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PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

VOLUME 83

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THE MARINE BIOLOGICAL LABORATORY

FORTY-FOURTH REPORT, FOR THE YEAR 1941—
FIFTY-FOURTH YEAR

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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 Mims, 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 annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 11.30 A.M., daylight saving time, in each year, 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. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio*, who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeritus, who shall be elected from the 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 Emeritus shall have all 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.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. 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 said 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.

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

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; 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. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have 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.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. 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.

IX. 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.

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

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1941.

The accounts have been audited by Messrs. Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

At the end of the year 1941, the book value of the Endowment Fund in the hands of the Central Hanover Bank and Trust Company, as

Trustee, was

General Fund,	Securities (Market $749,361.44)	$803,267.92
	Real Estate	74,835.98
	Cash	2,719.28
		$880,823.18
Library Fund,	Securities (Market $152,425.11)	$169,425.16
	Real Estate	20,000.00
	Cash	258.05
		$189,683.21

The income collected from these funds during the year was:

General Endowment	$32,306.81
Library	6,572.92
	$38,879.73

The income in arrears on these funds at the end of the year was:

General Fund	$ 6,300.00
Library	$ 5,231.00
	$11,531.00
Last year	$16,578.69

This reduction does not represent the payment of back interest, $6,750, being accounted for by interest written off on a foreclosure of a mortgage.

General Biological Supply House, Inc.: The dividends from General Biological Supply House, Inc. totalled $17,780, a decrease of $762 from 1940.

Bar Neck Property: The rental from the Bar Neck property which is based on the net profit of the garage was $4,719.28.

Retirement Fund: A total of $3,460 was paid in pensions. This fund at the end of the year consisted of:

Participations in mortgages	$ 7,464.99
Interest in Real Estate	2,301.88
Cash	5,046.91
	$14,813.78

Plant Assets: The land (exclusive of Gansett and Devil's Lane Tracts), the buildings, equipment and library represent an investment of $1,926,531.07
less reserve for depreciation 582,468.84
or a net of $1,344,062.23

Income and Expenses: The expenses including Reserve for Depreciation of $25,603.89 exceeded income by $9,482.01. There was expended from current funds for plant account a net of $16,248.50 and $1,748.86 net, was added to the Reserve Fund.

At the end of the year the Laboratory had no indebtedness on notes or mortgages. It owed on accounts payable $6,423.47, against which it had accounts receivable of $14,585.69 and cash in its general bank accounts of $10,619.85.

$45,623.38 was received during the year from the Rockefeller Foundation for the building of the library annex which completed its grant of $110,400, and there was expended during the year $67,714.81, which with the expenditures of 1940 brought the total cost of the building at the end of the year to $107,565.93, leaving a balance in the Building Fund account of $2,834.07.

$25,000 was received from The Carnegie Corporation of New York for the purchase of books and back sets and the improvement of the library.

Following is the balance sheet, the condensed statement of income and outgo and the surplus account all as set out by the auditors.

EXHIBIT A

Marine Biological Laboratory Balance Sheet
December 31, 1941

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee —Schedules I-a and I-b		$1,070,506.39	
Securities and Cash—Minor Funds—Schedule II		9,305.68	$1,079,812.07
Plant Assets:			
Land—Schedule IV	$ 111,425.38		
Buildings—Schedule IV	1,319,364.52		
Equipment—Schedule IV	185,610.60		
Library—Schedule IV	310,130.57		
	$1,926,531.07		
Less Reserve for Depreciation	582,468.84	$1,344,062.23	
Cash in Building Fund		2,834.07	
Cash in Reserve Fund		4,273.51	
Cash in Book Fund		25,000.00	$1,376,169.81

Current Assets:			
Cash		$ 10,619.85	
Accounts-Receivable		14,585.69	
Inventories:			
Supply Department	$ 38,658.35		
Biological Bulletin	12,228.56	50,886.91	
Investments:			
Devil's Lane Property	$ 45,375.48		
Gansett Property	998.70		
Stock in General Biological Supply House, Inc.	12,700.00		
Other Investment Stocks	17,770.00		
Securities, Real Estate, and Cash —Retiremend Fund—Schedule V	14,813.78	91,657.96	
Prepaid Insurance		3,761.21	
Items in Suspense		159.09	$ 171,670.71
Liabilities			
Endowment Funds:			
Endowment Funds—Schedule III	$1,068,780.68		
Reserve for Amortization of Bond Premiums	1,725.71	$1,070,506.39	
Minor Funds—Schedule III		9,305.68	$1,079,812.07
Plant Liabilities and Surplus:			
Donations and Gifts—Schedule III		$1,175,290.11	
Other Investments in Plant from Gifts and Current Funds		200,879.70	$1,376,169.81
Current Liabilities and Surplus:			
Accounts—Payable		$ 6,423.47	
Current Surplus—Exhibit C		165,247.24	$ 171,670.71

EXHIBIT B

Marine Biological Laboratory Income and Expense, Year Ended December 31, 1941

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 32,306.81		$ 32,306.81
Library Fund		6,572.92		6,572.92
Donations		100.00		100.00
Instruction	$ 9,593.55	9,770.00		176.45
Research	6,130.85	14,737.50		8,606.65
Evening Lectures	108.48		$ 108.48	
Biological Bulletin and Membership Dues	8,232.34	9,625.18		1,392.84

Supply Department—Schedule VI ..	33,600.35	30,527.78	3,072.57	
Mess—Schedule VII	23,502.23	21,460.17	2,042.06	
Dormitories—Schedule VIII	23,994.53	12,417.10	11,577.43	
(Interest and Depreciation charged to above 3 Departments—See Schedules VI, VII, and VIII)	24,117.65			24,117.65
Dividends, General Biological Supply House, Inc.		17,780.00		17,780.00
Dividends, Crane Company		500.00		500.00
Rents,				
Bar Neck Property		4,719.28		4,719.28
Janitor House	115.61	360.00		244.39
Danchakoff Cottages	367.13	700.00		332.87
Sale of Library Duplicates		87.43		87.43
Apparatus Rental		925.38		925.38
Sundry Income		157.15		157.15
Maintenance of Plant:				
Buildings and Grounds	25,221.68		25,221.68	
Chemical and Special Apparatus Expense	16,772.95		16,772.95	
Library Expense	7,776.09		7,776.09	
Workmen's Compensation Insurance	528.09		528.09	
Truck Expense	497.11		497.11	
Bay Shore Property	126.10		126.10	
Great Cedar Swamp	158.88		158.88	
General Expenses:				
Administration Expense	12,295.53		12,295.53	
Endowment Fund Trustee and Safe-keeping	1,014.45		1,014.45	
Bad Debts	706.52		706.52	
Reserve for Depreciation	25,603.89		25,603.89	
	$172,228.71	$162,746.70	$107,501.83	$ 98,019.82
Excess of Expense over Income carried to Current Surplus—Exhibit C		9,482.01		9,482.01
		$172,228.71		$107,501.83

EXHIBIT C

Marine Biological Laboratory, Current Surplus Account
Year Ended December 31, 1941

Balance, January 1, 1941		$172,107.36
Add:		
Reserve for Depreciation Charged to Plant Funds	$25,603.89	
Bad Debt Recovered	6.00	
Gain on Gansett Lots Sold	450.88	26,060.77
		$198,168.13

Deduct:			
Excess of Expense over Income for Year as shown in Exhibit B		$ 9,482.01	
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,			
Buildings	$ 4,167.02		
Equipment	3,298.84		
Library	9,188.24		
	$16,654.10		
Less Received for Plant Assets Disposed of	405.60	16,248.50	
Transfer to Plant Reserve Fund		3,977.18	
Pensions Paid	$ 3,460.00		
Less Retirement Fund Income	246.80	3,213.20	$ 32,920.89
Balance, December 31, 1941—Exhibit A			$165,247.24

Respectfully submitted,

LAWRASON RIGGS,
Treasurer.

V. REPORT OF THE LIBRARIAN

The appropriation to the Library for 1941 of $18,850.00 was expended as follows: books, $765.45; serials, $2739.53; binding, $1177.92; express, $90.60; supplies, $509.51; salaries, $7200.00; back sets, $3717.83; and sundries, $73.11, leaving a balance of $2663.48 (counting the duplicate sales which amounted to $87.43). From the sum held in reserve from the 1940 appropriation (see report of 1940) $2228.32 was used leaving a balance of $1748.86.

In December 1941 the gift of $25,000 from the Carnegie Corporation was appropriated to the Marine Biological Laboratory for Library development. This sum will not be subject to reversion to source and will be available whenever the foreign market for purchases is again open. In the meantime purchases are restricted to this country and to England where the rare sets we need are practically unavailable.

The Woods Hole Oceanographic Institution appropriated $800.00 to the Library for 1941 and a balance of $133.75 remained from the 1940 budget. An expended sum of $779.10 has been reported to the Director.

The Library receives currently 1297 serial publications: 409 (12 new) subscriptions to the Marine Biological Laboratory, 40 (6 new) to the Woods Hole Oceanographic Institution; 630 exchanges, 558 (13 new) with "The Biological Bulletin" and 72 (2 new) with the Woods Hole Oceanographic Institution publications; 210 gifts to the former institution and 8 to the latter. The Marine Biological Laboratory acquired 222 books: 86 by purchase of the Marine Biological Laboratory,

23 by purchase of the Woods Hole Oceanographic Institution, 7 presented by Dr. F. B. Hanson, 12 gifts from the authors, 58 gifts of publishers, 5 acquired by duplicate exchange, and 31 by miscellaneous donors. There were 27 back sets of serial publications completed: by purchase of the Marine Biological Laboratory, 13, by the Woods Hole Oceanographic Institution, 3, by exchange with "The Biological Bulletin," 5, by exchange of duplicates, 2, by gift, 4. Partially completed sets were 94: by purchase of the Marine Biological Laboratory, 12, of the Woods Hole Oceanographic Institution, 2, by exchange with "The Biological Bulletin," 5, with the Woods Hole Oceanographic Institution, 1, by exchange of duplicates, 34, and by gift, 40. Reprint additions number 3321: current of 1940, 1250; current of 1941, 552; and of previous dates, 1519. The present holdings of the Library are 49,179 bound volumes and 119,626 reprints.

The gifts of reprint collections have been very generous in the past two years. In 1940 Mrs. Metcalf presented 100 reprints and 2 books to be added to the Metcalf collection already here. Only 60 of these were duplicates. Dr. R. P. Bigelow gave us 1400 reprints, 450 of which were new for us. The Director of the Museum of Comparative Zoology presented a reprint of the "Origin and progress of the Anderson School of Natural History, Penikese Island, 1874." In 1941, Dr. Frank R. Lillie presented 29 reprints (4 not duplicated); Dr. Libbie Hyman, 270 (95 not duplicated); Dr. S. Wing Handford, 181 (72 not duplicated); Dr. E. W. Gudger, 23 (8 not duplicated); Dr. G. S. Dodds, 577 (192 not duplicated); Dr. Robert Chambers, 685 (221 not duplicated); Dr. G. N. Calkins, 750 (210 not duplicated); Dr. R. P. Bigelow, 3017 (873 not duplicated).

It seems not out of place at this date of sending in the 1941 report (March, 1942) to give important information that properly belongs in the 1942 report of the Library. A gift of a complete microfilming outfit has been presented and installed, and instruction given to a member of the library staff. Dr. Atherton Seidell of "Medicofilm Service" and the "Current List of Medical Literature" who conducts this service under the auspices of the "Friends of the Army Medical Library" not only presented the apparatus, one of his own construction, but brought the equipment in person and spent four days at the Library to set it up and to train a member of the staff so that she is able to carry on the work at once. In the near future an announcement of the service and a list of the journals available in this Library will be issued but in the meantime the acknowledgment of Dr. Seidell's generous gift will help to make known to our investigators this new service of the Library.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I present herewith a report of the fifty-fourth session of the Marine Biological Laboratory for the year 1941, with comments on matters which affect the session of 1942.

The war has brought about changes which have already profoundly affected the Laboratory and which will undoubtedly affect it still more in the future. We are faced with a marked decrease in attendance, a very considerable loss in income, and with the difficulty of obtaining supplies for research and for the upkeep of our buildings. But the fine spirit shown by our workers, both investigators and members of the permanent staff, is ample evidence that the Laboratory can adapt itself to its altered environment, and will continue to serve the cause of biological research and instruction.

1. *Attendance.* We shall miss many of our regular attendants who must remain at home to take their part in the accelerated teaching program, to engage in research problems suggested by the Defense Council, or to carry on other work directly connected with the war effort. A considerable number of students who had anticipated entering our courses will continue their studies in the summer sessions of their colleges. Others will enter military service. Many are prevented from coming to Woods Hole by the rubber and gas shortage. As long as the war continues we must expect further declines in attendance.

