic hormone (ACTH), cortisone acetate, and hydrocortisone acetate were obtained from the Nutritional Biochemical Laboratory, Cleveland, Ohio. Pituitaries were obtained by dissection from large adult females. All injections were given intraperitoneally. All enzyme activities are expressed in terms of μ moles of kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean.

Results

During the early stages of this investigation adult females were used exclusively since it was our intention first to study the activity of the enzyme, under various

Table I

Basal activity of enzyme in liver of adult Rana pipiens females (non-ovulated) during the breeding season

Date	Number of animals	Activity*
4/27/60–5/3/60	10	11.59 ± 1.99
5/4/60–5/11/60	21	19.68 ± 2.20
5/18/60–5/23/60	9	25.60 + 2.62

* μ moles kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean.

conditions, in the adult and ultimately to relate these findings to a study of the enzyme during embryonic development, starting with the ovarian egg. The results at first glance seemed to indicate that the enzyme activity in the female was extremely variable and that the frog was a rather poor experimental animal for this type of study. A more detailed analysis revealed that basal activity of the enzyme in the female was indeed variable but that the extreme variability was due to a progressive increase in enzyme activity following hibernation and during the breeding season. Table I summarizes these findings. It will be noted that a more than 100% increase in enzyme activity (11.59 ± 1.99 to 25.60 ± 2.62 μ moles kynurenine/hour/gm. dry weight of liver) occurred, in non-ovulated females, over an approximately 4-week period following hibernation.

A comparison of liver enzyme activity in females which had ovulated in nature with non-ovulated females revealed that sometime during or following the period of ovulation, enzyme activity radically decreased from a value of 25.10 ± 2.12 to 8.80 ± 1.00 (Table II).

It seemed probable that perhaps pituitary hormones were changing in activity

TABLE II

Basal activity of enzyme in liver of adult Rana pipiens ovulated females vs. non-ovulated females

State of ovulation	Number of animals	Activity*
Ovulated	17	8.80 ± 1.00
Non-ovulated	9	25.10 ± 2.12

* μ moles kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean.

during the breeding season and affecting the enzyme activity. Accordingly pituitaries isolated from mature females were injected into mature ovulated and non-ovulated females. The results are summarized in Table III. In ovulated females the control activities were of the low pre-breeding season values. Injection of pituitary lowered the enzyme activity to 4.45 ± 0.161. That this activity-lowering effect of pituitary injection was not due to events either preceding or occurring simultaneously with ovulation is shown by the results of pituitary injections on non-ovulated females (Table III). In this case the non-ovulated females, after injection of pituitaries, ovulated, but the liver enzyme activity remained at a high level. Further consideration will be given to this point in the discussion section of this paper.

Since the results indicated that enzyme activity in the female varied to a considerable extent during the breeding season, subsequent experiments were carried out on the male frog. Comparable suppression of enzyme activity following pituitary injections was also encountered in the male. In fact, enzyme activity in the male was almost identical with that of the female after this treatment, 4.99 ± 0.619 vs. 4.45 ± 0.161 in the female (Table III). It was interesting to note that basal activity in the male, 9.64 ± 1.26 (Table IV), very closely approximated the basal activity in female livers after ovulation (8.80 ± 1.00, Table II).

Table IV summarizes liver enzyme activity in the male frog after injections of various compounds known to increase activity in the normal intact mammal.

TABLE III

Activity of enzyme in liver of adult Rana pipiens after pituitary injections

Number, sex, and state of ovulation of animals	Treatment	Time (hrs.) assayed after injection	Activity*
14, females, ovulated	2–4 pituitaries in 1 ml. of 10% Holtfreter's solution	5	4.45 ± 0.161
6, females, ovulated	1 ml. of 10% Holtfreter's solution	5	7.41 ± 1.18
6, females, non-ovulated before injection	2 pituitaries in 1 ml. of 10% Holtfreter's solution	18	24.16 ± 2.53
6, females, non-ovulated before injection	1 ml. of 10% Holtfreter's solution	18	22.36 ± 1.79
6, males	5 pituitaries in 1 ml. of 10% Holtfreter's solution	14⅔	4.99 ± 0.619
6, males	1 ml. of 10% Holtfreter's solution	14⅔	9.80 ± 0.41

* μ moles kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean.

TABLE IV

Enzyme activity in liver of adult male Rana pipiens after various treatments

Number of animals	Treatment	Activity*
12	2 ml. L-histidine**	6.44 ± 0.66
12	2 ml. 0.65% NaCl	9.71 ± 0.97
6	ACTH***	7.56 ± 2.11
6	15% gelatin in 0.5% phenol****	9.65 ± 1.21
8	0.5 ml. cortisone acetate†	9.34 ± 1.00
8	0.5 ml. 0.65% NaCl††	9.59 ± 0.73
6	1 ml. hydrocortisone acetate†††	9.20 ± 0.98
6	1 ml. 0.65% NaCl	9.81 ± 1.47
6	3 ml. L-tryptophan††††	40.50 ± 4.90
6	3 ml. 0.65% NaCl	9.49 ± 0.53

* Micro moles kynurenine formed per hour at 38° C., per gram dry weight of liver, ± standard error of the mean. All animals sacrificed and livers assayed 4–6 hours after the last injection.

** Six hundred and thirty mg. in 12 ml. of 0.65% NaCl.

*** One-quarter ml. (8 units/ml. of 15% gelatin in 0.5% phenol); 0.1 ml. 24 hours later; then 0.25 ml. 16 hours later.

**** One-quarter ml.; 0.1 ml. 24 hours later; then 0.25 ml. 16 hours later.

† Ten mg./4 ml. of 0.65% NaCl; for 3 days at 24-hour intervals.

†† For 3 days at 24-hour intervals.

††† Eighty mg./10 ml. 0.65% NaCl.

†††† Seventy-five mg./15 ml. of 0.65% NaCl.

Injections of L-tryptophan produced a 427% increase in enzyme activity. Rather surprisingly, injection of the adrenal cortical hormones, cortisone acetate and hydrocortisone acetate, produced no significant change in enzyme activity in contrast to the increases in activity found in the mammal after their injection. Similarly, L-histidine and ACTH produced no increase as they do when injected into the mammal, and in fact the results suggest that these substances may produce an inhibition instead of an augmentation.

DISCUSSION

The observation that liver tryptophan pyrrolase activity increases in the female frog during the breeding season, to our knowledge, has no counterpart in the recorded observations of activity in the female mammal. Since no investigation, to our knowledge, has been carried out in the female mammal to check tryptophan pyrrolase activity during the estrous cycle it would be presumptuous to state that the amphibian, as exemplified by the frog, is in direct contrast to the mammal with respect to this observation. It is possible that a comparable investigation carried out in the mammal would lead to similar results. The mechanism of this increase remains to be elucidated.

It is, however, well known that in female frogs maintained at temperatures above 15–18° C. for several weeks there is a rapid consumption of the fat bodies and even of the eggs within the ovaries (Rugh, 1935). If female frogs are maintained at 20–25° C. for prolonged periods, abnormal eggs are produced after ovulation. It is possible that the ovarian eggs and fat bodies of female frogs which have not ovulated early in the breeding season begin to degenerate at the relatively warmer temperatures prevalent during the latter part of the season.

Concomitant with this degeneration one could assume a release of tryptophan (from the fat bodies and eggs into the maternal circulation) which is eventually transported to the liver where a tryptophan induction of enzyme activity would occur. While no figures are available, it is our observation that towards the end of the breeding season the percentage of non-ovulated females furnishing 95–100% fertilizable and normally developing eggs diminishes sharply. The return to relatively low enzyme activity following ovulation would offer partial support to this hypothesis. The failure of pituitary injections in non-ovulated females (although bringing about ovulation in these animals) to lower the enzyme activity would require under this hypothesis that the blood tryptophan concentration was sufficiently high to maintain appreciable enzyme activity (or continued induction) for some time after ovulation had occurred. Two experiments carried out in this laboratory indicate that one week after ovulation had been induced by pituitary injection, and the females stripped, the enzyme activity had dropped to its pre-breeding season value. Enzyme activity for these experiments was 8.90 μ moles of kynurenine per hour, per gm. dry weight of liver. Measurements of ovarian and fat body vs. blood tryptophan concentrations during the breeding season would be of value in testing the validity of this hypothesis.

It has been demonstrated by Sims and Bishop (1947) that the pituitary gland of *Rana pipiens* does undergo seasonal variations, increasing in gonadotropic potency between January and March. The possibility that pituitary hormones are increasing during the breeding season and affecting the adrenal cortical hormone balance, which in turn brings about an increase in enzyme activity, seems to be negated by the decrease in activity following pituitary injection. It is implicit in the hypothesis that the addition of pituitary should either augment enzyme activity or have no effect if the synthesis of enzyme is going on at a maximum rate before injection of pituitary. The most probable explanation for the pituitary effect is that the decrease of activity is due to growth hormone, for it has been demonstrated by Wood and Knox (1954) that growth hormone when injected into the mouse brings about a decrease in liver enzyme activity. Estrogenic hormones undoubtedly vary in concentration during the breeding season and should be tested for their effect on enzyme activity.

A third explanation for the increase in enzyme activity during the breeding season remains to be considered. It is possible that the feeding habits of the adult female are extremely variable and it is conceivable that the female upon breaking hibernation consumes a diet rich in tryptophan, leading to an induction of enzyme activity. Following ovulation the consuming of a diet low in tryptophan would in turn lead to a decrease in enzyme activity. A careful search of the literature has revealed no information as to the variation in the food habits of the female frog during this period of the year.

Recently Feigelson and Greengard (1961) have demonstrated that rat liver tryptophan pyrrolase is activated by an iron protoporphyrin present in liver microsomes. It has been further demonstrated (Greengard and Feigelson, 1961) that tryptophan treatment caused an approximately 20% increase in the iron protoporphyrin concentration of liver microsomes, and that the initial rise in enzyme activity after tryptophan treatment was due to an increased degree of saturation of the enzyme with respect to the activator. It is possible that the increases

noted in the enzyme activity of frog liver homogenates during the breeding season and following tryptophan administration and the suppression of activity following pituitary injection are due to changes in the iron protoporphyrin content of liver microsomes. Experiments carried out on the frog which are comparable to those of Feigelson and Greengard will elucidate the role of this activator under the conditions mentioned above.

The failure of the adrenal cortical hormones and of ACTH and L-histidine to produce an augmentation of enzyme activity is in direct contrast to the increases in activity found in the mammal after similar treatment (Knox and Mehler, 1951; Geschwind and Li, 1953). The data for ACTH and L-histidine suggest that these substances may in fact bring about a decrease in activity but further experiments are needed to substantiate this possibility. Although Carstensen *et al.* (1961) have demonstrated the production of aldosterone and corticosterone by *Rana catesbiana* adrenal tissue under the stimulation of bovine ACTH, there appears to be no published information regarding the adrenal cortical hormones in *Rana pipiens,* and it is possible that the amphibian cortical hormones are quite different from those of the mammal. Injections of frog adrenal homogenates would be extremely helpful in establishing this hypothesis.