2. *Income.* The prospect of a sharply reduced income in 1942 is a matter of grave concern. Although our income from all sources has been decreasing steadily for some time and was expected to drop still further, we did not anticipate the very considerable loss with which we are now faced. This loss is due in part to the decreased attendance resulting in smaller returns from investigators' and students' fees, and from the dormitories and the Mess. Interest on the Endowment Funds has grown smaller, and the return from other investments is less. It is estimated that the total income for 1942 may be $20,000 less than that of 1941.

3. *The Budget.* This situation forced the Executive Committee to revise the budget provisionally adopted in September 1941. Appropriations for the various departments have been greatly curtailed. The Library is provided with only enough to pay for the subscriptions to all journals currently received, and for binding. Very little is available for the purchase of back sets. The sum usually reserved for new apparatus

has also been cut, on the assumption that our present supplies are adequate for the time being. It should be noted that it is extremely difficult to buy new apparatus under any conditions. Reductions have also been made in the summer Collecting Crew, in the Chemical Room staff, and in the janitorial service. These changes should not materially affect actual research, but may cause occasional minor delays.

4. *Courses of Instruction.* It was evident to the Committee that the number of students who would apply for entrance to the courses in 1942 would probably be small, and that instruction could be maintained only at a considerable loss unless the expense, that is, the instructors' salaries, could be reduced. The members of the teaching staff were therefore asked if they would be willing to accept a 50 per cent cut, provided a total of only 50 students was admitted. The response to this unwelcome proposition was most heartening, the opinion being expressed that the courses should not be allowed to lapse even for a single summer. The Corporation is deeply indebted to the teaching staff for their willingness to carry on this fundamental part of our program at the risk of financial sacrifice.

5. *Use of Buildings by the Navy.* During the winter we received assurances that none of our buildings would be required by the rapidly expanding Coast Guard stationed at Woods Hole. But late in the spring the Navy Department asked for the exclusive use of the Mess, Homestead, Lecture Hall, and Botany Building. Subsequently the Apartment House was added to the list. These buildings will be occupied for the duration of the war. There was, of course, no hesitation on the part of the Committee in granting this request. To give up the buildings is a contribution to the war effort which we gladly make. The Lecture Hall and Botany could readily be relinquished for the work usually carried on in them can be transferred to other buildings which will not be fully occupied. More disturbing is the loss of the Mess and Apartment House. We are fortunate, however, in securing the Avery Hotel as a Mess. Although its kitchen facilities are not as complete nor as convenient as our own, they can be made to serve satisfactorily under the competent management of Miss Downing, our newly appointed matron.

6. *Gifts.* The Laboratory is deeply indebted to the Carnegie Corporation of New York for its gift of $25,000 to be used for the purchase of back sets of journals, of volumes to fill gaps in our present files of serials, and of classics in biology and allied sciences. By virtue of this timely contribution we are now able to satisfy the terms under which the Rockefeller Foundation granted us funds for the erection of the new wing of the Library. While it is difficult, under present conditions, to make rapid progress in completing our files, we are now assured that

whenever the needed volumes appear in the market, we can secure them. The value of the Library will be greatly enhanced by this gift.

Its usefulness is still further increased by the generosity of Mr. Atherton Seidell of the National Bureau of Health. Mr. Seidell, who has long been active in extending the use of microfilms, presented to the Laboratory a complete photographic and developing outfit for making these films, and helped to install it in one of the dark rooms in the basement of the new wing. He also provided us with a reading instrument. The Library is thus in a position to render valuable service to investigators who cannot secure the references they want without making a journey to some large institution. We are grateful to Mr. Seidell for his extremely useful gift.

7. *The Apparatus Department.* Last fall Dr. S. E. Pond resigned after serving nearly ten years as Technical Manager in charge of Chemicals and Scientific Apparatus, during which time he had greatly expanded the usefulness of his department. The Apparatus Committee, faced with the difficult task of finding a man to replace him, decided to divide the work into two sections, each under the direction of a part-time manager. Dr. E. P. Little, who has had supervision over the X-ray and photographic rooms, was put in charge of the apparatus, and Mr. K. S. Ballard, long a member of the Chemical Room staff, was put in charge of the chemicals and glassware. Dr. Little, with Dr. Sichel as consultant, takes up his work at a time when new apparatus is hard to come by, and when the present stocks must be made to serve—a difficult task but one in which we are confident he will succeed.

8. *Other Items.* Among various items of interest that may be mentioned are:

(*a*) The increase in the price of board at the Mess to $8.00 per week from $7.00, a rate which until now has stood unchanged for more than thirty years. The change was made necessary because last year the Mess was run at a loss.

(*b*) The construction of a new salt water intake pier, replacing the old one which was in imminent danger of collapse. A constant supply of sea water is the most essential part of our equipment for without it practically all research here would come to an end in a few hours. Fortunately, the work was begun in the fall when the necessary materials could still be secured.

(*c*) The sale of ten lots in the Gansett tract. At present only four parcels in this property remain unsold. The Devil's Lane tract, on the Shore Road to Falmouth, will provide space for many of our investigators and must be opened up as soon as there is a sufficient demand.

9. *Election of Officers and Trustees.* At the meeting of the Corporation held August 1, 1941 the following Trustee was elected Trustee Emeritus:

W. J. V. Osterhout, Rockefeller Institute

The new Trustees elected at that meeting were:

G. H. A. Clowes, Class of 1943
Columbus Iselin, Class of 1944

10. There are appended as parts of this report:

1. Memorials of deceased Trustees.
2. The Staff, 1941.
3. Investigators and Students, 1941.
4. A Tabular View of Attendance, 1937–1941.
5. Subscribing and Co-operating Institutions, 1941.
6. Evening Lectures, 1941.
7. Shorter Scientific Papers, 1941.
8. The General Scientific Meeting, 1941.
9. Members of the Corporation, 1941.

Respectfully submitted,

CHARLES PACKARD,
Director.

1. MEMORIALS OF DECEASED TRUSTEES

MEMORIAL TO DR. E. B. MEIGS

BY DR. R. S. LILLIE

Edward Browning Meigs was born in Philadelphia September 10, 1879, the son of Arthur Vincent Meigs and Mary Roberts Browning. He belonged to an old and distinguished American family of South English ancestry, and was a direct descendant of Vincent Meigs who came to America and settled in New Haven, Connecticut, in 1644. His father, grandfather, and great-grandfather were physicians; his great-great-grandfather, Josiah Meigs, was Professor of Mathematics and Natural Philosophy in Yale University in the 1790's. Scientific interests were strong in his ancestry. His father, a pediatrician, was interested in the chemical composition of milk and published papers on this subject; he also introduced a method of modifying cows' milk to make it suitable for infants. In Edward Meigs' memoir of his father he "finds it difficult to say whether more of my father's energy was devoted to the practice of medicine or to research."

Edward Meigs was the fourth physician in his family in the direct line, but his own interests were primarily scientific and he did not engage in practice. He graduated from Princeton University in 1900 and took his M.D. at the University of Pennsylvania in 1904. He was Assistant in Physiology in the same University during 1904–1906 and then spent a year abroad in study and research, working chiefly in Jena with the comparative physiologist, Wilhelm Biedermann. He also spent some time in Cambridge University, chiefly in association with Walter Fletcher and Gowland Hopkins, whose work on the physiology and biochemistry of muscular contraction was of special interest to him. He was instructor in Physiology in the Harvard Medical School during 1907–10. In 1910 he joined the Wistar Institute in Philadelphia in order to devote himself entirely to research. From 1915 until his death he was physiologist in the Bureau of Dairy Industry of the U. S. Department of Agriculture.

He first attended the Marine Biological Laboratory in 1904, and was elected a member of the Corporation in 1905. During the four summers of 1912–1915 he served as instructor in the Physiology course. He and his family have been summer residents of Woods Hole for a period of about thirty years, and his interest in the Laboratory has been constant.

In 1910 he married Margaret Wister of Philadelphia who with his two sons and two daughters survives him. He died November 5, 1940, after a prolonged illness.

Edward Meigs' early investigations were in the field of general physiology, especially the physiology of muscular contraction. Later, after he went to Washington, his work had reference chiefly to the physiology of milk production and related topics; problems connected with administration and the organization of research in this field also engaged much of his attention.

His early studies on the comparative histology and biochemistry of smooth and striated muscle, both vertebrate and invertebrate, were varied and extensive. His photographs of striated muscle fibres under high magnification are among the best that we have. His belief that changes of tension in muscle were a result of reversible changes of hydration in the fibrils led him to experiment on the influence of variations of osmotic pressure, chemical conditions and temperature on the water content and correlated state of contraction of different types of muscle. At one time he was greatly interested in physical models of muscular contraction, especially McDougall's model, in which increase of volume of inflation resulted in shortening. His interest in the relation of inorganic salts to contraction (as shown, e.g., in the potassium contraction of striated

muscle) led him to make comparative analytical studies of the salt content of smooth and striated muscle, vertebrate and invertebrate. This work had an indirect but important bearing on his later work in the Department of Agriculture on mineral metabolism in its relation to milk production. He recognized that the selective action of the muscle cell in accumulating its highly special salt content had the same physiological basis as the selective separation of salts by the mammary gland in milk secretion. Problems of permeability also interested him in relation to both the properties of muscle and the processes of secretion; and in work on artificial membranes he showed that impregnation of collodion films with insoluble calcium and magnesium salts formed membranes approaching living plasma membranes in their semipermeability, a property which in the living cell also is dependent on calcium. He found, however, that the behavior of smooth muscle in anisotonic Ringer's solution differed from that of striated muscle and indicated the presence of a much less diffusion-proof surface layer. This difference in physical properties he correlated with other evidence of a fundamental difference in the mechanism of contraction in the two types of muscle.

During Edward Meigs' work of twenty-five years in the Department of Agriculture he and his associates made varied and important contributions to the physiology of milk production. Mineral metabolism and vitamine supply in relation to milk production received special attention, and the results of this work were published in a long succession of special papers and reviews. He was also responsible for the general planning and direction of the work at the experimental farm at Beltsville, Maryland. Many problems of a highly practical kind also came up for consideration; for example, the incidence of mastitis in the experimental herd led to an investigation of the pathology of this condition and effective methods for its control were developed, including modification of certain types of milking machines which were found to be largely responsible. The work of these years is too varied to summarize briefly; some of its practical results are seen in the progressive improvement during recent years in the general methods employed in the dairy industry.

Much of Edward Meigs' success in this work came from the thoroughness, objectivity and freedom from bias that were characteristic of his scientific activity and outlook. He was highly tenacious in his convictions once they were formed, but his conclusions were always based on a clear-sighted and critical consideration of evidence, in the collection of which he spared no pains. Although weakened in his later years by illness, he maintained his scientific interest and activity to the last. His personal interests other than scientific were remarkably wide; he was

fond of nature and outdoor life, a great sailor, a man of imagination and culture, widely read, modest, loyal, high-minded and devoted to his friends. His characteristic generosity was well shown in his gift to the Marine Biological Laboratory in 1936 of the bathing beach property, including the bath house, on the Buzzards Bay front adjoining his family summer cottage. The use of this beach by members of the Laboratory, as well as of the tennis courts which occupy part of this property, is thus permanently assured.

Memorial to Dr. D. H. Tennent

By Dr. W. C. Curtis

In the premature death of David Hilt Tennent, the Corporation of the Marine Biological Laboratory has lost the crowning years in the life of a member distinguished for his accomplishment in research and even more for his quality as a man. Tennent was an investigator who proceeded without haste, yet unceasingly; for him quality, not quantity, of publication was the prime consideration, yet the volume of his published work is impressive. He was honored for his work by the Presidency of the American Society of Zoologists and by similar offices, and most notably by election to the National Academy of Science. He made outstanding contributions in his studies upon hybridization, fertilization and egg organization in Echinoderms, and in his later research upon photosensitization in which he took especial satisfaction since he regarded it as the most important work of his life. These contributions, which are familiar to workers in these fields, are not so well known to many investigators in other lines, because Tennent was the most modest and retiring of men. He had none of the flare for self-advertisement that carries some men so far on a modicum of worth. His every publication was marked not only by the critical nature of his observations and experiments but also by the meticulous care with which each phrase weighed to make sure it meant exactly what he had in mind, no more and no less. What he wrote or said publicly was always as exact as he could make it. Knowing the quality of the man and of his mind, I think one may feel that what he did is likely to stand until it becomes obsolete with the advance of knowledge, as so often happens although the historical importance of the work remains.