The frog appears to be an ideal organism for studying the mechanism of substrate induction in metazoa of this enzyme, for the results are not complicated by the possibility of an effect being mediated through the adrenal.

Summary

1. The effect of the injection of L-tryptophan, L-histidine, ACTH, cortisone acetate, hydrocortisone acetate, and pituitary on liver tryptophan pyrrolase activity was studied in adult male and female *Rana pipiens.*

2. L-tryptophan produced a 427% increase in activity but cortisone acetate and hydrocortisone acetate injections had no effect. ACTH and L-histidine injections produced a slight decrease in activity. Pituitary injections brought about a marked decrease in activity.

3. In adult females there was a progressive increase in enzyme activity following hibernation and during the breeding season. This progressive increase was followed by a sharp drop in activity following ovulation.

4. The results are discussed in terms of the mammalian data.

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POLYPHENOL OXIDASE IN THE TEGUMENTAL GLANDS IN RELATION TO THE MOLTING CYCLE OF THE ISOPOD CRUSTACEAN ARMADILLIDIUM VULGARE[1]

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Various hypotheses have been advanced to explain the function of the tegumental glands of Crustacea. The suggestions by early workers that they might be salivary glands, slime glands, sense organs, or excretory organs have been reviewed and disposed of by Yonge (1924, 1932), who believed that they might secrete the epicuticle. (Yonge called this layer the cuticle, but in this paper the terminology adopted by Richards, 1951, will be employed. According to this terminology, the outer, non-chitinous layer of the cuticle is the epicuticle and the inner chitinous layer is the procuticle.) Drach (1939), on the other hand, thought the epidermis secretes the epicuticle. Arguments for and against Yonge's view have been reviewed by Dennell (1947b, 1960), who suggested that since the tegumental glands are most abundant where the cuticle is tanned the most, they may produce the polyphenol oxidase involved in tanning.

This suggestion by Dennell has been investigated by Krishnan (1951). He used the nadi reagent as a test for oxidase and obtained a positive reaction in the tegumental glands of *Carcinus,* their ducts, and the pigment layer of the cuticle during middle and late intermolt when melanin is forming in the pigment layer. The reaction was thermo-labile and cyanide-sensitive. Also the glands and their ducts became dark brown in catechol during this same period. He concluded the tegumental glands secrete polyphenol oxidase for melanin production in the cuticle. Whether they also secrete polyphenol oxidase earlier for tanning is not known. Apparently Krishnan did not study this earlier stage in the molting cycle.

In order to discover whether the tegumental glands secrete polyphenol oxidase for tanning, the molting cycle of the pillbug, *Armadillidium vulgare,* has been studied and polyphenol oxidase has been looked for in the tegumental glands of the legs at every stage of the molting cycle.

Materials and Methods

Several hundred specimens of *Armadillidium vulgare* collected in a garden in Seattle, Washington, were maintained in the laboratory in large jars partly filled with moist dirt. Pieces of carrot and potato were placed in each jar for food.

Tegumental glands were obtained for study by dissection of pereiopods in 0.35 *M* NaCl. A separate leg was removed from a living animal for each test and used immediately. The glands of one segment were tested with the reagent and

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the glands of another segment of the same leg were tested with the reagent plus inhibitor. The glands are very abundant in the carpus and merus, less abundant in the ischium, and small and scarce in the basis. They seem to be absent from the propus and dactylus. To ensure uniformity, only animals in molting stage C were used for the tests for polyphenol oxidase. The dissections were made in 0.35 *M* NaCl because this concentration has a freezing point depression close to 1.18°, the average value for *Armadillidium* according to Parry (1953). No visible changes in the glands take place in this solution, whereas the secretory droplets in the glands burst in hypotonic solutions.

The nadi reagent was prepared immediately before use by mixing 0.1 ml. of 1% a-naphthol in 40% ethanol and 0.1 ml. of 1% N,N-dimethyl-p-phenylenediamine hydrocholoride with 2.5 ml. of buffer. Acetate, phosphate, carbonate, and glycine buffers were used to provide a range in pH from 4 to 11.

A stock of *Armadillidium vulgare* adults was also maintained, one animal to a jar, in 100-ml. jars, each having a shallow layer of moist dirt and potato and carrot for food. By making daily observations on each animal, criteria for recognizing the different molting stages were developed, as described below.

Results

Molting stages. The stage designations developed by Drach (1939) for the Decapoda and some of the modifications of these designations developed by Charniaux-Legrand (1951) for the Amphipoda have been used.

The half of the body posterior to the suture separating the fourth and fifth free thoracic segments molts first, and the half of the body anterior to this suture molts a few days later. Since the molting stages of the two halves of the body can be determined independently, one can designate the molting stage of an animal by stating the stages of each half; for example, anterior: stage C^a, posterior: stage $D_1{}^p$. The letters a and p indicate anterior and posterior.

The following descriptions apply to either half.

Stage A. Immediately after the molt. The body half appears dark brown or black, and the cuticle is soft, shiny, and slightly sticky. Grains of dirt may adhere to it. The legs are not functional, but the animal can walk with the legs of the other half of the body. At first the half in stage A is only about half as long as the other half, but it begins to expand immediately. At the end of stage A, expansion is completed, the cuticle is becoming gray in color, and the legs are beginning to be functional. Duration of stage A: 1–2 hours.

Stage B. The cuticle is gray in color. It has only begun to harden; the terga can be depressed without cracking. The body half has expanded to its full, new size. The pereiopods are functional but their cuticle, as measured in optical section, has not yet reached its definitive thickness (Fig. 1). The animal normally eats its exuviae during this stage. Duration of stage B: several hours.

Stage C. The body has achieved its definitive coloration and hardness. In the early part of this stage the cuticle of the pereiopods becomes progressively harder, beginning distally. Stage C could be subdivided in terms of this progressive hardening. At the same time the cuticle of the pereiopods becomes progressively thicker (compare Figs. 1 and 2). Duration of stage C: about 15–60 days.

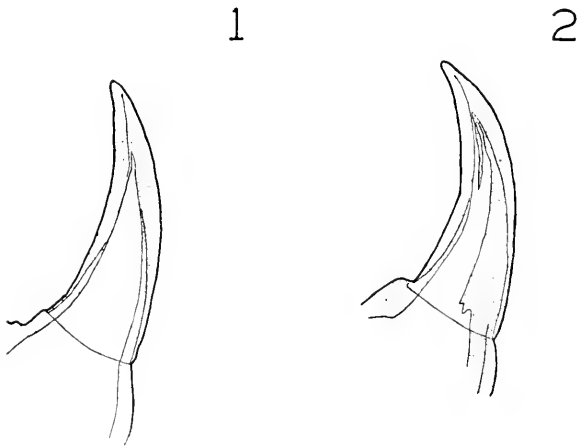

FIGURE 1. Tip of left first pereiopod in stage B. 280 ×.
FIGURE 2. Tip of right first pereiopod of same specimen 10 days later. Stage C.

Stage D. Preparation for the molt. This stage can conveniently be subdivided, as follows:

D_1. New claws begin to form in the pereiopods, as described by Charniaux-Legrand (1951) in amphipods (Fig. 3). While the posterior half of the body is in stage D_1 a pair of white plates develops on each of thoracic sterna 1 to 4. Duration of stage D_1: a few days to a week.

D_2. The new claws have become amber-colored, and the pre-exuvial layers of the new cuticle are secreted over the entire body half. While the anterior half of the body is in stage D_2 the white sternal plates disappear. Duration of stage D_2: about one day.

D_3. As in amphipods, nothing corresponding to stage D_3 of decapods is readily discernible in *Armadillidium*.

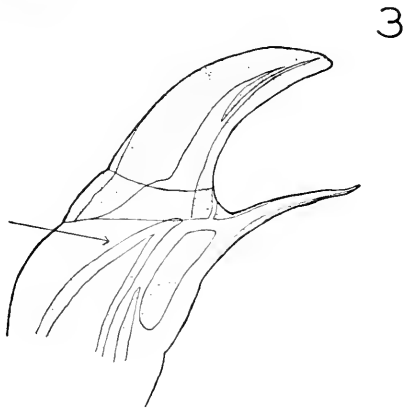

FIGURE 3. Tip of right first pereiopod in stage D_1. The tip of the new dactylus (arrow) can be seen inside the old. The leg was too opaque for the proximal part of the new dactylus to be seen.

D_4. The body half can be compressed easily with the fingers, and the old cuticle can be flaked off. Duration of stage D_4: a few hours.

Stage E. Ecdysis. Molting may occur at any hour, day or night.

Nadi test at different stages of the molting cycle. The tegumental glands of the legs were tested with the nadi reagent in phosphate buffer at pH 7.4 in every stage of the molting cycle and at different intervals within stages C and D. In every case, deep blue color developed in the secretory droplets within a few minutes in the absence but not in the presence of 0.002 *M* NaCN.

Additional tests for polyphenol oxidase. In addition to NaCN, two other inhibitors, NaN_3 and salicylaldoxime, were used with the nadi reagent, and the effect of heat was tested. According to Keilin (1936), catechol oxidase (polyphenol oxidase) from mushrooms was not inhibited by 0.002 *M* NaN_3 at pH 7.3, but 68% inhibition was obtained at pH 5.9. On the other hand only 0.001 *M* NaN_3 produced 80–90% inhibition of cytochrome oxidase at pH 6.5–7.5. In order to test for this same kind of behavior by the oxidase of the tegumental glands,

TABLE I

Results of nadi tests on tegumental glands at different pHs

Without inhibitor										
pH 4	5	6	6.8	7	7.2	7.4	8	9	10	11
−	−	+	+	+	+	+	+	+	±	±
With 0.02 *M* NaN_3										
pH	5	6	6.8	7	7.2	7.4	8	9	10	11
	−	−	−	−	+	+	+	+	±	−

the nadi reagent in 0.002 *M* NaN_3 was used in buffers over a pH range from 4 to 11. For comparison with cytochrome oxidase, the nadi-positive granules of the leg muscles of *Armadillidium* were used. The two tissues could be examined together on the same slide. It was found that 0.002 *M* NaN_3 completely prevented the development of blue color in the granules of the muscles throughout the entire range, pH 6 to pH 8, in which indophenol blue developed in muscles in the control slides. The same concentration of NaN_3 had no discernible effect on the reaction in the tegumental glands at any pH, however. Accordingly, the series was repeated using 0.02 *M* NaN_3. This time, inhibition was obtained in the secretory droplets of the tegumental glands below pH 7.2, as shown in Table I. Without azide, blue color developed in all tests from pH 6 to pH 9. At pH 10 and pH 11, blue color sometimes developed in some of the glands. With 0.02 *M* NaN_3, no blue color developed at pH 5, 6, 6.8, or 7, and normal blue color developed as rapidly as in the controls at pH 7.2, 7.4, 8, and 9. Blue color sometimes developed in some of the glands at pH 10, never at pH 11. A pH of 4 was not tested.