In his work as a teacher of undergraduates and as a director of graduate students, Tennent was no less effective. The same thoroughness and determination to do his best characterized his teaching as it did his research. Tennent and I were graduate students together at the Johns Hopkins and fellow members of Drew's Invertebrate staff at the

Marine Biological Laboratory. I well recall his first lecture to the Invertebrate class. He was so scared the chalk rattled against the board, but he did better than he thought and after that first summer the rest of us felt we must keep up with him. At the Hopkins he was not satisfied with his first year's seminar lectures. To be safe the next year, as he confided to me later, he went to the laboratory the evening before each lecture, turned on the lights in the empty seminar room and put himself through a dress rehearsal of his lecture to be given the next morning. It was this kind of determination and performance that characterized all his work. It had to be done as well as he could do it. I was told that E. A. Andrews went so far as to say at the time that Tennent was the best assistant he had ever had. Only those of us who were assistants to Andrews can fully appreciate what that meant as to quality of performance. Thus, Tennent had the instinct of workmanship at the beginning of his career.

Tennent always commanded the loyalty and admiration of graduate students to a marked degree. His great disappointment was that he did not train more students who were able to find places commensurate with their ability. Those in his confidence knew that he often longed for a position where he might have had more "disciples." He trained many women of ability, but for the most part, in this *man's* world, they could not find positions worthy of their competence. His summers at the Tortugas Laboratory gave him opportunities to extend the kind of contacts he might have had in larger measure throughout the year in some institutions. I have often heard of what a stimulus he was to the younger investigators at Tortugas summer after summer.

On the personal side, Tennent was always quiet and reserved, though in his later years as well as in his youth a delightful companion to those who knew him well. He may have seemed austere to those who knew him casually. Yet he had a keen sense of humor for all his quietness. I never knew a man whose sense of obligation to do what he thought just and right seemed to me stronger nor a man whom I would trust further. Thinking of him personally, one felt that here was a man to whom the abused and meaningless phrase "a gentleman and a scholar" might be applied with meaning.

To Mrs. Tennent and to his son, who as a scientist follows in his father's footsteps, we extend our deepest sympathy.

2. THE STAFF, 1941

Charles Packard, Director, Assistant Professor of Zoology, Institute of Cancer Research, Columbia University.

ZOOLOGY

I. Investigation

Gary N. Calkins, Professor of Protozoology, Emeritus, Columbia University.
E. G. Conklin, Professor of Zoology, Emeritus, Princeton University.
Caswell Grave, Professor of Zoology, Emeritus, Washington University.
Frank R. Lillie, Professor of Embryology, Emeritus, The University of Chicago.
C. E. McClung, Professor of Zoology, Emeritus, University of Pennsylvania.
S. O. Mast, Professor of Zoology, Johns Hopkins University.
T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.
G. H. Parker, Professor of Zoology, Emeritus, Harvard University.
Lorande L. Woodruff, Professor of Protozoology, Yale University.

II. Instruction

T. H. Bissonnette, Professor of Biology, Trinity College.
P. S. Crowell, Jr., Instructor in Zoology, Miami University.
A. M. Lucas, Associate Professor of Zoology, Iowa State College.
W. E. Martin, Assistant Professor of Zoology, DePauw University.
N. T. Mattox, Instructor in Zoology, Miami University.
J. S. Rankin, Jr., Instructor in Biology, Amherst College.
A. J. Waterman, Assistant Professor of Biology, Williams College.

Junior Instructors

W. J. Bowen, Assistant Professor of Biology, University of North Carolina.
E. R. Jones, Jr., Professor of Biology, College of William and Mary.

EMBRYOLOGY

I. Investigation

(*See Zoology*)

II. Instruction

Hubert B. Goodrich, Professor of Biology, Wesleyan University.
W. W. Ballard, Assistant Professor of Biology and Anatomy, Dartmouth College.
Donald P. Costello, Assistant Professor of Zoology, University of North Carolina.
Viktor Hamburger, Assistant Professor of Zoology, Washington University.
Oscar Schotté, Associate Professor of Biology, Amherst College.

PHYSIOLOGY

I. Investigation

William R. Amberson, Professor of Physiology, University of Maryland, School of Medicine.

HAROLD C. BRADLEY, Professor of Physiological Chemistry, University of Wisconsin.
WALTER E. GARREY, Professor of Physiology, Vanderbilt University Medical School.
M. H. JACOBS, Professor of Physiology, University of Pennsylvania.
RALPH S. LILLIE, Professor of General Physiology, The University of Chicago.
ALBERT P. MATHEWS, Professor of Biochemistry, University of Cincinnati.

II. INSTRUCTION

Teaching Staff

ARTHUR K. PARPART, Associate Professor of Biology, Princeton University, in charge of course.
ROBERT BALLENTINE, National Research Council Fellow, Rockefeller Institute, New York.
KENNETH C. FISHER, Assistant Professor of Experimental Biology, University of Toronto.
RUDOLF T. KEMPTON, Professor of Zoology, Vassar College.
C. LADD PROSSER, Assistant Professor of Zoology, University of Illinois.
F. J. M. SICHEL, Assistant Professor of Physiology, University of Vermont, College of Medicine.

BOTANY

I. INVESTIGATION

S. C. BROOKS, Professor of Zoology, University of California.
B. M. DUGGAR, Professor of Physiological and Economic Botany, University of Wisconsin.
D. R. GODDARD, Assistant Professor of Botany, University of Rochester.
E. W. SINNOTT, Professor of Botany, Barnard College.

II. INSTRUCTION

WM. RANDOLPH TAYLOR, Professor of Botany, University of Michigan.
B. F. D. RUNK, Instructor in Botany, University of Virginia.
WILLIAM J. GILBERT, Department of Botany, University of Michigan.

GENERAL OFFICE

F. M. MACNAUGHT, Business Manager.
POLLY L. CROWELL, Assistant.
GLADE ALLEN, Secretary.

RESEARCH SERVICE AND GENERAL MAINTENANCE

SAMUEL E. POND, Technical Mgr.
G. FAILLA, X-ray Physicist.
ELBERT P. LITTLE, X-ray Technician.
J. D. GRAHAM, Glassblower.
T. E. LARKIN, Superintendent.
LESTER F. BOSS, Technician.
W. C. HEMENWAY, Carpenter.
R. S. LILJESTRAND.

LIBRARY

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.) Librarian.
Deborah Lawrence, Secretary.
Mary A. Rohan, S. Mabell Thombs, Assistants.

SUPPLY DEPARTMENT

James McInnis, Manager.
Milton B. Gray, Collector.
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Geoffrey Lehy, Collector.
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F. N. Whitman, Collector.
Ruth S. Crowell, Secretary.
Grace Harman, Secretary.

INVESTIGATORS AND STUDENTS

Independent Investigators, 1941

Addison, William H. F., Professor of Normal Histology and Embryology, University of Pennsylvania, School of Medicine.
Allee, W. C., Professor of Zoology, The University of Chicago.
Amberson, William R., Professor of Physiology, University of Maryland, School of Medicine.
Baker, Horace B., Professor of Zoology, University of Pennsylvania.
Ballard, W. W., Assistant Professor of Biology and Anatomy, Dartmouth College.
Ballentine, Robert, Procter Fellow, Princeton University.
Barron, E. S. Guzman, Assistant Professor of Biochemistry, The University of Chicago.
Bartlett, James H., Jr., Associate Professor of Theoretical Physics, University of Illinois.
Berger, Charles A., Professor of Cytology, Director of Biological Laboratory, Fordham University.
Bissonnette, T. H., Professor of Biology and Head of Biology Department, Trinity College.
Bliss, Alfred F., Lecturer in Biophysics, Columbia University.
Boche, R. D., Instructor in Zoology, University of Pennsylvania.
Bowen, W. J., Assistant Professor, University of North Carolina.
Boyd, Milford J., Assistant Professor of Biochemistry, University of Cincinnati.
Bradley, Harold C., Professor of Physiological Chemistry, University of Wisconsin.
Brill, Edmund R., Graduate Student in Biology, Harvard University.
Bronfenbrenner, J. J., Professor of Bacteriology and Immunology, Washington University.
Brooks, Matilda M., Research Associate, University of California.
Brooks, Sumner C., Professor of Zoology, University of California.
Brown, Dugald E. S., Professor of Physiology, New York University.
Budington, R. A., Professor of Zoology, Emeritus, Oberlin College.
Bullock, Theodore H., Sterling Fellow in Zoology, Yale University.
Cable, R. M., Associate Professor of Parasitology, Purdue University.
Calkins, Gary N., Professor Emeritus of Protozoology, Columbia University.
Carothers, E. Eleanor, Research Associate in Zoology, University of Iowa.
Chambers, Edward, Research Worker, Washington Square College, New York University.
Chambers, Robert, Research Professor of Biology, Washington Square College, New York University.

CHASE, AURIN M., Instructor, Princeton University.
CLAFF, C. LLOYD, Research Associate in Biology, Brown University.
CLARK, ELEANOR LINTON, Voluntary Research Worker, University of Pennsylvania.
CLARK, ELIOT R., Professor and Director of Department of Anatomy, University of Pennsylvania, School of Medicine.
CLARK, LEONARD B., Assistant Professor of Biology, Union College.
CLAUDE, ALBERT, Research, Rockefeller Institute.
CLOWES, G. H. A., Director of Research, Eli Lilly and Company.
COLE, KENNETH S., Associate Professor of Physiology, Columbia University.
COLWIN, ARTHUR L., Instructor, Queens College.
COLWIN, LAURA HUNTER, Instructor, Vassar College.
CONKLIN, EDWIN G., Professor of Biology, Emeritus, Princeton University.
COOPER, KENNETH W., Instructor, Princeton University.
COOPER, RUTH, Research Worker, Princeton University.
COPELAND, MANTON, Professor of Biology, Bowdoin College.
CORNMAN, IVOR, Graduate Student, University of Michigan.
COSTELLO, DONALD P., Assistant Professor of Zoology, University of North Carolina.
CROWELL, SEARS, Assistant Professor of Zoology, Miami University.
CURTIS, W. C., Professor of Zoology, University of Missouri.
DAM, HENRIK, Associate Professor of Biochemistry, University of Copenhagen.
DAVSON, HUGH, Associate Professor of Physiology, Dalhousie University.
DEWEY, VIRGINIA C., Manufacturers' Research Fellow in Protozoology, Brown University.
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FORBES, JAMES, Assistant Professor of Biology, Fordham University.
FURTH, JACOB, Associate Professor of Pathology, Cornell University Medical College.
GARREY, W. E., Professor of Physiology, Vanderbilt University, School of Medicine.
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HARTMAN, FRANK A., Professor of Physiology, Ohio State University.
HARVEY, E. NEWTON, Professor of Physiology, Princeton University.

Harvey, Ethel B., Research Investigator, Princeton University.
Hayashi, Teru, Graduate Assistant, University of Missouri.
Haywood, Charlotte, Associate Professor of Physiology, Mount Holyoke College.
Heilbrunn, L. V., Associate Professor of Zoology, University of Pennsylvania.
Hendricks, Eliott M., University of Cincinnati.
Hibbard, Hope, Professor, Oberlin College.
Hill, Samuel E., Professor of Biology, Russell Sage College.
Hopkins, Dwight L., Professor, Mundelein College.
Howe, H. E., Editor, Industrial and Engineering Chemistry, Washington, D. C.
Hunninen, Arne V., Professor of Biology, Oklahoma City University.
Hunter, George W., Assistant Professor of Biology, Wesleyan University.
Hutchens, John O., Johnston Scholar, Johns Hopkins University.
Illick, J. Theron, Associate Professor of Zoology, Syracuse University.
Jacobs, M. H., Professor of General Physiology, University of Pennsylvania.
Janowitz, Olga, Instructor, Potomac School, Washington, D. C.
Jenkins, George B., Professor of Anatomy, Emeritus, George Washington University.
Jones, E. Ruffin, Jr., Professor of Biology, College of William and Mary.
Kaylor, Cornelius T., Instructor in Anatomy, Syracuse University, College of Medicine.
Kempton, Rudolf T., Professor of Zoology, Vassar College.
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Lerner, Edwin M., II., Harvard University.
Liebmann, Emil, Research Fellow, Tulane University.
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Mathews, Albert P., Professor of Biochemistry, Emeritus, University of Cincinnati.
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Mavor, James W., Professor of Biology, Union College.
McClung, C. E., Professor of Zoology, Emeritus, University of Pennsylvania.
Menkin, Valy, Assistant Professor of Pathology, Harvard University Medical School.
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Meyerhof, Prof. O. H., Research Professor of Biochemistry, University of Pennsylvania.
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Milne, Lorus J., Associate Professor of Biology, Randolph-Macon Woman's College.
Mitchell, Philip H., Professor of Biology, Brown University.

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Moore, Walter G., Instructor, Loyola University of the South.
Morgan, Isabel M., Assistant, Rockefeller Institute.
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Navez, Albert E., In Charge of Tutorial System, Milton Academy.
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O'Brien, John P., Johns Hopkins University.
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Rona, Elizabeth, Holder of a Carnegie Fellowship, Geophysical Laboratory, Carnegie Institution.
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Scott, Allan, Assistant Professor, Union College.
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College.
Shapiro, Herbert, Instructor in Physiology, Vassar College.
Shaw, Myrtle, Senior Bacteriologist, New York State Department of Health.
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Sichel, F. J. M., Assistant Professor of Physiology, University of Vermont, College of Medicine.
Slifer, Eleanor H., Assistant Professor, State University of Iowa.
Smith, Jay A., Instructor in Physiology and Pharmacology, Chicago Medical School.
Speidel, Carl C., Professor of Anatomy, University of Virginia.
Steinbach, H. Burr, Assistant Professor of Zoology, Columbia University.
Stern, Kurt G., Research Assistant Professor, Yale University.

Stewart, Dorothy R., Associate Professor of Biology, Skidmore College.
Stokey, Alma G., Professor of Botany, Mount Holyoke College.
Stunkard, Horace W., Professor of Biology, New York University.
Sturtevant, A. H., Professor, California Institute of Technology.
Tashiro, Shiro, Professor of Biochemistry, University of Cincinnati.
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Velick, Sidney F., Research Fellow, Yale University.
Wald, George, Faculty Instructor, Harvard University.
Walker, Roland, Instructor in Biology, Rensselaer Polytechnic Institute.
Warner, Edward N., Instructor in Zoology, Ohio State University.
Waterman, Allyn J., Assistant Professor of Biology, Williams College.
Watterson, Ray L., Graduate Teaching Assistant in Zoology, University of Rochester.
Wenrich, D. H., Professor of Zoology, University of Pennsylvania.
Whiting, Anna R., Guest Investigator, University of Pennsylvania.
Whiting, P. W., Associate Professor of Zoology, University of Pennsylvania.
Wichterman, Ralph, Assistant Professor of Biology, Temple University.
Wilbur, Karl M., Research Associate, Washington Square College, New York University.
Wilhelmi, Raymond W., Graduate Assistant, New York University.
Willier, Benjamin H., Professor of Zoology and Chairman of the Department of Biology, Johns Hopkins University.
Wolf, E. Alfred, Associate Professor of Biology, University of Pittsburgh.
Wolf, Opal M., Assistant Professor, Goucher College.
Woodruff, Lorande L., Professor of Protozoology and Director of the Osborn Zoological Laboratory, Yale University.
Woodward, Alvalyn E., Assistant Professor of Zoology, University of Michigan.
Woodward, Arthur A., Jr., Graduate Assistant, New York University.
Wrinch, Dorothy, Lecturer in Chemistry, Johns Hopkins University.
Yntema, Chester L., Assistant Professor of Anatomy, Cornell University Medical College.
Zinn, Donald J., Graduate Student, Yale University.
Zweifach, Benjamin W., Research Associate, New York University.

Beginning Investigators

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Alsup, Fred W., Graduate Student, University of Pennsylvania.
Aquila, Sister M., Graduate Student, Villanova College.
Benedict, Dora, Cohasset, Massachusetts.
Bernheimer, Alan W., Graduate Student, University of Pennsylvania.
Birmingham, Lloyd, Graduate Teaching Assistant, University of Rochester.
Bongiovanni, Alfred M., Investigator, Villanova College.
Burt, Richard L., Junior Research Fellow, Brown University.
Cole, Roger M., Teaching Fellow, Harvard University.
Compton, Alfred D., Jr., Graduate Student, Yale University.
Duncan, George W., Halstead Fellow in Surgery, Johns Hopkins University.
Elizabeth, Sister Miriam, Associate Professor of Biology, Chestnut Hill College.
Gabriel, Mordecai L., Assistant in Zoology, Columbia University.
Genevieve, Sister Mary, Graduate Student, Villanova College.
Gilbert, William J., University of Michigan.
Glancy, Ethel A., Tutor, Queens College.

Goldin, Abraham, Graduate Student, Columbia University.
Grand, C. G., Research, Washington Square College, New York University.
Green, James Watson, Graduate Student, University of Pennsylvania.
Groupe, Vincent, Graduate Student, University of Pennsylvania.
Haas, Ward J., Undergraduate, Massachusetts Institute of Technology.
Hamilton, Pauline G., Research Assistant and Graduate Student, University of Pennsylvania.
Harris, Daniel L., Instructor, University of Pennsylvania.
Hassett, Charles, Graduate Assistant, Johns Hopkins University.
Hayes, E. R., Graduate Assistant in Anatomy, Ohio State University.
Hendley, Charles D., Assistant in Biophysics, Columbia University.
Hendricks, Anne L., Medical Student, University of Cincinnati.
Henry, Richard J., Student, University of Pennsylvania, School of Medicine.
Henson, Margaret, Teaching Fellow in Biology, Washington Square College, New York University.
Hickson, Anna K., Research Chemist, Eli Lilly and Company.
Hinchey, M. Catherine, Graduate Student, University of Pennsylvania.
Hopkins, Marjorie G., Graduate Assistant, Mount Holyoke College.
Horn, Annabelle Broomall, Graduate Student, University of Pittsburgh.
Houck, C. Riley, Sterry Junior Fellow, Princeton University.
Hyman, Chester, Research Assistant, New York University.
Klotz, John W., Graduate Student, University of Pittsburgh.
Levin, Erwin, Student, Ohio State University.
McNutt, W. S., Jr., Assistant in Biology, Brown University.
Melkon, Bernard, University of Pennsylvania.
Metz, Charles B., Teaching Fellow in Embryology, California Institute of Technology.
Molter, John A., Graduate Student, University of Pennsylvania.
Moog, Florence, Graduate Student, Columbia University.
Muir, Robert M., Rackham Predoctoral Fellow, University of Michigan.
Oesterle, Paul Daniel, Biology Instructor, Chestnut Hill College.
Recknagel, Richard, Graduate Student, University of Pennsylvania.
Rollason, Herbert D., Jr., Graduate Assistant, Williams College.
Saunders, Grace, Teaching Fellow, Washington Square College, New York University.
Shelanski, Louis, Graduate Student, University of Pennsylvania.
Stern, Joseph R., Research Assistant, University of Toronto.
Thivy, Francesca, Graduate Student, University of Michigan.
Troedsson, Pauline H., Student, Columbia University.
Weisiger, James R., Graduate Fellow in Physiological Chemistry, Johns Hopkins University.
Wiercinski, Floyd J., Research Assistant, University of Pennsylvania.
Williams, John L., Graduate Assistant, New York University.
Wilson, Walter L., Graduate Student, University of Pennsylvania.
Zarudnaya, Katya, Student, Radcliffe College.
Zimmerman, George L., Student, Swarthmore College.
Zingher, Joseph M., College of the City of New York.
Zorzoli, Anita, Graduate Student, Washington Square College, New York University.

Research Assistants

Baker, Linville A., Research Assistant in Physiological Chemistry, Eli Lilly and Company.
Bradford, Audrey A., Student, Vassar College.
Brownell, Katharine A., Research Assistant, Ohio State University.

Cohen, Irving, Research Assistant, New York University.
Conger, Martha, New York State Department of Health.
Crawford, John D., Milton Academy.
Donovan, Mary K., Graduate Student, Villanova College.
DuBois, Arthur B., Milton Academy.
Dytche, Maryon, Graduate Assistant in Physiology, University of Pittsburgh.
Erlanger, Margaret, Instructor, West Virginia University.
Ferguson, Frederick P., Teaching Assistant, University of Minnesota.
Finkel, Asher J., Research Assistant, The University of Chicago.
Garner, Harold, Research Assistant, The University of Chicago.
Gelback, Elizabeth L., Assistant, Yale University.
Goldinger, J. M., Research Assistant, The University of Chicago.
Hager, Russell P., Research Assistant, University of Pennsylvania.
Hamilton, Howard L., Research Assistant, Johns Hopkins University.
Heilbrunn, Constance, Philadelphia, Pennsylvania.
Hohwieler, Harold J., Graduate Assistant, Washington University.
Jacobs, Joye, Assistant in Physiology, University of Maryland, School of Medicine.
Jandorf, Bernhard J., Research Assistant, Eli Lilly and Company.
Keefe, Eugene L., Research Assistant, Washington University.
Marmont, George, Research Associate in Physiology, College of Physicians and Surgeons, Columbia University.
Martin, Rosemary D. C., Demonstrator, University of Toronto.
Maurer, Jane, Yale University Medical School.
Mead, Frank W., Ohio State University.
Meed, Margaret R., Graduate Student, Brown University.
Mullins, Lorin J., Assistant in Physiology, University of Rochester.
Nash, Gerry H., Research Assistant, New York University, College of Medicine.
Nelson, Gordon H., Assistant, Milton Academy.
Netsky, Martin, Medical Student, University of Pennsylvania.
Ormsbee, Richard A., Laboratory Assistant, Brown University.
Osterud, Dorothy W., Research Assistant, New York University.
Perkins, Patricia J., Graduate Student, University of Cincinnati.
Philips, Frederick S., Seessel Fellow in Biology, Yale University.
Ronkin, Raphael R., Graduate Student, University of California.
Rothstein, Aser, Graduate Assistant, University of Rochester.
Schaffel, Milton, Research Assistant, University of Pittsburgh.
Shapiro, Henry, Williams College.
Slaughter, Joseph C., Research Assistant, State University of Iowa.
Spiegelman, S., Research Assistant, Columbia University.
Spratt, Nelson T., Jr., Research Assistant, Johns Hopkins University.
Stebbins, Robert B., Assistant, Washington Square College, New York University.
Sullivan, Pauline F., Teacher, Choate School, Brookline, Massachusetts.
Sutro, Peter J., Student, Harvard University.
Trinkaus, J. Philip, Cramer Fellow, Dartmouth College.
Tseng, C. K., Fellow in Botany, University of Michigan.
Warren, Alathier A., Assistant, Harvard University Medical School.
Wasserman, Edward, Undergraduate Assistant, Wesleyan University.
Weaver, Margaret A., Graduate Student, University of Texas.

Library Readers, 1941

Auger, Carlton, Rockefeller Institute for Medical Research.
Baker, Gladys E., Instructor in Department of Plant Science, Vassar College.
Ball, Eric G., Assistant Professor of Biochemistry, Harvard University Medical School.
Beck, L. V., Instructor in Physiology, Hahnemann Medical College.

Belda, Walter H., Professor, Fordham University.
Bloch, Robert, Osborn Botanical Laboratory, Yale University.
Bodian, David, Assistant Professor of Anatomy, Western Reserve University Medical School.
Bowen, Earl, Head of Department of Biology, Gettysburg College.
Castelnuavo, Ejina, University of Missouri.
Cox, Edward H., Professor of Chemistry, Swarthmore College.
Gates, R. Ruggles, Professor of Botany, University of London.
Granick, Sam, Assistant, Rockefeller Institute for Medical Research.
Gray, Irving E., Associate Professor of Zoology, Duke University.
Gurewich, Vladimir, Cornell University Medical College.
Hall, Thomas S., Instructor, Laurenceville School.
Humm, Frances D., Research Fellow in Embryology, Yale University.
Kreezer, George L., Assistant Professor of Psychology, Cornell University.
Levy, Milton, Assistant Professor, New York University, College of Medicine.
Loewi, Otto, Research Professor of Pharmacology, New York University, College of Medicine.
MacKnight, Robert H., Instructor, University of North Carolina.
Magnus-Levy, Adolf, Yale University.
Oster, Robert H., Assistant Professor of Physiology, University of Maryland School of Medicine.
Rabinowitch, Eugene, Research Associate, Massachusetts Institute of Technology.
Rahn, Hermann, Instructor, University of Wyoming.
Reiner, John M., Room 516, 303 Fourth Avenue, New York, New York.
Rothen, A., Associate, Rockefeller Institute for Medical Research.
Sevag, Manasseh G., Assistant Professor of Biochemistry, University of Pennsylvania, School of Medicine.
Shwartzman, Gregory, Head of Department of Bacteriology, Mount Sinai Hospital.
Solberg, Archie N., Assistant Professor of Biology, University of Toledo.
Stowell, Robert E., Research Assistant, Barnard Free Skin and Cancer Hospital.
Young, William C., Associate Professor of Primate Biology, Yale University.

Students, 1941

BOTANY

Abbott, Charles C., Student, Haverford College.
Bayard, Ellen B., University of Connecticut.
Bull, Nancy B., Student, Wellesley College.
Enzenbacher, Jean A., Smith College.
Frantz, Ruth E., Elmira College.
Katz, Elaine J., Student, Goucher College.
Leuchs, Augusta V. H. A., Graduate Student, Radcliffe College.
Muir, Robert M., University of Michigan.
Salvin, Samuel B., Assistant, Harvard University.
Smith, Frederick B., Professor of Soils, University of Florida.
Stanton, Connie Lee, Bryn Mawr College.
Stenger, Rev. Frederick R., Professor of Biology, St. Mary of the Lake Seminary.
Thorne, Robert F., Dartmouth College.
Waldron, Jacqueline M., Student, American University.
Williams, Robert H., Instructor in Botany, Cornell University.