Salicylaldoxime is a reagent for copper and therefore should inhibit the copper-containing enzyme polyphenol oxidase. The tegumental glands were tested with

the nadi reagent with and without 0.001 *M* salicylaldoxime and with and without 0.01 *M* salicylaldoxime. At 0.001 *M*, blue color developed about half as rapidly (14–15 minutes) as in the controls (5–6 minutes). At 0.01 *M*, no color developed at all.

The nadi test was also negative in glands that had been incubated at 65° C. for 30 minutes.

Fresh tegumental glands were also treated with 1% 3,4 DL dihydroxyphenylalanine (dopa) in phosphate buffer, pH 6.8. After three hours at 22° C., the secretory droplets were black. None of the other tissues had become colored.

Discussion

Studies on molting and related phenomena in crustaceans are sometimes hampered by the difficulty of obtaining the desired stages in the molting cycle when needed. By using *Armadillidium* and the criteria for recognizing the different stages as described in this paper, however, one can obtain any stage at any time. For instance, in a stock of only 60 animals an average of one will molt each day. To designate short periods within each of the primary stages, substages or number of hours before or after an event can be used. George and Sheard (1954) have used the latter method in their description of changes in the epidermis and cuticle of *Porcellio scaber*.

From the evidence presented in this paper, the tegumental glands seem definitely to contain polyphenol oxidase. The nadi test is rather uncertain and variable—poor staining was obtained if the stock solutions were more than a month or less than a week old! But inhibition of the reaction by cyanide, azide, salicylaldoxime, and heat clearly indicates the presence of an enzyme. Inhibition by azide was exactly as predicted from Keilin's work with catechol oxidase of mushrooms, except that a higher concentration of inhibitor was needed.

The most specific test for polyphenol oxidase used was treatment with dopa, which is oxidized to dopa quinone by this enzyme. The dopa quinone then becomes melanin by polymerization.

The tegumental glands of the legs of *Armadillidium* are similar histologically to tegumental glands of other parts of the body and to tegumental glands of other crustaceans. Their function is probably to secrete polyphenol oxidase into the epicuticle from where it diffuses inward and catalyzes sclerotization in the epicuticle and exocuticle (Dennel, 1947b). The evidence for this is three-fold. First, the glands develop and degenerate with each molting cycle, and the peak in their activity occurs some time before molting (Yonge, 1932; Gorvett, 1946). It may be that the peak coincides with the appearance of polyphenol oxidase in the epicuticle, but further work will have to be done to determine whether this is the case. Second, the ducts of the glands extend to or through the epicuticle, and these ducts are most abundant where the cuticle is tanned the most (Dennell, 1947b, 1960). The presence of the ducts extending through the procuticle to the epicuticle is significant because the epicuticle is the part of the cuticle where polyphenol oxidase appears first (Dennell, 1947b). However, in the insect *Sarcophaga,* in which the enzyme also appears first in the epicuticle, there is evidence that it originates in the epidermis, diffuses through the procuticle, and somehow accumulates in the epicuticle (Dennell, 1947a). Third, as demonstrated in this paper, the tegu-

mental glands do contain polyphenol oxidase. In addition to this evidence, direct evidence that the glands release polyphenol oxidase into the epicuticle at or before the time when it is used in tanning would be desirable before one concluded that they do so. It may be that the enzyme is involved in the metabolism of the glands and that the secretory product is some other material. Both mucus (Farkas, 1927) and lipid (Yonge, 1932) have been reported in tegumental glands, and there is evidence that tyrosinase (polyphenol oxidase) may function as a terminal oxidase in respiration, at least in plants and insects (Dawson and Tarpley, 1951; Patterson, 1958).

The presence of polyphenol oxidase in the tegumental glands of the legs of *Armadillidium* throughout the molting cycle is not inconsistent with the idea that the enzyme is released at a particular time. The enzyme may be in storage most of the time. However, it raises the question of why *Carcinus* is different in this respect. Krishnan found polyphenol oxidase only during middle and late intermolt in *Carcinus*. Another difference in *Carcinus* is that the tegumental glands also apparently secrete polyphenol oxidase into the pigment layer, where it catalyzes melanin production (Krishnan, 1951). Presumably this does not occur in *Armadillidium,* which lacks a pigment layer.

Undoubtedly some of the different types of tegumental glands perform different functions. Gorvett (1946) has described six types in isopods, and he presents evidence that the lobed glands of the uropods and lateral plates secrete bad-tasting and bad-smelling material that protects against spiders (Gorvett, 1951, 1952, 1956). Other types may have yet different functions.

Summary

1. Criteria are presented for distinguishing the molting stages of *Armadillidium vulgare*.

2. An oxidase is present in secretory droplets in the tegumental glands of the legs throughout the entire molting cycle.

3. This oxidase seems to be the polyphenol oxidase involved in sclerotization of the cuticle.

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SEASONAL VARIATIONS IN O_2-CONSUMPTION OF UCA PUGNAX

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Within the past few years studies on O_2-consumption of the fiddler crab, *Uca pugnax*, have provided descriptions of diurnal and lunar-tidal rhythms (Brown, Bennett and Webb, 1954). Webb and Brown (1958) have described the persistence of lunar-tidal rhythms of O_2-consumption for as long as three weeks in the laboratory. Both of these investigations were carried out during the summer months when these animals regularly leave their burrows at the time of low tide. Because of the marked stability exhibited by the lunar-tidal rhythm and the fact that the period of the rhythm is not one normally associated with human activity in the laboratory, it was of interest to study this rhythm at various times of year. We were particularly concerned with two aspects: (1) any seasonal variations in either the diurnal or the lunar-tidal rhythms, and (2) the degree of interdependence between the diurnal rhythm and the lunar-tidal one as indicated, for example, by the extent to which a change in one occurred simultaneously with a change in the other.

Materials and Methods

These experiments were carried out at the Marine Biological Laboratory at Woods Hole, Mass., and the animals used were collected at Chappaquoit Beach, or at Silver Beach, both on Cape Cod, Mass. During the summer months collections were made at approximately weekly intervals, the animals being picked up as they ran on the beach near the time of low tide. All of these collections were made at Chappaquoit Beach, the last one in 1959 on September 30. On January 7, 1960, and again on January 29, animals were obtained from the Supply Department of the Marine Biological Laboratory. On both of these occasions the animals were dug from their burrows at Silver Beach, which is about one mile from Chappaquoit and where low tide occurs within a few minutes of its occurrence at Chappaquoit. In the laboratory the animals were kept in large white enamel pans with a small amount of sea water which was changed daily. Only males, of the species *Uca pugnax*, ranging in weight from 2 to 4 grams were used. The animals were kept in a laboratory where they were exposed to the normal diurnal changes in illumination, and thus they experienced decreasing photoperiods until late December, and in January and February, an increasing one.

Oxygen consumption was measured by means of Brown respirometers (Brown, 1957) with the conditions the same as those described by Webb and Brown (1958). A maximum of six barostats was used at any one time. Thus the maximum num-

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ber of animals represented on any one day is 24, and the minimum is four (one barostat). Throughout most of the study three barostats (12 animals) were being used. The lever system was such that an excursion of 1.4 mm. on the record was equivalent to 1 ml. of O_2 consumed. Animals usually remained in the respirometers three or four days. Only complete days of data were used; thus the portions of records obtained between the time of setting up the respirometers and the first midnight were excluded, as were the terminal portions from midnight of the last day of each run.

Results

Data are available for 11 semi-monthly periods from July 25, 1959, to February 18, 1960, and for two such periods from June 25 to July 25, 1960. The mean values of O_2-consumption and the ranges for the mean cycles are presented in

Table I

Respiratory rates and cycle ranges for U. pugnax

Calendar period	Mean rate (ml./kg./hour)	Diurnal range (% of mean rate)	Lunar range (% of mean rate)
A. 7/25– 8/8	81	40%	24%
B. 8/9 – 8/24	80	33%	26%
C. 8/24– 9/8	80	28%	31%
D. 9/8 – 9/22	70	36%	37%
E. 9/22–10/6	63	28%	48%
F. 10/11–10/25	60	43%	22%
G. 10/25–11/8	48	34%	20%
H. 11/19–12/2	43	33%	22%
I. 12/4 –12/18	44	28%	27%
J. 1/9 – 1/23	46	36%	33%
K. 2/4 – 2/18	40	31%	21%
L. 6/25– 7/9	77	33%	24%
M. 7/10– 7/25	86	31%	27%

Table I. It is evident that there occurs a marked reduction in rate of respiration throughout the autumn season. The values can be roughly separated into three categories: June 25 through September 8, when the values are highest and quite constant; September 8 through November 8, when a decreasing series is obtained; November 8 through February 18, during which time the values show little or no change and are low.

It is also clear from Table I that the range of a 15-day mean cycle bears no consistent relationship to the mean hourly rate or to the season of the year. Practically the entire gamut of fluctuations in range of the solar-day cycle is encompassed within the first three periods during which the mean rate remains essentially unchanged.

In Figure 1 is seen a series of solar-day curves, each calculated for a 15-day period, plotted as deviations from the mean for the period. The periods are those recorded in Table I. It can be seen that throughout the series the form of the solar-day curve remains relatively stable. It exhibits the highest values during the

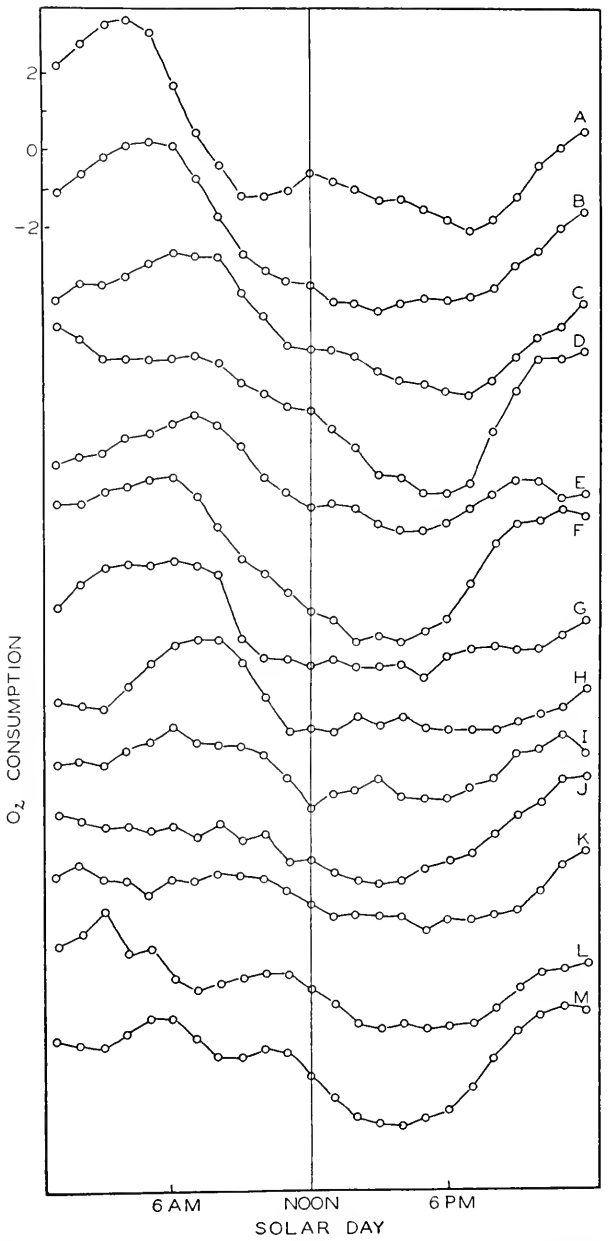

FIGURE 1. Mean diurnal variations in O_2-consumption of *Uca pugnax* for 13 15-day periods during 1959–60. The curves are for the periods appearing in Table I where each period is identified by the same letter as the corresponding curve in the Figure.

hours between 10 P.M. and noon, and the lowest values during afternoon or early evening. There are variations in position of the maximum but no obvious pattern to these variations. In Figure 2 these data are summarized in the form of four curves, one for each of the following periods: July 25 to October 25, 1959; October 25, 1959, to January 23, 1960; February 2 to 18, 1960; and June 25 to July 25, 1960. It is obvious that the absolute amplitude is reduced during the winter months but as was seen in Table I the absolute rate is decreased also. Thus when amplitude (range) is expressed as a percentage of the mean for the period, there is no consistent seasonal trend.

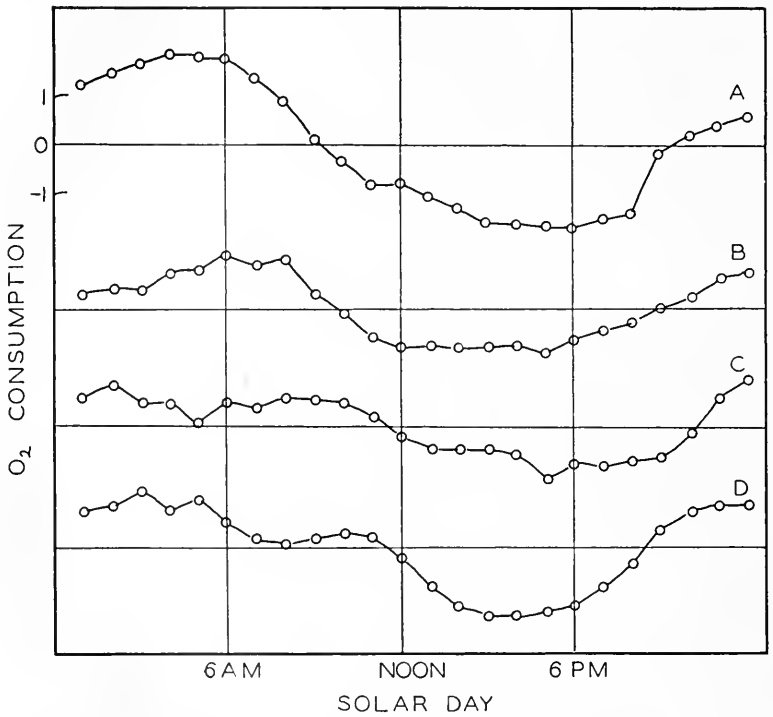

FIGURE 2. Mean diurnal variation in O_2-consumption of *Uca pugnax* for four periods throughout the year (see text for explanation).

When 15 days of data are analyzed for a lunar-day rhythm and each day is used only once, there will be present in the resulting curve a residual of the diurnal rhythm since any particular point of the solar day will have scanned only twelve hours of the lunar day. In cases where the rhythm is obviously a lunar-tidal one, with two peaks symmetrically placed in a lunar day, one can synchronize lunar zenith with lunar nadir and thus obtain complete scanning of the now approximately 12-hour day. Alternatively, one can calculate a "residual" diurnal curve and use it to make appropriate corrections on the observed 15-day lunar curve. A residual diurnal curve for each 15-day period was obtained by using the mean diurnal values which include the 15-day period for which the correction was to be

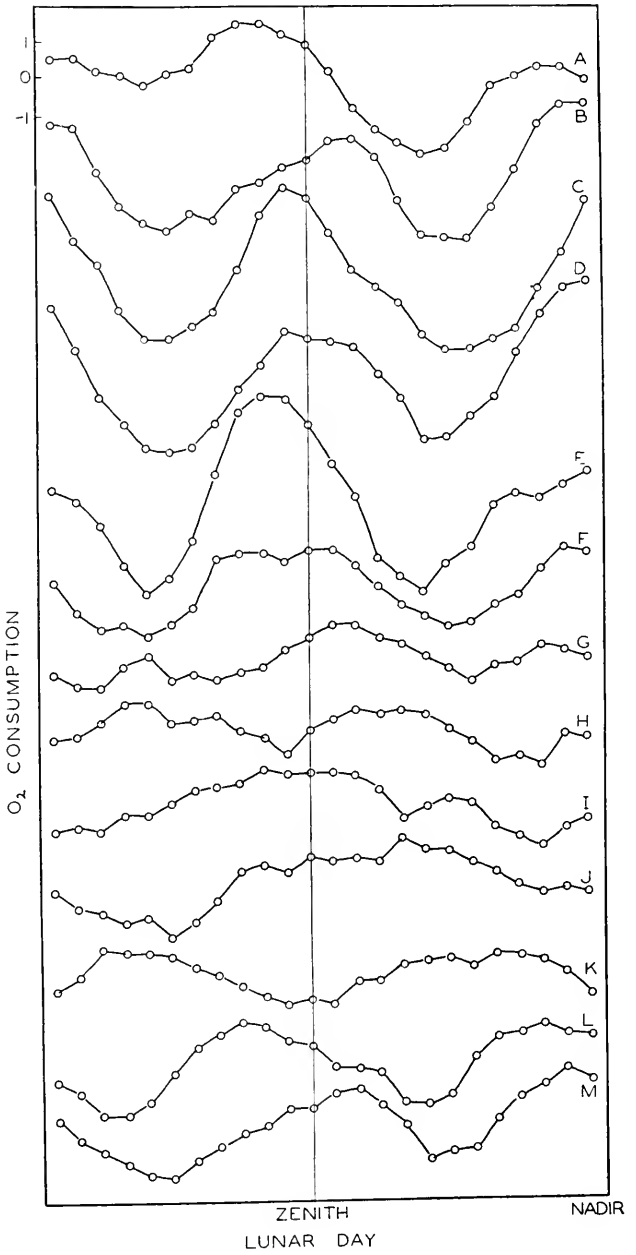

FIGURE 3. Mean lunar-day variation in O_2-consumption of *Uca pugnax* for 13 15-day periods. The letters identify the dates of the periods as found in Table I.

used. The mean diurnal values are repeated as if they represented successive days but are synchronized for lunar time and a mean value for each hour of the lunar day is thus obtained. These values then represent what would be expected if a diurnal rhythm without lunar component were analyzed for a lunar-day rhythm on a 15-day basis. The hourly values thus obtained were subtracted from the corresponding values of the observed 15-day lunar curve.

In Figure 3 is seen the series of corrected mean lunar-day curves for the same 15-day periods which have been described in Table I and Figure 1. It is seen that the first four periods (through September 22) exhibit quite symmetrically bimodal curves with maxima at or near zenith and nadir. There is a tendency for the zenith peak to alternate between an hour or two before and an hour or two after

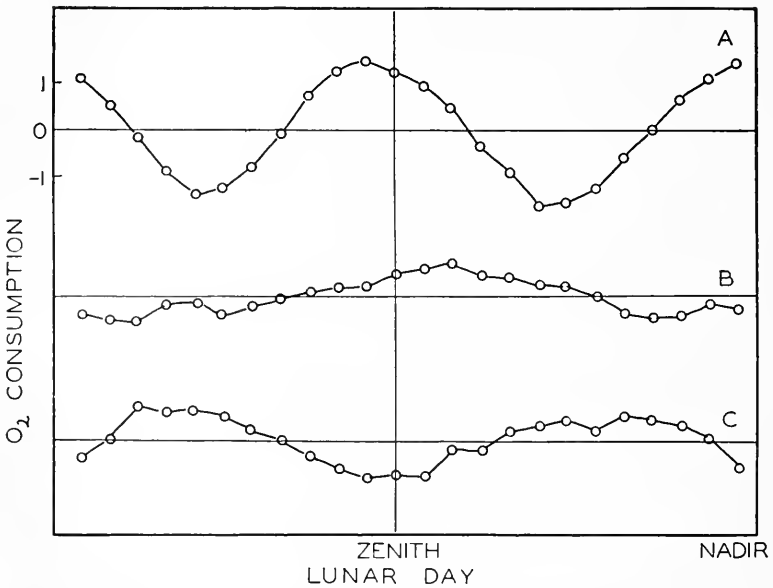

FIGURE 4. Mean lunar-day variation in O_2-consumption of *Uca pugnax* for three periods throughout the year (see text for further explanation).

zenith. During late September and early October (Curves E and F) there is some decrease in the regularity of the curves but they are still clearly bimodal and the peaks are still close to the times of zenith and nadir. Curve G (October 22 to November 6) shows a clear maximum at between one and two hours after zenith; the maximum is smoothly approached during a six-hour period on either side of it. The other twelve hours of the lunar "day" are irregular with apparent secondary maxima about three hours before lunar nadir and about five hours after it. The curves for the periods from November 19 through January 23 (Curves H, I, J) are irregular and of low absolute amplitude although, like the diurnal rhythms, the relative amplitudes show no consistent trend. These three curves on the whole seem to be unimodal in character with high values predominating near the time of lunar zenith. Curve K, Figure 3, representing the period February 2

to 18, is bimodal and in this respect resembles the "summer" curves. However, the phase relations in February are different from those observed in the summertime so that now the minima coincide with lunar zenith and nadir. The final two curves in Figure 3 are those obtained during June and July, 1960; they are bimodal with maxima near the times of zenith and nadir. Here again the tendency toward alternation of peaks around zenith is evident.