EMBRYOLOGY

Bergstrom, William H., Student, Amherst College.
Birmingham, Lloyd W., University of Rochester.

Cole, Roger M., Teaching Fellow, Harvard University.
Covalla, Miriam J., Instructor, Seton Hill College.
Crumb, Cretyl, Wellesley College.
Eldred, Earl, Teaching Assistant, Northwestern University.
Gibbs, Elizabeth, Wheaton College.
Gross, John B., Student, DePauw University.
Hasse, Gordon W., Graduate Assistant, University of Illinois.
Hendricks, Anne L., University of Cincinnati.
Hollister, Patricia, Student, Sarah Lawrence College.
Hopkins, Marjorie G., Graduate Assistant, Mount Holyoke College.
Josephson, Neil, Assistant, Wesleyan University.
Karczmar, Alexander G., Columbia University.
Kezer, Leonard J., Biology Instructor, State Teachers College, Newark, N. J.
Kieffer, Richard F., Jr., Franklin and Marshall College.
Kirkpatrick, Elizabeth M., Connecticut College.
Lambie, Morris W., Student, Harvard University.
Lerner, Eleanor D., Graduate Student, Brooklyn College.
Lintlicum, Anne H., Student, Goucher College.
Martin, Richard G., Harvard University.
Martin, T. Sterling, Oberlin College.
Medlicott, Mary, Graduate Assistant, Mount Holyoke College.
Morgan, Thomas W., Washington and Jefferson College.
Mothes, Arlene M., Student, Massachusetts State College.
Muchmore, William B., Oberlin College.
Plough, Irvin C., Student, Amherst College.
Power, Maxwell E., Graduate Assistant, Yale University.
Regnery, David C., Laboratory Instructor, Stanford University.
Reiner, Elliot R., Laboratory Assistant, University of Alabama.
Royle, Jane G., Graduate Student, Bryn Mawr College.
Saunders, Grace S., Teaching Fellow, Washington Square College.
Smithcors, J. Frederick, Rutgers University.
Svihla, George, Research Assistant in Zoology, University of Illinois.
Timanus, Alice L., Student, Converse College.
Wasserman, Edward, Undergraduate Assistant, Wesleyan University.
Zinghier, Joseph M., College of the City of New York.

PHYSIOLOGY

Algire, Glenn H., Graduate Student, University of Maryland.
Burns, J. E., Jr., Graduate Assistant, Wesleyan University.
Coe, Frederick W., Ohio Wesleyan University.
Deyrup, Ingrith J., Barnard College.
Egan, Richard W., Research Assistant, Canisius College.
Gordon, Harold T., Teaching Fellow, Harvard University.
Green, James W., Student, Davis-Elkins College.
Gregg, John R., Undergraduate Assistant, University of Alabama.
Harrison, Robert W., Graduate Assistant, Wesleyan University.
Hartmann, J. Francis, Assistant, Cornell University.
Henry, Jane E., Gettysburg College.
Jacobs, Jack, Teaching Fellow, Washington Square College.
Keezer, William S., Indiana University.
Lein, Joseph, College of the City of New York.
Lorenz, Philip B., Swarthmore College.
Perkins, Patricia J., University of Cincinnati.
Rollason, Herbert D., Jr., Graduate Assistant, Williams College.
Rosenblum, Eugene D., Brooklyn College.

Schepartz, Bernard, Student, Ohio Wesleyan University.
Schlosser, Adele P., Vassar College.
Stern, Joseph R., Research Assistant, University of Toronto.
Tuttle, L. Constance, Graduate Assistant, Mount Holyoke College.
Weiner, Herbert M., Student, Harvard University.
Zimmerman, George L., Swarthmore College.

ZOOLOGY

Anderson, Dorcas J., Graduate Assistant, Purdue University.
Andrews, Thomas J., Massachusetts State College.
Aram, Hartley H., State University of Iowa.
Batchelor, William H., Student, Harvard College.
Beardsley, Margaret, Smith College.
Berg, Philip W., State University of Iowa.
Brainerd, John W., Student, Harvard University.
Brown, Henrietta B., Student, Tufts College.
Burke, Roger K., Undergraduate Assistant, Springfield College.
Byerrum, Richard, Student, Wabash College.
Carpenter, Elizabeth, Mount Holyoke College.
Cole, LaMont C., University of Chicago.
Corder, Henry R., Graduate Assistant, Wesleyan University.
Cornish, Helen R., Teacher of Biology, Virginia Intermont College.
Culberson, Arthur W., Williams College.
Dodd, Samuel G., Wesleyan University.
Dole, Dorothy K., Assistant in Zoology, Vassar College.
Garman, Elizabeth M., Student, New Jersey College for Women.
Gillette, Roy J., Graduate Assistant, Washington University.
Gilligan, Catherine, Teacher, Hyde Park High School.
Goffin, Mary F., Seton Hill College.
Gross, Louise E., Student, Smith College.
Hahn, Rhea J., Student, Radcliffe College.
Harris, Norman D., Teacher of Biology, Berkshire School.
Hauschka, Theodore S., Harrison Fellow, University of Pennsylvania.
Heaps, Marian E., Teacher of Biology, Pennsgrove.
Hinde, Howard P., Graduate Assistant, Yale University.
Humm, Douglas G., Graduate Assistant, Yale University.
Kielich, F. Randolph, Laboratory Assistant, Canisius College.
King, Ellen E., Student, Sarah Lawrence College.
Kohler, Charles E., Rutgers University.
Lumb, Ethel S., Graduate Student, University of Missouri.
Mahr, Merle M., Graduate Assistant, New York University.
Mead, Albert R., Assistant in Zoology, Cornell University.
Miller, Helena A., Student, Radcliffe College.
Miner, Howard D., Jr., Student, Wabash College.
Osmun, John V., Graduate Student, Amherst College.
Perkins, David D., University of Rochester.
Pond, Sidney, Wesleyan University.
Powers, Rev. William T., Instructor in Zoology, DePaul University.
Randall, Walter C., Graduate Assistant, Purdue University.
Roberts, Beryl J., Teacher, City of Boston—School Department.
Roberts, Henry S., Jr., Fellow, Duke University.
Roberts, Wesley F., Graduate Assistant, Northwestern University.
Robinson, Margaret, Graduate Student, Radcliffe College.
Ross, Lucille J., Student, Barnard College.
Schlechter, Helena L., Wilson College.

Senyard, Juanita, Laboratory Assistant, Oberlin College.
Talmage, Roy Van Neste, Instructor in Zoology, University of Richmond.
Tuttle, Ruth F., Student, Wheaton College.
Weber, Ann M., Undergraduate Laboratory Assistant, Montclair State Teachers College.
Wieder, Herbert, Student, Hamilton College.
Wilber, Charles G., Graduate Assistant, Johns Hopkins University.
Williams, Robert W., Harvard University.
Wilson, Mae E., Teacher of Biology, Los Angeles City Schools.

TABULAR VIEW OF ATTENDANCE

	1937	*1938*	*1939*	*1940*	*1941*
Investigators—Total	391	380	352	386	337
Independent	256	246	213	253	197
Under Instruction	74	53	60	62	59
Research Assistants	61	81	79	71	50
Library Readers					31
Students—Total	133	132	133	128	131
Zoology	57	54	55	55	55
Protozoology (Not given in 1941)	16	10	12	7	—
Embryology	35	34	36	34	37
Physiology	16	22	21	22	24
Botany	9	12	9	10	15
Total Attendance	524	512	485	514	468
Less Persons Registered as both students and investigators	13	16	14	7	7
	511	496	471	507	461
Institutions Represented—Total	165	151	162	148	144
By Investigators	134	125	132	112	102
By Students	79	67	72	79	72
Schools and Academies Represented					
By Investigators	3	4	2	1	5
By Students	2	1	2	2	2
Foreign Institutions Represented					
By Investigators	16	14	8	2	3
By Students	—	3	1	1	1

SUBSCRIBING AND CO-OPERATING INSTITUTIONS

1941

American University
Amherst College
Barnard College
Biological Institute, Philadelphia, Pennsylvania
Bowdoin College
Brooklyn College
Brown University
Bryn Mawr College
Canisius College
California Institute of Technology
College of Physicians and Surgeons
Columbia University
Cornell University
Cornell University Medical College
DePauw University
Duke University
Fordham University
Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Indiana University
Industrial and Engineering Chemistry, of the American Chemical Society
Johns Hopkins University

Sarah Lawrence College
Eli Lilly & Co.
Loyola University of the South
Josiah Macy Jr. Foundation
Massachusetts State College
Mount Holyoke College
Mount Sinai Hospital, New York City
Mundelein College
New York State Department of Health
New York University
New York University College of Medicine
New York University Washington Square College
Northwestern University
Oberlin College
Ohio State University
Ohio Wesleyan University
Princeton University
Purdue University
Radcliffe College
Rockefeller Foundation
Rockefeller Institute for Medical Research
Russell Sage College
Rutgers University
Seton Hill College
Smith College
Springfield College
State University of Iowa
Syracuse University
Tufts College
Tulane University
Union College
University of Chicago
University of Cincinnati
University of Illinois
University of Maryland Medical School
University of Michigan
University of Missouri
University of Pennsylvania
University of Pennsylvania School of Medicine
University of Pittsburgh
University of Rochester
University of Toledo
University of Virginia
Vanderbilt University Medical School
Vassar College
Villanova College
Wabash College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Wheaton College
Yale University
Yale University Medical School

THE FRIDAY EVENING LECTURES, 1941

Friday, June 27
DR. F. R. LILLIE ..... "How Feathers Are Made."
Friday, July 11
PROFESSOR K. S. LASHLEY ..... "The Integration of Neurology and Psychology."
Friday, July 18
DR. ERIC PONDER ..... "Red Cell Structure in the Light of Shape Transformations."
Friday, July 25
DR. DOROTHY WRINCH ..... "The Native Protein."
Friday, August 1
DR. ERNST MAYR ..... "Speciation in Birds."
Friday, August 8
DR. PHILIP B. ARMSTRONG ..... "Function in the Developing Gastrointestinal Tract of Amblystoma punctatum in Relation to Embryonic Determination and Differentiation."
Friday, August 15
DR. FRANZ SCHRADER ..... "The Mitotic Movements of Chromosomes."

Friday, August 22
Dr. Otto Meyerhof ..... "The Nature, Function and Distribution of the Phosphogens in the Animal Kingdom."

Friday, August 29
Dr. Rudolf Schoenheimer ..... "The Dynamic State of the Body Constituents."

ADDITIONAL LECTURES, 1941

Thursday, July 3
Dr. G. S. Avery ..... "Current Approaches to the Plant Hormone Problem."

Thursday, July 31
Dr. P. S. Galtsoff ..... "Seals in Alaska."

Wednesday, August 20
Dr. Henrik Dam ..... "The Biological Significance of Vitamin K."

Wednesday, August 27
Mr. George G. Lower ..... "Local Marine Life" in color.

Thursday, August 28
Dr. Milton Levy ..... "Chemical Studies of the Chick Embryo."

SHORTER SCIENTIFIC PAPERS, 1941

Tuesday, July 8
Dr. Eric G. Ball ..... "The Source of Pancreatic Juice Bicarbonate."
Mr. Arthur J. Dziemian ..... "The Permeability and the Lipid Content of the Erythrocytes in Experimental Anemia."
Dr. Rita Guttman and
Dr. K. S. Cole ..... "Electrical Rectification in Single Nerve Fibers."
Dr. Herbert Shapiro ..... "Metabolism and Fertilization in the Starfish Egg."

Tuesday, July 15
Dr. L. B. Clark ..... "A Suggested Mechanism by which the Moon Influences Reproduction in the Atlantic Palolo Worm."
Dr. Paul S. Galtsoff ..... "Accumulation of Manganese and the Sexual Cycle in Ostrea virginica."
Dr. R. M. Cable and
Dr. A. V. Hunninen ..... "Studies in the Life History of Siphodera, a Trematode Parasite of the Toadfish."
Dr. H. W. Stunkard ..... "Pathology and Immunity to Infection by Heterophyd Thematodes."

Tuesday, July 22

Dr. G. H. Parker ..... "The Melanophore System of Teleosts."

Dr. R. L. Watterson ..... "Some Aspects of Pigment Deposition in Feather Germs of Chick Embryos."

Dr. H. L. Hamilton ..... "The Influence of Hormones on the Differentiation of Melanophores in Birds."

Dr. Hermann Rahn ..... "The Distribution and Development of the Melanophore Hormone in the Pituitary of the Chick."

Tuesday, July 29

Dr. Kenneth C. Fisher ..... "The Fractionation of Cellular Respiration by Narcotics."

Dr. T. C. Evans and
Mr. J. C. Slaughter ..... "Radiosensitivity of Arbacia Sperm under Different Conditions."

Tuesday, July 29

Dr. A. M. Chase ..... "Effect of Azide on Cypridina luciferin."