In Figure 4 are seen curves summarizing the data of Figure 3. Curve A, Figure 4 represents July 25 to October 25; Curve B is for October 25 to January

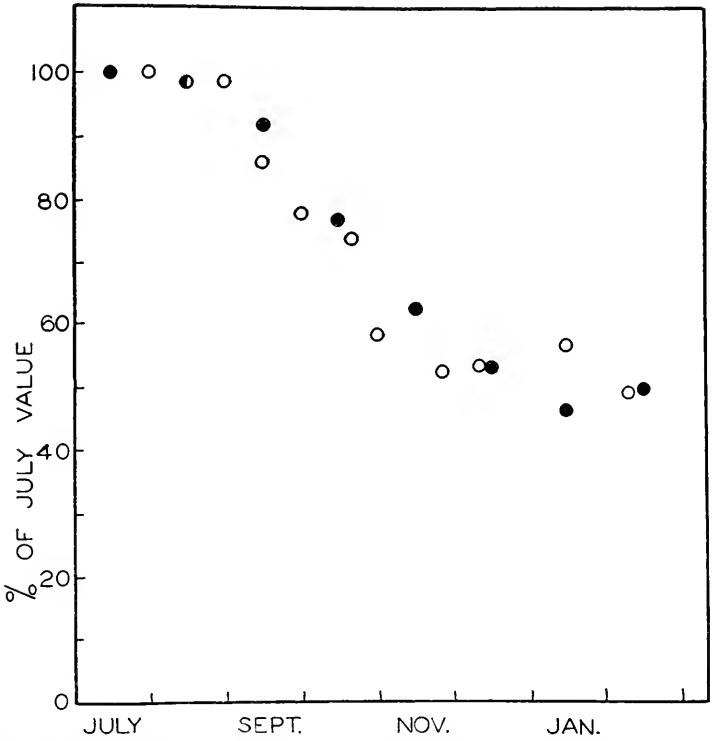

FIGURE 5. Mean monthly outdoor air temperature (solid circles) and mean rates of O_2-consumption of *Uca pugnax* (open circles). (The data on temperature were provided by Dr. Charles Packard of the Marine Biological Laboratory.)

23 and clearly reveals the unimodal nature of the curve for this period; Curve C is for February 2 to 18. From this figure it is clear that three distinct types of lunar day curves are obtained at three different times of the year.

Figure 5 contains data for the mean monthly outdoor air temperature at Woods Hole, Mass., for the months July, 1959, through February, 1960, and the mean rates of O_2-consumption calculated for each 15-day period throughout the same months. In both cases the July value is taken as 100% and the subsequent values are plotted as the appropriate per cent of that value. It is obvious that any two curves drawn through the two sets of points would be practically indistinguishable.

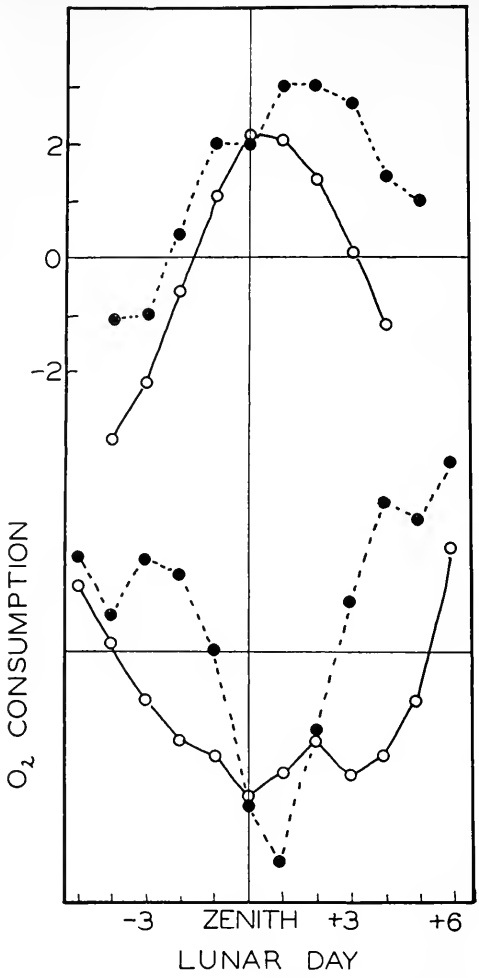

FIGURE 6. Variations in O_2-consumption of *Uca pugnax* for the hours around lunar zenith on four different days.

Figure 6 represents the values obtained, on four different days, during November and December, for the nine-hour period over lunar zenith.

DISCUSSION

The question of the extent to which maintenance of animals in the laboratory contributes to the difference in rates observed in these studies deserves consideration. It will be recalled that collections were made on the following dates: September 30, 1959, January 7, 1960, and January 29, 1960. Therefore, at the end of the test periods October 11 to 25, January 9 to 23, and February 2 to 18, animals had been in the laboratory for 25, 16 and 20 days, respectively. Yet the rates obtained in the

last two periods are considerably lower than those found for the period October 11 to 25. On the other hand, the rates observed in January and February are not significantly different from those observed in the December period. The animals used in December had been in the laboratory for 79 days by the end of the test period. Clearly it is not the length of time in the laboratory that is the major factor in the control of respiratory rates.

An interesting correlation is evident when the mean rates of O_2-consumption for successive periods are compared with mean monthly outdoor air temperature for the corresponding months, as is done in Figure 5. Since the respiratory rates were determined under constant temperature conditions, the remarkable parallel between the two series of points cannot indicate a direct cause and effect relationship. However, the obvious correlation with one factor in the physical environment (which may serve as a representative of a whole complex of annually fluctuating factors), together with the equally obvious lack of correlation with the immediate history of the animals, strongly suggests a contribution of some uncontrolled (and unrecorded) environmental factor to the apparent annual rhythm in respiratory rate.

It is realized that the results reported here are in apparent disagreement with relationships between summer and winter rates of respiration for *Uca pugnax* previously reported by Vernberg (1959) and Teal (1959). In both cases when *Uca* were tested at the same temperatures during summer and winter the rates were higher in the winter time. The animals used by both of these investigators came from different geographical areas than did those used in our experiments. Different methods of measuring O_2-consumption were used, and their methods involved much shorter test times. The maximum time for which their animals were exposed to the measurement situation would be about 8 hours and this only for the lower temperatures. In our case the first 8 to 16 hours of recording were not included in the calculations. Until some of these more obvious differences in conditions have been examined experimentally it seems unprofitable to do more than acknowledge the difference in results.

In addition to the apparent annual cycle in mean rates of O_2-consumption, the results reported here reveal variations in the form of the lunar rhythm. As was seen in Figures 3 and 4, three types of curves were encountered in going from July to February. The curve obtained in the summertime and that for February are both bimodal, one being the inverse of the other; in the intervening period a unimodal curve was obtained.

The apparently unimodal rhythm observed during the transitional period is very probably the result of averaging together for any one period curves, from single days, which show varying degrees of inversion. Evidence bearing on this question was presented in Figure 6 which shows data for the period centering on lunar zenith for four different days. It is quite clear that on two of these days distinct maxima were recorded at lunar zenith while on the other two distinct minima occurred. The relatively few days during the transitional period for which clear inversions are evident can easily be explained when it is realized that even a single record represents the sum of four animals' respiration. As an increasing proportion of the population shows an inversion of the summer pattern at lunar zenith, the chances of getting all the animals being recorded at any one time exhibiting the inverted pattern are increased. This interpretation is supported by the observation that in

February the records show inversion on more than 50% of the days while in less than 25% of the records is there a peak at lunar zenith.

Since the animals tested in December and January show similar patterns for the lunar-day rhythm, although one group has been in the laboratory for 79 days and the other for only 16, it is clear that the conditions of maintenance in the laboratory are not sufficient to explain the observed changes in the rhythm. One condition that was common to all the animals, whether in the laboratory or in the field, was the absence of illumination periods limited to the time of low tide. From early October it was not possible to collect crabs except by digging. Since they remained in their burrows they would not be exposed to all of the changes associated with low tide to which they are exposed in the summertime. These animals would presumably be in continuous darkness. The animals in the laboratory, on the other hand, would have experienced light periods longer than those to which they are normally exposed, and lacking in any tidal component. It is possible, therefore, that the absence of illumination periods associated with the tides permitted this inversion of the form of the rhythm. It is to be noted, however, that there is no evidence of a loss of precision of the period; the majority of the animals are in synchrony during February. The simplest explanation seems to be that the animals receive information concerning the time of lunar zenith and nadir. The difference between summer and winter respiratory patterns might reflect a difference in pattern of the "information" or it might represent a different sign of response on the part of the animals.

Regardless of interpretations as to cause, it is clear from the observations reported here that animals, while showing no obvious changes in the diurnal rhythm, have exhibited alterations in the lunar-day rhythm of respiration. There is no evidence of change in the period of the rhythm but there is evidence of "preferred" phase relations to lunar zenith and nadir.

Summary

1. Records of the O_2-consumption of *Uca pugnax* were obtained during the summer, autumn, and winter months and analyzed for diurnal and lunar-day rhythms.

2. An apparent annual rhythm in mean rate of respiration is reported.

3. The respiratory rate during December, January, and February was found to be approximately 50% of that during June, July, and August. During September, October, and November, a gradual decrease in rate was observed.

4. The form of the diurnal rhythm of respiratory rate showed no changes associated with time of year. Similarly, no seasonal changes in phase relations were observed.

5. The range of both diurnal and lunar-day rhythms showed fluctuations but these were not clearly associated with time of year.

6. The lunar-day rhythm was found to show changes as follows: June through September the curve was bimodal with maxima at lunar zenith and nadir; beginning in October and continuing through January the curve was unimodal with a maximum near lunar zenith; in February the curve was bimodal but with minima at lunar zenith and nadir.

7. The unimodal curve of the transitional period is interpreted as being the resultant curve of a mixed population.

8. The possible relationship of exogenous factors to these seasonal variations is discussed.

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THE JUVENILE HORMONE. II. ITS ROLE IN THE ENDOCRINE CONTROL OF MOLTING, PUPATION, AND ADULT DEVELOPMENT IN THE CECROPIA SILKWORM

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In the history of every hormone there is a more or less prolonged period when the factor is recognizable only as a certain "activity" within a living system. Progress at this stage is largely dependent on the development of a method of biological assay which is simple, selective, and quantitative.

In the case of the juvenile hormone of insects, the pioneering studies that led to the discovery of the hormone simultaneously directed attention to a method for its assay. This method, as developed by Wigglesworth (1936, 1948, 1958), is performed on mature nymphs of *Rhodnius*. In brief, a fifth instar nymph is given a blood meal to provoke the molt to the adult stage. If one now implants active corpora allata (or, by parabiosis, transfuses blood containing juvenile hormone), the nymph transforms into an adult which retains certain nymphal characteristics. By the use of this assay, Wigglesworth (1940) found the corpus allatum of *Rhodnius* to be active in the immature nymph, inactive in the mature nymph, and active again in the adult.

Numerous investigators, following Wigglesworth's lead, have utilized the "larval assay" in testing the endocrine activity of corpora allata. The literature includes studies of the following genera: *Bombyx* (Bounhiol, 1938; Fukuda, 1944; Ichikawa and Kaji, 1950), *Dixippus* (Pflugfelder, 1939, 1958), *Tenebrio* (Radtke, 1942), *Galleria* (Piepho, 1942, 1950b), *Melanoplus* (Pfeiffer, 1945), *Drosophila* (Vogt, 1946), *Gryllus* (Poisson and Sellier, 1947), *Oncopeltus* (Novák, 1951), and *Calliphora* (Possompès, 1953). The conclusions derived in all these studies have confirmed the fact that the corpora allata undergo substantial changes in activity during the course of metamorphosis.

At the Harvard laboratory we also have tried to make use of the larval assay in testing for juvenile hormone. A survey of all of our experiments performed during the past fifteen years fails to reveal a single instance in which the implantation of active corpora allata interfered with the transformation of fifth stage Cecropia larvae into normal pupae. For reasons that are not fully understood, the larval assay does not work in the case of the Cecropia silkworm.

Solution of our problem came from an unexpected direction. As described in the previous paper of this series, the "pupal assay" was accidentally discovered in 1947; unlike the larval assay, it proved to be an extremely sensitive test for juvenile hormone (Williams, 1952a, 1959).

In the present study the pupal assay has been used as a principal tool in a study

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of the role of juvenile hormone in the growth and metamorphosis of the Cecropia silkworm.

Material and Methods

The experiments were performed on larvae, pupae, and adults of the Cecropia silkworm, and on pupae and adults of the Cynthia silkworm. In addition to methods described previously, the following procedures were utilized.

1. *The pupal assay for juvenile hormone*

Pairs of corpora allata-corpora cardiaca complexes were excised from larval, pupal, or adult donors, and placed in Ringer's solution. In the early experiments the corpora allata were dissociated from the closely attached corpora cardiaca. This tedious maneuver proved inconsequential (Williams, 1959) and was generally omitted in later experiments.

The glands were tested for endocrine activity by implanting them into the abdomens of female Cecropia pupae that had been stored at 6° C. for at least four months. For reasons considered below, pupae that had already initiated adult development were not suitable as test animals. The implantation was accomplished as follows:

Each test pupa was anesthetized with carbon dioxide, and a disc of integument excised from the tip of its abdomen. By means of forceps, one to eight corpora allata were placed among the strands of fat-body deep inside the abdomen. Crystals of an equal part mixture of phenylthiourea and streptomycin sulfate were spread in the wound, along with sufficient Ringer's solution to fill the body cavity. The wound was capped with a plastic window which was sealed in place with melted wax. Finally, the pupa was returned to air and stored at 25° C.

Within a few days the wound was healed by a deposit of blood cells on the plastic window and the centripetal spread of the simple epidermis from around the margins of the wound. Adult development was ordinarily initiated after about ten days; the zero day of development was recognized by the beginning of the retraction of the regenerate epidermis underlying the plastic window (Schneiderman and Williams, 1954).

On about the fifth day of development the first indication of a positive test for juvenile hormone was evident under the window. Here one could witness the formation of a new pupal cuticle which soon became tan and sclerotized. A few days later, the old pupal cuticle became thin and crisp, due to a precocious activation of the molting-fluid and the localized resorption of endocuticle (Passonneau and Williams, 1953). Commonly, one could detect the discharge of a white sludge of meconium into the molting-fluid—an event that has rarely been observed in the course of normal metamorphosis.

Development was allowed to proceed for a total of twenty-one days or until the molting-fluid was partially resorbed. The pupal exuvia was then peeled away with forceps. The insect was immersed in Ringer's solution and subjected to detailed external and, in many cases, internal examination.

A positive test for juvenile hormone is signaled by the preservation of pupal characters (Williams, 1952a, 1956, 1959)—a finding which will be considered in detail in the section on Results.

2. *Excision of larval corpora allata*

Matched pairs of larvae were sacrificed and the corpora allata dissected from their heads, as previously described in the case of adult corpora allata (Williams, 1959, page 327). In certain experiments it was necessary to excise the corpora allata without killing the larval donors. By adaptations of a method suggested by Dr. William Van der Kloot (unpublished observations), a surgical approach through the underside of the neck was utilized, as follows:

A 3-cm. length of dowel was attached to a small base-board so that the dowel stood vertically above the perforated plate of the anesthesia funnel. The top end of the dowel was grooved to fit the dorsum of the larval head capsule. The larva was deeply anesthetized and placed in the anesthesia funnel so that the underside of the neck was stretched and flexed over the top end of the dowel. The head capsule was held in the groove by small clips so that the thorax and anterior abdominal segments hung vertically. In this way the blood was pressed from the neck region and the latter was flattened and essentially bloodless.

The ventral midline of the neck was lifted with forceps and a single V-shaped incision was made through the integument with microscissors. Under a dissecting microscope the operation was carried out through the incision, first on one side of the neck and then on the other. With blunt probes the muscles of the neck were pressed apart and the corpora allata located and excised.

At the conclusion of the operation the flap of skin was spread in place. The animal was stored in a capped cardboard container at 5° C. until the next day. It was then returned to room temperature and placed on a netted branch of wild-cherry leaves.

3. *Excision and transplantation of wing-discs*

The thorax plus first abdominal segment was isolated, opened along the mid-dorsal line, and spread and pinned under Ringer's solution. After the removal of the viscera, the two pairs of wing-discs were located between the body wall and the complex musculature of the meso- and metathorax.

Each disc, together with its peripodal sac, was trimmed away from surrounding structures and placed in a dish of insect Ringer. In some cases the dissection was continued by cutting away the peripodal sac. The discs were implanted under abdominal windows of previously chilled pupae, as described above.

Experimental Results

1. *Quantitative aspects of the pupal assay for juvenile hormone*

When a living, active corpus allatum is implanted into a test pupa, the gland survives and becomes the site of synthesis and secretion of juvenile hormone. If several active glands are implanted, a corresponding number of synthetic centers are established. So, in theory, one should be able to vary the rate of secretion and accumulation of juvenile hormone by varying the number of implanted glands. The developmental reactions of the test animal should then reflect the quantitative aspects of the pupal assay.

In experiments of this type, advantage was taken of the high activity that one routinely observes in tests of the corpora allata of freshly emerged Cecropia moths

(Williams, 1959). Four (two pairs) adult corpora allata were implanted into each of 24 test pupae; a second group of 24 pupae each received only a single implant (one-half pair). The hosts were then placed at 25° C. to await the onset of development.

Three to four weeks later, a spectacular difference was evident between the two groups of animals. The individuals that received the four implants showed a generalized inhibition of adult differentiation, as signaled by the formation of a new pupal cuticle throughout broad areas of head, thorax, and abdomen. Indeed, some of these animals could properly be described as second pupal stages in which only

TABLE I

Developmental status of previously chilled Cecropia pupae after receiving implants of corpora allata. The endocrine activity of the implants is scored in intensities ranging from zero to five

Intensity of reaction	Anatomical characters			
	Head	Thorax	Abdomen	Internal anatomy
0	Adult	Adult	Adult	Adult
1	Normal adult, except for small facet-free crescents in eyes*	Adult with pupal cuticle occasionally in midline of prothoracic tergum*	Adult except for small zone of pupal cuticle at base of genitalia*	Adult
2	Normal adult, except for facet-free crescents in eyes	Pupal cuticle in midline of tergum	Zones of pupal cuticle anterior to intersegmental membranes; genitalia slightly arrested with localized pupal cuticle	Adult with slight suppression of gonad development
3	Covered with pupal cuticle with pubescence only on frons; antennae show incomplete barbs and pupal cuticle locally on shaft	Large zones of pupal cuticle on tergum and legs; wings show scales, but incomplete pigmentation	Generally covered with pupal cuticle showing sparse pubescence; genitalia very inhibited and covered with pupal cuticle	Gonads show considerable arrest; flight muscles show only early development; gut retains pupal configuration; prothoracic glands persistent
4	Pupal cuticle on head, antennae, and palps; antennae show only traces of segmentation or of subdivision into barbs	Pupal cuticle throughout with only localized islands of pubescence; wings generally white, fleshy and friable	Pupal cuticle throughout; genitalia show no development	Only traces of development in gonads, gut, and muscles; fat-body friable and in chunks; prothoracic glands persistent
5	Pupal throughout except for small pigmented eyes	Pupal cuticle throughout; sometimes also at base of wings and around margins of forewings	Wholly pupal	Pupal; prothoracic glands persistent

* Pupal cuticle forms in all zones where the integument has been injured.

traces of adult characteristics had been differentiated. By contrast, the pupae that had received only a single corpus allatum ordinarily developed into adult moths which showed few abnormalities except for the formation of a new pupal cuticle under the plastic window where the pupal integument had been excised in the implantation procedure.

These results have been duplicated and extended on a large scale during the past fourteen years. Under most conditions and circumstances, the standardized pupal assay has proved to be a sensitive, selective, and semi-quantitative test for the concentration of juvenile hormone.

Under the standardized conditions of the assay, each concentration of juvenile hormone ordinarily produces a certain pattern of inhibition. In practice, it was possible to subdivide the intensities of the reactions into six categories. This scoring system, summarized in Table I, permitted the pupal assay to be used in a study of corpus allatum activity at successive stages in the life history of the Cecropia silkworm.

2. *Endocrine activity of corpora allata*

A. Larval corpora allata

Corpora allata were removed from matched pairs of Cecropia larvae at stages ranging from the middle of the third instar to the end of the fifth instar. The two pairs of glands were, in each case, implanted into a single female test pupa, as described under Methods.