Tuesday, August 5

Dr. Victor Schechter ..... "Aging Phenomena, and Factors Influencing the Longevity of Mactra Eggs."

Dr. Frederick S. Philips ..... "Comparison of Regional Respiratory Rates of the Chick Embryo During Early Stages of Development."

Dr. Mathilda M. Brooks ..... "Further Interpretations of the Effects of CO and CN on Oxidations in Living Cells."

Tuesday, August 12

Dr. William Trager ..... "Studies on Conditions Affecting the Survival *in vitro* of a Malarial Parasite."

Dr. Charles Hassett ..... "The Effect of Dyes on the Response to Light in Peranema."

Dr. J. D. Hutchens ..... "Utilization of Ammonia by Chilomonas paramoecium."

Miss Virginia Dewey and
Dr. G. W. Kidder ..... "The Possibility of Thiamine Synthesis by Ciliates."

Tuesday, August 19

Dr. David Nachmansohn ..... "Electrical Potential and Activity of Choline Esterase in Nerves."

Dr. Albert Claude ..... "Chemical Composition of Mitochondria and Secretory Granules."

Dr. Dorothy Wrinch ..... "Native Proteins and the Structure of Cytoplasm."

GENERAL SCIENTIFIC MEETINGS, 1941

Tuesday, August 26

Dr. Caswell Grave ..... I. "Further Studies of Metamorphosis of Ascidian Larvae."
II. "The 'Eye Spot' and Light Responses of the Larva of Cynthia partita."

Mr. Lloyd Birmingham ..... "Regeneration in the Early Zooids of Amaroucium constellatum."

Dr. Ivor Cornman ..... "Characteristics of the Acceleration of Arbacia Egg Cleavage in Hypotonic Seawater."

Dr. Ethel Browne Harvey ..... "Material Inheritance in Echinoderm Hybrids."

Dr. E. S. Guzman Barron and
Dr. J. M. Goldinger ..... "Intermediary Carbohydrate Metabolism of Sperm and Eggs of Arbacia before and after Fertilization."
tion."

Mr. J. D. Crawford,
Miss D. Benedict,
Mr. A. B. DuBois and
Dr. A. E. Navez ..... "On Contraction of the Venus Heart."

Dr. Alfred M. Lucas and
Mr. James Snedecor ..... "Co-ordination of Ciliary Movement in the Modiolus Gill."

Dr. Lorus J. Milne ..... "Preparing an Animated Diagram of Somatic Mitosis."

Dr. E. Newton Harvey ..... "Stimulation by Intense Flashes of Ultra Violet Light."

Dr. T. C. Evans,
Dr. G. Failla,
Mr. J. C. Slaughter and
Dr. E. P. Little ..... "Influence of the Medium on the Radiosensitivity of Arbacia Sperm."

Dr. C. Ladd Prosser and
Mr. G. L. Zimmerman ..... "Comparative Pharmacology of Myogenic and Neurogenic Hearts."

Dr. Lois E. TeWinkel ..... "Structures Concerned with Yolk Absorption in the Dogfish, Squalus acanthias."

Dr. W. H. F. Addison ..... "The Distribution of Elastic Tissue in the Arterial Pathway to the Carotid Bodies in the Dog."

Dr. Richard G. Abell and
Dr. Irvine H. Page ..... "Behavior of the Arterioles in Hypertensive Rabbits, and in Normal Rabbits Following Injection of Angiotinin."

Wednesday, August 27

Dr. M. H. Jacobs and
Dr. Dorothy R. Stewart ....."Catalysis of Ionic Exchanges by Bicarbonates."

Dr. Dorothy R. Stewart and
Dr. M. H. Jacobs ....."The Role of Carbonic Anhydrase in the Catalysis of Ionic Exchanges by Bicarbonates."

Mr. Martin G. Netsky and
Dr. M. H. Jacobs ....."Some Effects of Desoxycorticosterone and Related Compounds on the Mammalian Red Cell."

Dr. Herbert Shapiro and
Dr. Hugh Davson ....."Permeability of the Arbacia Egg to Potassium."

Dr. A. K. Parpart ....."Lipo-protein Complexes in Arbacia Eggs."

Dr. S. E. Hill ....."Relation Between the Action Potential and Protoplasmic Streaming in Chara and Nitella."

Dr. Aurin M. Chase ....."Observations on Luminescence in Mnemiopsis."

PAPERS READ BY TITLE

Mr. Fred W. Alsup ....."Photodynamic Studies on Arbacia Eggs."

Dr. T. C. Evans ....."The Effect of Roentgen Radiation on the Jelly of the Nereis Zygote."

Dr. R. Ruggles Gates ....."Tests of Nucleoli and Cytoplasmic Granules in Marine Eggs."

Dr. Russell P. Hager ....."Sex-linkage of Stubby (sb) in Habrobracon."

Mr. E. R. Hayes ....."The Elasmobranch Interrenal; a Preliminary Note. The Interrenal Body of Alopias vulpinus (Bonnaterre)."

Dr. Dwight L. Hopkins ....."The Cytology of Amoeba Verrucosa."

Dr. George W. Hunter, III and
Mr. Edward Wasserman ....."Observations on the Melanophore Control of the Cunner Tautogolabrus adspersus (Walbaum)."

Miss Florence Moog ....."The Influence of Temperature on Reconstitution in Tubularia."

Dr. Clinton M. Osborn ....."Factors Influencing the Pigmentation of Regenerating Scales on the Ventral Surface of the Summer Flounder."

Dr. G. H. Parker ....."Hypersensitization of Catfish Melanophores to Adrenaline by Denervation."

DR. LEONARD P. SAYLES ....."Implants Consisting of Young Buds, Formed in Anterior Regeneration in Clymenella, Plus the Nerve Cord of the Adjacent Old Part."

DR. A. A. SCHAEFFER ....."Chaos Nobilis Penard in Permanent Culture."

DR. ALLYN WATERMAN ....."Ectodermization of the Larva of Arbacia."

DR. RALPH WICHTERMAN ....."Studies on Zoochlorella-free Paramecium Bursaria."

MR. FLOYD J. WIERCINSKI ....."An Experimental Study of Intracellular pH in the Arbacia Egg."

DR. E. ALFRED WOLF,
MISS MARYON DYTCHE AND
MR. MILTON SCHAFFEL ....."Heat Produced by Respiring Whole Blood of Tautoga onitis and Mustelus canis."

DR. C. L. YNTEMA ....."Effect of Differences Between Stages of Donor and Host Upon Induction of Auditory Vesicle from Foreign Ectoderm in the Salamander Embryo."

DEMONSTRATIONS

Wednesday, August 27

MR. L. F. BOSS AND
DR. M. H. JACOBS ....."Stabilized Source of Current for Lamps and Other Purposes."

DR. E. R. CLARK AND
MRS. ELEANOR LINTON CLARK ...."Behavior of Giant Cells as Observed in the Living Mammal."

DR. E. N. HARVEY AND
DR. F. J. M. SICHEL .....1. "Apparatus for Intense Flashes of Ultra Violet Light, and the Killing of Small Organisms."
2. "Apparatus for High Speed Photography with the Microscope."

DR. KURT G. STERN ....."An Air-driven High-speed Centrifuge for Optical Observations."

DR. IVOR CORNMAN ....."Disruption of Mitosis in Colchicum by Colchicine."

DR. SEARS CROWELL .....1. "Nematostella, a Simple Anemone, Suitable for Laboratory Work in General Zoology."
2. "The Nematosomes of Nematostella."

DR. JOHN KEOSIAN .....1. "Apparatus of Simple Construction for Use in Microchemical Work."
2. "A Spot Test Method for the Quantitative Determination of Magnesium in Tenths of a Microgram."

DR. ELEANOR H. SLIFER ....."A Mutant Drosophila Melanogaster with Extra Sex Combs."

DR. LOIS E. TEWINKEL ....."Structures Concerned with Yolk Absorption in the Dogfish."

DR. ERIC LOEWENSTEIN ....."Demonstration of Fluorophotometer, and Discussion of Fluorometric Methods of Determining Biological Substances."

DR. LAURENCE IRVING,
DR. P. F. SCHOLANDER,
MR. C. LLOYD CLAFF,
MR. GEORGE EDWARDS AND
MR. NIELS HAUGAARD .....Section A

Sensitive volumetric apparatus devised by Dr. P. F. Scholander, as used in the continuous measurement of respiration and in the measured delivery of small quantities of liquid.

1. "A Respirometer with Mechanical Circulation of Air, Sensitive to 0.3 mm^3., Used to Observe Long Continuous Measurements of O_2 Consumption in Water of Animals of Approximately ½ Gram Body Weight." *Edwards.*
2. "A Respirometer Similar in Construction to the Above, But Lacking a Mechanical Circulation, Sensitive to 0.15 mm^3., Used to Observe Long Continuous Measurements of the O_2 Consumption of Protozoa (5,000–10,000 Organisms)."
3. "A Micrometer Calibrating Burette, Sensitive to 0.3 mm^3., Used for the Delivery of Small Quantities of Liquid." *Claff and Edwards.*
4. "A Micro-micrometer Calibrating Burette, Sensitive to 0.02 mm^3., Used for the Delivery of Minute Quantities of Liquid." *Claff.*
5. "The Small Gear Pump Used for the Circulation of Air in the Respirometer, Devised by Dr. Scholander, Constructed Under the Direction of C. Lloyd Claff by Mr. Carl A. Moeller." *Claff.*

Section B

"A System Suitable for Aquatic Animals, Sensitive to 0.02 cc., Used to Measure the O_2 Consumption of Fishes of 20–200 Grams Body Weight at 10 Minute Intervals for Periods Lasting for 12 to 24 Hours." *Haugaard.*
(The glassblowing was done by Mr. J. D. Graham.)

9. MEMBERS OF THE CORPORATION, 1941

1. Life Members

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Maryland.
Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York.
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REGENERATION OF THE REPRODUCTIVE SYSTEM FOLLOWING BINARY FISSION IN THE SEA-CUCUMBER, HOLOTHURIA PARVULA (SELENKA) [1]

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Although it is a familiar fact that sea-cucumbers possess an extraordinary capacity for regeneration and often reproduce asexually, very little is known concerning the normal cellular basis of such reconstitution Of particular interest in this regard is the reported regeneration of gonads in adult sea-cucumbers and the question of the origin of germ cells. Investigators commonly state that following autotomy of the viscera or binary fission involving a loss of gonadal tissue, the sea-cucumbers rebuild the structures which they lack, yet we are indebted to Deichmann (1922) for the only specific data relative to the condition of the gonad. In *Holothuria parvula* (Selenka) and *Holothuria difficilis* (Semper) which have undergone transverse fission she reports briefly that "In specimens where all other organs were nearly as well developed as in undivided specimens, genital organs were absent, or very feeble, while an equally sized normal, undivided animal had a one centimeter long tuft." Since the entire original reproductive system remains in the anterior fission half, the poorly developed gonads within large specimens could be explained by any one or by a combination of the following processes: (1) an extreme periodic retrogression of the gonad which may or may not be related to asexual reproduction, (2) a delay in the normal development of the reproductive system of some individuals possibly related to an early onset of asexual reproduction, or (3) the late regeneration of a gonad within a posterior fission half which has reconstituted all other parts. None of these phenomena has been demonstrated for any echinoderm.

It is proposed, therefore, to investigate the extreme variation existing in the reproductive system of *Holothuria parvula* (Selenka) by means of dissection and microscopic examination. Data on the gonad

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will be obtained from three different groups: (I) whole animals in which all other systems are apparently typical, (II) anterior fission halves which are regenerating new posterior ends, and (III) posterior fission halves which are regenerating new anterior ends.

Materials and Methods

Holothuria parvula (Selenka) is a convenient holothurian for the laboratory and for histological studies, since it rarely exceeds a length of about 6–7 cm. (For general anatomy and taxonomy consult Deichmann, 1930.) It is common over most of the West Indian region. Specimens were collected at Dry Tortugas in late June and July of 1937, and again in 1938 with the help of Dr. B. R. Coonfield. For comparative purposes, additional specimens were obtained in January from the Bermuda Biological Station through the courtesy of Dr. J. G. H. Wheeler.

In order to kill the animal with the viscera intact a small amount of Bouin's fluid was first injected into the coelomic cavity and the entire specimen was then immersed in the solution. Within a few minutes it was slit open, the coelomic cavity thoroughly washed out with fresh Bouin's, and the animal stored in Bouin's until dissected. As a rule, a good fixation of the reproductive system was obtained.

Three hundred and forty specimens representing both the summer and winter collections were used for the present study. They included 170 that were apparently whole, while 75 were obviously regenerating an anterior end, and 95 were regenerating a posterior end. All specimens were first dissected and the reproductive systems studied *in toto* with the aid of a dissecting scope ($\times 22$). Graded stages in the development of the gonad were then selected from specimens regardless of their size. Thirty-four of these were studied histologically by means of serial sections. A variety of stains was used including alum hematoxylin, Mallory's triple, iron hematoxylin, and alum cochineal. For a sharp nuclear stain, the iron hematoxylin was best. Counter stains of eosin and cotton blue [2] were particularly helpful in bringing out the cytoplasmic inclusions peculiar to the germ cells.