Before considering the results of the assays, it is worth recalling that the growth and metamorphosis of the corpora allata are synchronized with the growth and

TABLE II

Activity of Cecropia corpora allata implanted into chilled Cecropia pupae (2 pairs of glands into each test pupa)

Stage of donors	No. of tests	Intensity of hormone reaction						Average Index*
		0	1	2	3	4	5	
3d instar	5	1	2	0	2	0	0	1.6
Late 3d	2	0	1	1	0	0	0	1.5
3d molting	9	0	3	5	1	0	0	1.8
Early 4th instar	11	0	2	2	6	1	0	2.5
4th	6	1	1	2	2	0	0	1.8
Late 4th	6	1	2	2	1	0	0	1.5
4th molting	10	1	5	2	2	0	0	1.5
Early 5th instar	12	0	4	0	7	1	0	2.4
5th	19	4	9	3	3	0	0	1.3
Late 5th	13	2	2	6	3	0	0	1.8
1st day spinning	19	2	15	2	0	0	0	1.0
2nd day spinning	12	2	10	0	0	0	0	0.8
Finished spinning	7	0	6	2	0	0	0	1.1
Early prepupa	10	4	6	0	0	0	0	0.6
Fresh pupa	10	10	0	0	0	0	0	0
Unchilled pupa	7	7	0	0	0	0	0	0
Chilled pupa	29	29	0	0	0	0	0	0
1–2 day adult development	5	5	0	0	0	0	0	0
9–11 day	6	6	0	0	0	0	0	0
14th day	18	13	1	2	2	0	0	0.6
17th day	4	3	1	0	0	0	0	0.25
Adult (freshly emerged)	24	0	0	2	5	12	5	3.8

* For definition, see text.

metamorphosis of the animal as a whole (for review see Pflugfelder, 1958). And since four glands were routinely implanted into each test pupa, the assay did not compensate for the changing mass of endocrine tissue. What the pupal assay recorded was the endocrine activity of the four implants, irrespective of size, rather than the activity per unit mass of endocrine organ.

Over a period of several years a total of 131 assays were performed on larval corpora allata. In the results tabulated in Table II, a certain scatter is observed in the activity recorded at each stage. In order to derive an overall index of activity

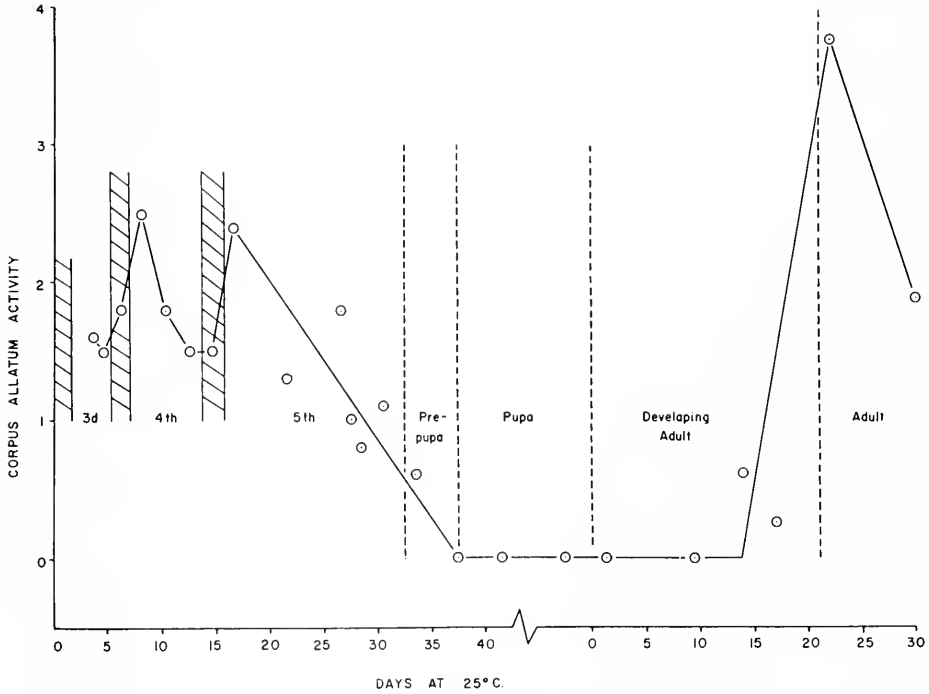

FIGURE 1. Changes in the endocrine activity of the corpora allata of the Cecropia silkworm during the third, fourth, and fifth instars of larval life and during metamorphosis. The average indices of corpus allatum activity (derived from "pupal assays") are plotted as a function of the stage of development of the donor animals. The discontinuity in the x-axis signals the storage of donor pupae at 6° C. for 10 to 20 weeks; the cross-hatched zones correspond to the periods of larval molting.

at each stage, a zero response was counted as zero, a one response as one, a two as two, and so on. The total was then divided by the number of experimental animals to yield an "average index" of corpus allatum activity at each stage.

In the plot of these average indices in Figure 1, attention is first directed to what appears to be a cycle of corpus allatum activity during the third and fourth larval instars. Their endocrine activity is apparently minimal just prior to the molt, maximal shortly after the molt, and subject to a steady decline during the instar itself. As illustrated in Figure 1, this decline is particularly striking during the final (fifth) larval instar.

Late in the fifth instar, when the activity of the corpora allata has declined to a critically low level, a dramatic change occurs in the silkworm's behavior. It ceases to feed, empties its gut, and then begins a two-day period of spinning. This impressive change in behavior is the earliest sign of approaching pupation.

B. Prepupal corpora allata

The onset of the prepupal stage is signaled by a detachment and retraction of the epidermis from the larval cuticle and by the initiation of the cytological events which, during a period of five days, transform the larva into a pupa. In Figure 1 we note the surprising finding that the corpora allata show a low but definite activity during the prepupal period—a result which will be considered below in further detail.

C. Pupal corpora allata

The decline in corpus allatum activity continues during the prepupal period. Then, immediately after the pupal ecdysis, the glands for the first time are found to be completely inactive.

As illustrated in Figure 1, the inactivity of pupal corpora allata persists during the entire pupal period, irrespective of whether the pupae are stored at high or low temperatures. In my entire series of assays I have never obtained a positive test for the pupal corpora allata of Cecropia, Polyphemus, Cynthia, or any other lepidopteran. In a univoltine species such as Cecropia, the pupal stage extends from midsummer until the following spring. So, during a period of about eight months, the corpora allata are apparently inactive.

D. Corpora allata during adult development.

Adult development, once initiated, requires three weeks at 25° C. The corpora allata continue to be inactive during the first two weeks of this period. On the fourteenth day of development one begins to record renewed activity in the glands of a certain proportion of individuals (Table II and Fig. 1). Within the final week of adult development the corpora allata recover their endocrine activity. Indeed, in the pupal assay the glands of freshly emerged male or female moths show higher activity than at any other stage in the life history.

In the absence of functional mouth-parts, the Cecropia moth lives for only 7 to 10 days at 25° C. During this brief period the glands of most individuals undergo a marked decline in activity to a level approximately one-half of that a week earlier.

3. *The role of juvenile hormone in pupation*

As noted in Section 2B, the corpora allata show a low but definite activity during the prepupal period. This is an extremely surprising result since the prepupal stage is distinguished by metamorphic changes on an unprecedented scale. The experiments, about to be described, were designed to test the physiological significance of the low concentration of juvenile hormone that is apparently present during the prepupal period.

In an initial series of experiments performed in collaboration with Dr. William H. Telfer, the corpora allata were excised from a group of eight Cecropia larvae early in the fifth instar. The loss of the glands seemed inconsequential. Feeding and growth continued and two weeks later the animals spun normal cocoons. But, within the following ten days, only two of the eight animals transformed into normal pupae. The other six formed strange creatures in which a considerable number of tissues and organs had overleaped the pupal stage by undergoing precocious adult differentiation.

One of these animals is illustrated in Figure 2. The head shows the pigmented, faceted, compound eyes of the adult. The antennae exhibit the segmentation and

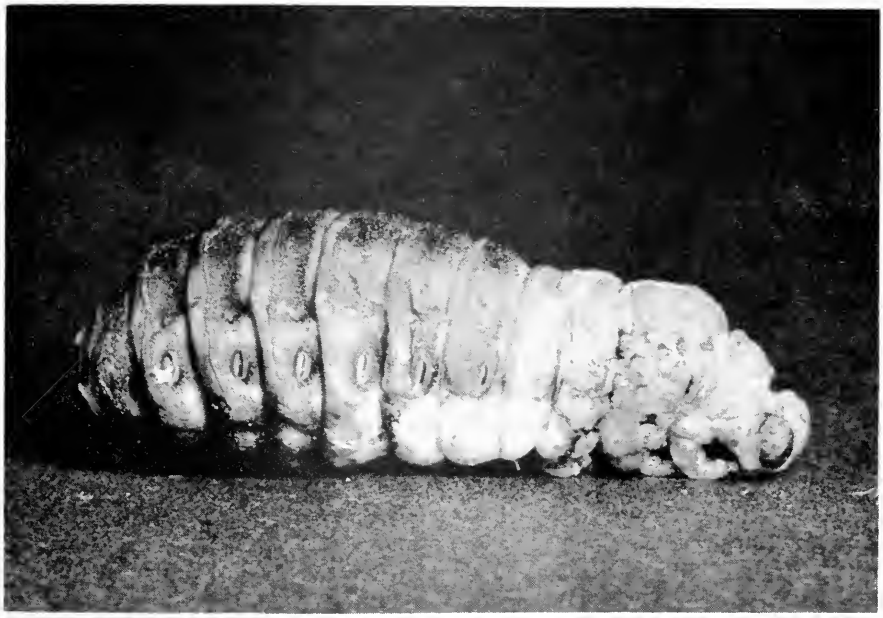

FIGURE 2. By excision of the corpora allata during the final larval instar, this animal was caused to undergo pupation in the absence of juvenile hormone. A large number of larval tissues and organs have undergone precocious adult differentiation without traversing the pupal stage.

subdivisions characteristic of early adult development. The thorax is covered with a mixture of rugose pupal cuticle and smooth cuticle of the adult type. The adult patagia have developed at the base of the fore-wings. The sclerotization of the thoracic tergum is adultoid. The thoracic pleura and sternum are covered for the most part by a smooth, adult-type cuticle. The legs show segmentation and the differentiation of tarsal claws and pulvilli. The proximal ends of the wings are covered by adult cuticle. The cuticle of the abdomen is wholly pupal, except in the immediate region of the genitalia; the latter are represented, not by imaginal discs, but by miniature adult genitalia which show an early elaboration of the various valves and adult structures. Dissection revealed that the fat-body was similar to that of a pupa after the initiation of adult development. Moreover, the ovaries

TABLE III

Effects of removal of corpora allata from fifth instar Cecropia larvae

State at time of operation	No. of animals	Results	
		Normal pupa	Pupal-adult
Young 5th instar	3	0	3
Mid 5th instar	9	5	4
Late 5th instar	5	2	3
Spinning	13	12	1

showed the differentiation of ovarioles to a stage corresponding to that encountered in early adult development.

The experiment was repeated on a larger scale utilizing animals at successive stages in the fifth instar. The results in Table III record the production of pupal-adult mixtures in all three animals from which the corpora allata were extirpated early in the fifth instar. Excision in the mid or late fifth instar gave rise to mixed forms in about half the animals. And when the operation was further postponed to the period of spinning, only one of thirteen animals developed into a mixed form.