The Reproductive System of H. parvula

The gonad of *H. parvula* develops in and from the left side of the dorsal mesentery, just behind the calcareous ring (Figure 1). It may be divided into three major portions: (1) a single tuft of gonad tubules, (2) the gonad-basis from which these tubules originate and (3) nests

[2] I am indebted to my colleague, Ruth Jones, for this helpful suggestion.

of germ cells imbedded within a restricted region of the dorsal mesentery just anterior to the basis (Figure 1, GC).

In the normal young specimens of my collection, the gonad consists only of nests of germ cells. This is true for all specimens whose volume was less than 0.7 cc. and whose shape, diameter and development of tube feet clearly separated them from the regenerating halves of large specimens. All these normal young individuals had a maximum diameter of 6 mm. or less. In specimens whose volume was just above 0.7 cc. and whose maximum diameter was 7 and 8 mm., I found the first indication of tubule formation. The tubules always arise at the posterior level of the germ cell field in the form of small vesicles projecting into the coelom on the left side of the mesentery (similar to Figure 5). Apparently, they arise consecutively, for if more than one is present, they are graded in size, the posterior one being the largest. As these vesicles develop, they increase in size, and change their shape to that of a tubule which may or may not branch dichotomously. The origin of the tubules in *H. parvula* is therefore similar to the fundamental pattern described for other sea-cucumbers and the nests of germ cells are analogous to the strand-like "germinal cord" found in *Mesothuria intestinalis* by Théel (1901).

As the tubules increase in number, the basis from which they arise enlarges and likewise bulges into the coelom as a single large vesicle. By the time four tubules have originated from the gonad-basis, a gonoduct is present in the dorsal mesentery of all specimens of my collections. It takes its origin from the anterior face of the gonad-basis and extends obliquely forwards and upwards to open on the surface of the body wall. In fully grown specimens one can find the opening in a depression located in a mid-dorsal position about 7 mm. behind the base of the tentacles. Among five apparently whole (non-regenerating) specimens from Tortugas, each of which possessed only three immature tubules, the gonoduct extended to the body wall in three, was incomplete in one, and entirely absent in the other. It may be completely developed when only one gonad tubule has appeared. In the Tortugas collections seven such specimens possessed a gonoduct, while four did not. Thus considerable variation exists, but the gonoduct never develops until at least one gonad tubule has made its appearance.

The Extreme Variation of the Reproductive System in the Large Animals

While it might be expected that increase in the size and complexity of the maturing reproductive system would be correlated with an increase in size of the maturing specimens, it is surprising to find within the largest

specimens of the collection a graded series of stages typical of its development. The great variation in the reproductive systems of three large specimens is strikingly shown by comparing Figure 1 with Figures 2 and 4, and in two medium-sized specimens, by comparing Figure 6 with Figure 5. Each one of the specimens is much larger than the minimum size at which this sea-cucumber typically matures, yet the gonoduct may or may not be present and the gonad varies from a group of mature tubules to mere nests of germ cells imbedded in the dorsal mesentery. The poorly developed reproductive systems are like those in very young, immature specimens with a volume of less than 1 cc. With the exception of the reproductive system, there are no other striking variations in the anatomy of these large specimens. Without dissection they would unquestionably pass as fully matured individuals. Apparently the size of the gonad is not necessarily correlated with the size of the animal except during the typical development of the reproductive system in young specimens.

Variation in the Gonad Due to Periodic Resorption of Discharged Tubules

Several investigators have reported the fact that in certain sea-cucumbers the gonad tubules reach maturity, discharge their gametes, and then undergo regression. If this tubule resorption extended even periodically to the entire gonad, such retrogression could well explain the variation in the size of the gonads. Examination of the midsummer material revealed 11 clear-cut cases of resorption actually in progress.

Plate I [3]

Anterior ends of 5 preserved specimens (The Dry Tortugas, July 10). Left body wall has been removed to show the water vascular ring (W), anterior portion of the alimentary canal (A), with a portion of the dorsal mesentery including the gonad site.

Figure 1. Mature ovary of a very large specimen (7.0 cc.). O, oviduct; GC, nests of germ cells imbedded in the dorsal mesentery, and ovary consisting of 9 tubules originating from a common gonad-basis. × 8.

Figure 2. Extremely simple gonad in a very large specimen (7.2 cc.). Note absence of gonoduct. Nests of germ cells are present in the mesentery, but do not show in the photograph. × 8.

Figure 3. Gonad region of Figure 2, enlarged. × 20.

Figure 4. Nests of germ cells imbedded in the dorsal mesentery at the gonad site. No gonoduct nor tubules. Large specimen (5.3 cc.). × 8.

Figure 5. The origin of the first tubule at the extreme posterior end of a well-defined gonad primordium. Medium-sized specimen (3.3 cc.). × 8.

Figure 6. Mature testes. R, advanced resorption of the posterior tubule. Medium-sized specimen (3.5 cc.). × 8.

[3] The writer is indebted to Mr. John Spurbeck, The Johns Hopkins University Department of Biology, for aid in photography.

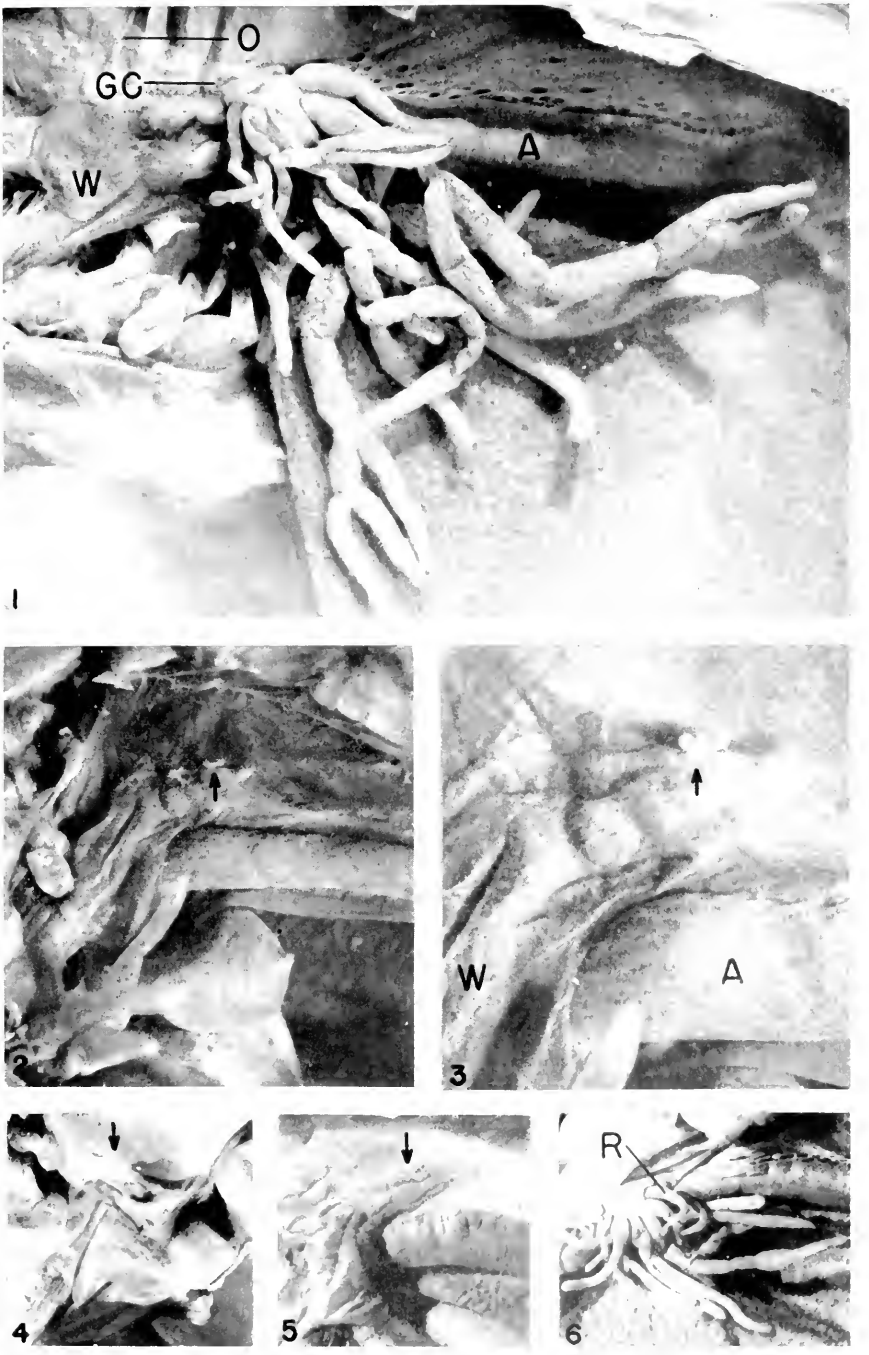

PLATE I

There were also nine cases among the specimens collected at Bermuda in January and there were many specimens in each collection in which certain posterior tubules had apparently been completely resorbed. Figure 6 shows a typical case of seven graded tubules followed by two others which are shorter than the seventh. The ninth tubule (R) was brown and finely wrinkled in contrast to the white smooth walls of the other tubules. Histological examination shows that this last tubule has been invaded by large numbers of phagocytic cells.

For our present purpose, it is important to stress that regardless of the number of tubules that are being resorbed within unregenerating specimens, the retrogression never extends to the three most anterior tubules, nor to the gonoduct. These anterior tubules are of course very small, but they never show any signs of being resorbed. Their small size is due wholly to their immaturity. Not a single stage in the resorption of a gonoduct was observed. Once formed, the gonoduct is evidently a permanent structure. The combination of a very few immature tubules plus a well-developed gonoduct apparently represents the extreme retrogression of the gonad in whole specimens (Group I). In the light of these data it is highly improbable that the extremely simple gonad which is occasionally found in specimens of maximum size can be due to a periodic gonad retrogression. That a periodic resorption of the oldest tubules produces considerable variation in the gonad is obvious, but we must look elsewhere for an understanding of the extreme variations found in animals of maximum size.

Variation in the Gonad and Asexual Reproduction

If asexual reproduction begins at an early stage in some individuals, it is possible that the normal development of the gonad might be delayed. It is pertinent to consider this possibility since Crozier (1917) working at Bermuda came to the conclusion that "if *H. captiva* (= *H. parvula*, Deichmann, 1930) undergoes division normally, it can occur only in very young stages." Deichmann (1922), on the other hand, found that 40 of the 82 specimens collected by Mortensen at Buccoo Bay, Tobago, B. W. I., were regenerating either a new anterior or a new posterior end. She reports no differential as to size. She also states (1930) that even some of the type specimens which are in the Museum of Göttingen give evidence of regeneration.

My findings are in accord with those of Deichmann. Out of 117 specimens collected at Bermuda (January), 51 were obviously regenerating either a new posterior or anterior end and another 15 were probably in very late stages of regeneration. Identifications in the field at

Tortugas (June–July) revealed that 10 to 15 per cent were regenerating one end of the body. This figure would have been much greater if a dissection had been made of each specimen, for the very early and the very late stages in regeneration are difficult to recognize externally. In the first instance, sufficient new tissue has not appeared, while in the second, the normal dark pigmentation and proportions of the body have been restored. Not only is this additional evidence that *H. parvula* normally makes use of binary fission in reproduction, but a study of the sizes of the regenerating Tortugas specimens indicates that fission normally occurs only in specimens ranging from about 30 to 60 mm. in length and from 5 to 14 mm. at their greatest diameter. Since none of the small specimens of my collections gave any indication of a recent fission, the evidence is against the retardation of normal gonad development by the early introduction of an asexual generation.

It is of interest to consider at this point whether the condition of the gonad bears any particular relation to the onset of fission among the large specimens. Dissection of specimens which are obviously regenerating new posterior ends (Group II) will provide the pertinent data. Since such individuals have fairly recently undergone transverse fission, a study of their gonads should reveal any correlation that exists. The examination of 50 Tortugas specimens regenerating posterior ends showed that development of the gonad was more advanced than in the collection as a whole, yet all stages of gonad development were represented. Since the data cited above show that fission occurs only among the larger specimens it is to be expected that in the ordinary course of events the gonads in Group II would be the best developed. Five of them possessed gonads consisting only of germ cell nests, while four had several immature tubules. In two specimens, resorption had progressed beyond that seen in any of the unregenerating specimens. In these, a large gonad-basis and a well-developed gonoduct remained but no tubules were present. Most of the remaining 39 specimens possessed seven to 12 well-developed tubules. Apparently no particular condition of the gonad seems to favor asexual reproduction. This holds for the Bermuda winter collection as well as the Tortugas summer collection. Evidently the few extremely immature gonads found in large-sized specimens of Group II must be related to the same factors which bring about this condition in apparently whole animals.