These findings suggest that the juvenile hormone plays a definite role in the endocrine control of pupation. Evidently, the low concentration of juvenile hormone in the mature larva and prepupa is necessary to prevent the precocious adult differentiation of larval tissues and organs.

4. *Precocious adult differentiation of larval wing-discs*

Wing-discs were excised from fourth and fifth instar Cecropia larvae and implanted into previously chilled Cynthia pupae just before the latter initiated adult development. In this manner the discs were exposed to the ecdyson of the developing host under conditions where juvenile hormone was absent. After the hosts had completed adult development, the implants were recovered and subjected to detailed examination.

As is true of implants of all epidermal tissues, the implants took the form of cyst-like structures with the integumentary surface facing inward. Each cyst was cut open and a record made as to whether it had formed a rugose pupal cuticle or a smooth, scale-covered cuticle of the adult type.

TABLE IV

Metamorphosis of larval wing-discs implanted into previously chilled pupae

Initial status of discs	No. of discs	No. of hosts	Differentiation of implants		
			Pupal	Pupal-adult	Adult
4th instar	20	10	5	8	7
Early 5th	2	2	0	2	0
Late 5th	9	4	4	1	5
Spinning 5th	4	2	0	4	0

The results are summarized in Table IV. Of the 35 wing-discs that were studied, 12 showed adult cuticle, 9 showed pupal cuticle, and 15 showed a mixture of pupal and adult cuticle. Certain cysts showed the differentiation of the articular sclerites distinctive of the adult wings.

It is noteworthy that precocious adult differentiation was readily obtained in wing-discs as early as the fourth instar. Consequently, when exposed to ecdyson in the absence of juvenile hormone, these implants omitted the fifth larval instar as well as the entire pupal stage. Here again we see that a certain low concentration of juvenile hormone is prerequisite for the transformation of a Cecropia larva into a normal pupa.

These results are reminiscent of Schaller's (1952) finding that larval honeybees undergo precocious adult development when decapitated prior to pupation. Moreover, Nayar (1954) has reported that pieces of larval integument undergo precocious adult differentiation when transplanted to pupae of *Ephestia*, *Galleria*, or *Pieris*. Here again we see that in many species a certain low concentration of juvenile hormone is necessary for the normal transformation of a larva into a pupa. This fact has long been suspected (Piepho, 1951; Wigglesworth, 1954, 1959; Schneiderman and Gilbert, 1959; Novák and Červenková, 1960), but is documented for the first time in the present investigation.

Discussion

1. *Juvenile hormone and adult development*

The experimental results direct attention to a prolonged period during which the corpora allata appear to be totally inactive in the secretion of juvenile hormone. This period begins immediately after pupation and continues throughout the entire pupal stage. Since the prothoracic glands are also inactive at this same time (Williams, 1952b) the pupal stage is characterized by subthreshold titers of both ecdyson and juvenile hormone.

After eight months of pupal diapause, the prothoracic glands recover their endocrine activity. Ecdyson is secreted and adult development begins. Meanwhile, the corpora allata continue to be inactive—a condition which persists throughout the first two-thirds of adult development.

The pupal assay derives its sensitivity from the fact that the early phase of adult development takes place only if juvenile hormone is absent. If the hormone is supplied by the implantation of active corpora allata, the net result is to block adult differentiation and to promote the formation of a second pupal instar. In effect, we duplicate the endocrine conditions peculiar to larval life in that ecdyson and juvenile hormone are caused to act side-by-side. The pupa molts into a further pupal instar just as, under the same circumstances, the larva molts to a further larval instar.

2. *Juvenile hormone and pupation*

The endocrine stimulus for pupation is the action of ecdyson in the presence of a low but finite titer of juvenile hormone. If the corpora allata are excised so that the larva approaches pupation in the absence of juvenile hormone, then a large number of larval tissues overleap the pupal stage and undergo precocious adult develop-

ment. The same result was observed when larval wing-discs were subjected to *in vivo* culture in the presence of ecdyson and the absence of juvenile hormone. Here again we see that the presence of a low concentration of juvenile hormone at the time of pupation serves as a brake on the precocious acting-out of the life-plan.

3. *Juvenile hormone and larval development*

According to the pupal assays, the corpora allata undergo a cycle of endocrine activity during successive larval instars. If the glands are extirpated so that an immature larva approaches a larval molt in the absence of juvenile hormone, the result, once again, is precocious metamorphosis in response to ecdyson (Bounhiol, 1938; Piepho, 1943; Fukuda, 1944; Williams, 1946).

In Figure 1 it is of particular interest to note that the corpora allata show a marked decline in activity during the intermolt period and are minimally active just prior to the molt itself. By virtue of the declining titer of juvenile hormone, one would anticipate some measure of progressive differentiation between successive larval instars.

This inference is confirmed by the striking heteromorphic changes (Snodgrass, 1954) which the Cecropia silkworm undergoes during the larval period. Thus, the first instar is jet black and covered with spinose tubercles; the second instar is yellow with black tubercles and spots; the third instar is greenish yellow with black spots and red, blue, and yellow spinose tubercles; the fourth instar is bluish green with pairs of red, yellow, and blue spinose tubercles; the fifth instar is bright green, shows distinctive red and yellow tubercles on the thoracic segments and a considerable reduction in the spines on all tubercles. In summary, each larval instar shows distinctive characteristics which, irrespective of the insect's overall size, permit one to recognize the individual instars with ease. It is no exaggeration to say that the magnitude of these changes between successive larval instars equals or exceeds those commonly encountered in hemimetabolous insects at the time of metamorphosis.

If larval heteromorphosis is attributable to the declining titer of juvenile hormone prior to the larval molts, then, by augmenting the concentration of juvenile hormone, one at least in theory should be able to obtain fifth stage larvae which retain the form of the first stage. Such experiments have not been performed. However, according to the unpublished observations of Dr. Judith Willis, the Cecropia silkworm, under certain environmental conditions, repeats the second, third, or fourth larval instar without change in form. Presumably, in all these cases, the corpora allata fail to undergo the normal decline in activity prior to the extra molt.

As pointed out by Snodgrass (1954), spectacular degrees of larval heteromorphosis are frequently encountered among predatory or parasitic species of insects, examples being known among the Neuroptera, Coleoptera, Strepsiptera, Lepidoptera, Hymenoptera, and Diptera. Evidently, the heteromorphic larval molts are here preceded by an even greater decline in corpus allatum activity than that encountered in the Cecropia silkworm.

4. *Theory of juvenile hormone action*

A comprehensive theory of the action of juvenile hormone must account for the following findings: (1) Ecdyson is a potent growth hormone either in the presence

or absence of juvenile hormone. (2) In all of the phenomena here under consideration, juvenile hormone is active only in the presence of ecdyson. (3) When juvenile hormone is absent, ecdyson promotes, not only the synthetic acts prerequisite for growth, but also the new synthetic acts that are necessary for metamorphosis. (4) When ecdyson acts in the presence of juvenile hormone, growth continues, but new synthetic acts are blocked to a degree proportional to the concentration of juvenile hormone.

From this summary we learn that the role of juvenile hormone is to modify the cellular reactions to ecdyson. It appears to do so by opposing progressive differentiation without interfering with growth and molting in an unchanging state. In some unknown manner, it blocks the de-repression and de-coding of fresh genetic "information" without interfering with the acting-out of information already at the disposal of the cells.

5. *The cytological actions of juvenile hormone*

Though we are unable to state how juvenile hormone controls the flow of fresh genetic information, certain clues may be derived from the data already at hand. Thus, in the case of the pupal-adult transformation, it is clear that the events which are sensitive to juvenile hormone take place at the very outset of adult development. So, if the implantation of corpora allata is delayed until the fifth day of the twenty-one days of adult development, it is already too late for juvenile hormone to have any effect. To be maximally effective, juvenile hormone must be present during the initiation of adult development; *i.e.*, during precisely the period when ecdyson is secreted by the prothoracic glands and acts throughout the pupa.

Evidently, the targets of juvenile hormone are certain very early events, including mitotic divisions, which are the normal cellular reactions to ecdyson at the outset of adult development. Whatever these cellular or subcellular events may be, we can state that they occur early, that they show a rapid loss of sensitivity to juvenile hormone, and that, if unopposed by juvenile hormone, they commit the cells to developmental reactions accompanied by metamorphosis.

It is worth recalling that, even at the outset of adult development, the various pupal tissues show a great range of sensitivities to juvenile hormone (Table I). Consequently, a cytological and cytochemical comparison between tissues of high and low sensitivity may yield additional information as to the mode of action of juvenile hormone. This approach seems particularly cogent in the case of the pupal epidermis whose sensitivity to juvenile hormone is vastly amplified at the site of an integumentary injury.

Summary

1. By means of a standardized "pupal assay" for juvenile hormone, the endocrine activity of the corpora allata was found to undergo large and systematic changes during the postembryonic development of the Cecropia silkworm.

2. In each of the larval instars that were studied, the glands are least active just prior to the larval molt and most active shortly after the molt. Larval molting therefore appears to take place in the presence of a declining titer of juvenile hormone—an endocrine situation which apparently permits the striking changes in

morphology and pigmentation which occur in successive larval stages of the Cecropia silkworm.

3. The corpora allata show low but definite activity at the time of pupation. If the glands are extirpated so that pupation occurs in the absence of juvenile hormone, many larval tissues overleap the pupal stage and undergo precocious adult differentiation. Therefore, the low concentration of juvenile hormone in the mature larva plays a definite role in the endocrine control of pupation.

4. The corpora allata are inactive throughout the entire pupal stage. This inactivity persists during the first two-thirds of adult development. During the final week of adult development, the glands recover their endocrine function and are maximally active by the time of emergence of the adult moth.

5. The absence of juvenile hormone proves to be an obligatory feature of the initial phase of adult differentiation. If active corpora allata are implanted into a pupa so that adult development is initiated in the presence of juvenile hormone, the pupa develops and molts into a creature which is a mixture of pupa and adult. The higher the titer of juvenile hormone, the more extensive is the preservation of pupal characters.

6. Juvenile hormone is effective in blocking adult differentiation only when it is present during the first five days of adult development. Consequently, the target of juvenile hormone appears to be certain early events which are the normal reactions to ecdyson at the outset of adult development. If unopposed by juvenile hormone, these events commit the cells to developmental reactions accompanied by metamorphosis.

7. A comprehensive theory is presented for the action of juvenile hormone in the Cecropia silkworm. According to this theory, juvenile hormone modifies the cellular reactions to ecdyson by opposing the de-repression, de-coding or acting-out of fresh genetic information prerequisite for progressive differentiation but not prerequisite for growth in an unchanging state.

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