Evidence for the Regeneration of Gonads

Since the presence of extremely immature gonads in large-sized specimens cannot be accounted for on the basis of gonad resorption, nor of

gonad retardation, the condition must be due to the regeneration of a gonad within a posterior half resulting from the process of transverse fission. If this were true, it would be reasonable to suppose that specimens which are obviously regenerating new anterior ends would show the earliest stages in such regeneration. On the contrary, data obtained from the dissection of 75 specimens of Group III, and the microscopic examination of five specimens, give no indication of the re-establishment of any part of the reproductive system.

Direct evidence of gonad regeneration must therefore be sought among the specimens of Group I, which includes some animals that are actually fission halves in very late stages of regeneration. In these specimens, the complete sterility found in Group III should persist, or very early stages in gonad regeneration should be found. From the dissection alone, one might be led to think that sterility did exist. Out of 80 large (30 to 60 mm.) specimens from Tortugas, which were apparently not regenerating, there were 14 which possessed only an opaque spot or spots in the dorsal mesentery instead of a gonad (Figure 4). Histological analysis shows, however, that these opaque spots are nests of germ cells imbedded in the mesentery. They are in every respect identical with the germ cells found in this same region in the very young, immature sea-cucumber where we recognize them to be the first morphological indication of a reproductive system. Therefore, complete sterility does not exist except in specimens of Group III, all of which have undergone fission comparatively recently.

Furthermore, using only the medium to large-sized animals of Group I which in every other respect are apparently full grown, one can select a complete series of stages which will represent the typical development of the reproductive system: first, the germ cell nest mentioned above (Figure 4); then, the addition of one or two minute tubules springing from the gonad-basis (Figures 5 and 2); then, a gonoduct extending half way from the gonad-basis to the dorsal body wall; and finally, a complete gonoduct. If a great number of specimens were collected, one could confine himself entirely to the very largest specimens, yet obtain data on the development of the reproductive system as complete as that provided by selecting various sizes of the very small sea-cucumbers. This complete series of stages in the development of a reproductive system and the absence of sterility in large-sized animals, coupled with the indirect evidence mentioned above, establishes the fact that *H. parvula* can regenerate the reproductive system as completely as any other system.

It is obvious that experimental transections would provide direct evidence on regeneration. Such experiments would also determine

whether or not there is any preparatory step preceding normal fission involving germ cell migration. These experiments were tried, but unfortunately the lag in the regeneration of the gonad was too great to obtain positive results at Tortugas. My first attempts failed because experimental animals did not live longer than two weeks in our aquaria. It was discovered later that operated animals, returned to the sea floor in wire cages filled with coral, lived and immediately started to regenerate. Three weeks after the operations all systems in the new anterior end were being re-established with the exception of the reproductive system. Externally the newly regenerated portion extended as a small knob of tissue between 2 and 3 mm. in length, though the size of the regenerating halves varied from 13 to 25 mm. in length. New anterior ends regenerated at approximately the same rate as did new posterior ends. Judging from the scanty evidence provided by these specimens, at least several months must be required to re-establish completely normal proportions for the newly regenerated ends. Since this usually is accomplished before rudiments of a new reproductive system make an appearance, a much longer period must elapse before the gonad tubules are regenerated. From a study of a preserved collection, Deichmann (1922) was also of the opinion that the "genital organs seem to develop very late."

This tardy regeneration of the gonads is of particular value, however, in providing evidence that an alternation of generations does not necessarily occur. As reported above, among the 50 Tortugas specimens regenerating new posterior ends, five possessed poorly developed gonads consisting of germ cell nests only. This condition is evidence that two successive fissions have occurred. The second one has taken place before sufficient time has elapsed for the complete regeneration of the gonad. It is evident that under natural conditions the asexual mode of reproduction may be repeated without the intervention of the sexual process.

The Origin of the Germ Cells in Sea-Cucumbers

The germ cells of the gonad tubules normally arise from an aggregation of germ cells imbedded in a restricted region of the dorsal mesentery in all sea-cucumbers so far analyzed. *H. parvula,* however, can develop after fission new and fertile gonads from the dorsal mesentery at a point far posterior to the primary gonad. In order to determine whether the primary group of germ cells has made any direct contribution posteriorly to the site of the secondary gonad, a careful study was made of the dorsal mesentery within posterior halves just after fission had oc-

curred. If gonad tubules are entirely dependent upon the germ cells found in this restricted region of the dorsal mesentery, it might be expected that immediately before transverse fission occurred some natural preparatory step might occur. This could take the form of a backward extension of the primary aggregation of germ cell nests, in which case it would be visible under low powers of magnification. On the other hand, it might take the form much more difficult to trace, namely, the migration of individual germ cells. When the dissecting microscope revealed no evidence of such a contribution, serial sections were prepared in order to search for individual germ cells. In all five specimens so prepared, none was found. If the primary store of germ cells made any contribution to the posterior fission half, such cells must have lost every characteristic which had so sharply differentiated them from other cells of the mesentery at their original site.

When these negative results are coupled with certain histological data of a positive nature, the need for a revision in the traditional history of the germ cells in sea-cucumbers is apparent in so far as these regenerating specimens are concerned. Nothing is known concerning the early history of the germ cells in sea-cucumbers until late in larval life. At this time certain enlarged cells in a restricted region of the epithelium covering the dorsal mesentery burrow into the body of the mesentery to establish a fund of germ cells. The germ cells are then contributed to the gonad tubules as they arise and establish a germinal epithelium in each. No one has ever suggested that germ cells may also arise from cells in the mesenterial epithelium of the adult, yet my observations on regenerating *H. parvula* all point in this direction. It is not within the scope of the present paper to present cytological details related to the origin of germ cells from cells in the coelomic epithelium, but attention may be called to the main features: (1) In specimens which possess only germ cells at the gonad site, the coelomic epithelium in this region of the dorsal mesentery is hypertrophied on the left side, (2) single cells and sometimes groups of several cells, each possessing a large nucleus and much cytoplasm, may be seen projecting into the mesentery from the left coelomic epithelium, and (3) at more posterior levels of this restricted region of the mesentery, groups or nests of similar cells lie more deeply imbedded in the mesentery, asymmetrically related to the left coelomic epithelium but isolated from it. In each group, some cells can be identified unmistakably as germ cells. Even in later stages when the regenerating gonad consists of a group of very well-developed tubules, sections through the dorsal mesentery just anterior to the tubules will show proliferation from the left epithelium of the mesentery. I

cannot at present give the precise origin of the particular cells in the epithelium which invade the mesentery as germ cells.

Regeneration of the Gonad in Other Sea-Cucumbers

The experiments on *Thyone briareus* (Lesueur) (Kille, 1939) constitute the only other investigations on gonad regeneration in sea-cucumbers which have included any histological details. The capacity of this genus to regenerate gonads within a four-month period was tested by extirpation. When tubules only were removed nests of germ cells were retained, imbedded in the dorsal mesentery at the anterior end of the gonad-basis. New tubules arose at an abnormal rate to re-establish a complete gonad. Those instances of gonad regeneration briefly noted in the literature as occurring within a few months after loss of the main mass of tubules are probably cases of this kind. For example, in *Thyone briareus* and *Cucumaria grubi,* Torelle (1909) reported that "the reproductive organs are the last to regenerate in individuals in which these organs have been extruded." Bertolini (1932), working with *Holothuria tubulosa* D. Ch., stated that the gonad is probably regenerated, though it was impossible for her to tell whether the gonad that was expelled was the whole gonad or only part of it.

If the nests of germ cells as well as the tubules are extirpated, *Thyone briareus* does not regenerate a gonad within the period of four months. The long period required for gonad regeneration in *H. parvula* emphasizes the possibility that the failure of *T. briareus* to completely reconstitute a gonad under these conditions might be due to insufficient time. On the other hand, genera do vary in respect to capacity for regeneration. It would not be unusual to find that *H. parvula* which frequently reproduces by fission could regenerate the entire reproductive system including germ cells, but that *Thyone briareus,* which reproduces by sexual means only, could not.

Summary

1. The extreme variation in the reproductive system of large specimens of the sea-cucumber, *Holothuria parvula,* is investigated by means of dissection and microscopic examination.

2. As a result of frequent transverse fission, specimens are found in three conditions irrespective of the variation in the reproductive system: (I) apparently whole animals, (II) anterior fission halves which are obviously regenerating new posterior ends, and (III) posterior fission halves which are obviously regenerating new anterior ends.

3. Sterility existed in all animals of Group III, but in none of Group I.

4. In small specimens of Group I, the degree of development of the reproductive system is correlated with the size of the animal, but in specimens of medium size or larger all stages in the typical development of the system occur.

The simplest gonad consists of nests of germ cells imbedded in the dorsal mesentery which originate from cells in the hypertrophied left epithelium.

5. Extremely simple gonads and the absence of a gonoduct in large specimens of Groups I and II cannot be attributed to periodic resorption nor to a retarded development related to asexual reproduction, though resorption of the oldest tubules does account for considerable variation in the size of the gonad.

6. It is concluded that the simple gonads in large specimens represent early stages in the late regeneration of the reproductive system within posterior fission halves which have already reconstituted all other systems.

7. The condition of the gonad in Group II shows that no correlation exists between a particular condition of the gonad and the occurrence of fission, and that under natural conditions the asexual mode of reproduction may be repeated without the intervention of the sexual process.

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PELAGIC LARVAL STAGES OF THE SAND CRABS EMERITA ANALOGA (STIMPSON), BLEPHARIPODA OCCIDENTALIS RANDALL, AND LEPIDOPA MYOPS STIMPSON

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Introduction

In a previous report (Johnson, 1940) the larvae of *Emerita analoga* were dealt with from the standpoint of dispersal of pelagic stages as correlated with and as indicators of water currents off the California coast. That report was made possible only through a detailed study of the pelagic developmental stages which provided a key to the exact identification essential to investigation of the distribution.

The present report will cover mainly the descriptive details of the larval developmental stages of which there are five.

A study of the early larval stages of two other less abundant sand crabs, namely *Lepidopa myops* Stimpson and *Blepharipoda occidentalis* Randall, was undertaken by the senior author, and is here reported briefly for comparison. This study was made in order to ascertain whether or not the larvae of these related forms are sufficiently similar to those of Emerita to cause confusion when piecing together the developmental series by collecting the later zoeal stages from the plankton as was done for Emerita. That no such confusion is possible is seen from the strikingly different structure of the zoeal stages of each as shown in the figures.

Emerita analoga

Emerita analoga is abundant in the intertidal zone of many sandy beaches along the coast of southern California and according to Schmitt (1935) its range of distribution is from Oregon (Holms); Drake Bay, California, to Magdalina Bay, Lower California; and from Salvery, Peru, to Lota, Chile. A second Pacific species, *Emerita rathbunae* Schmitt, occurs from La Paz, Lower California, to Capon, Peru, but because of the remoteness from our region its pelagic larvae are not likely to be confused with larvae of *Emerita analoga* caught in our waters.

[1] Contributions from the Scripps Institution of Oceanography, New Series No. 170.

The larvae of two species of Emerita, namely *Emerita talpoida* of the eastern North American coast and *Emerita asiatica* of the Asiatic coast, have previously been investigated, both under the generic name *Hippa*. Of these, the former was studied incompletely by Smith (1877) and in greater detail by Faxon (1879) and the latter in detail by Menon (1933), who also makes comparisons between the two species.

As might be expected within the same genus, the structure of these larvae is very similar to that of our local species. The major differences will be noted in the course of discussion.

The eggs are carried attached to the abdominal appendages during the incubation period and the larvae, which quickly become pelagic, hatch in the first zoeal stage. A figure of this stage and also early cleavage stages of the eggs has been given by Johnson and Snook (1927).

Distribution of Larvae

In Table I is given an analysis of the occurrence of Emerita larvae for cruises covering the period April 8 to December 18. The seasonal distribution here suggested would indicate that the bulk of larvae are

TABLE I

Frequency of occurrence of Emerita larvae taken during the routine cruises of the "E. W. Scripps" for periods indicated

1938	Number of stations	Per cent successful for larvae	Zoea I	Zoea II	Zoea III	Zoea IV	Zoea V
April 8–12	7	0.0					
June 7–18	25	4.0			1		
August 16–26	34	38.0	9	30	12	12	1
October 26–November 5	28	36.0			6	25	
December 9–18	27	22.0			4	17	1

hatched during July and early August. This appears also to be in keeping with the conclusion of MacGinitie (1938) that the height of the mating season falls in May and June, though the duration of egg carriage by the female has not been determined. The wide distribution (Figure 1) indicates that the pelagic period is a relatively long one enabling the larvae to become dispersed and transported from their place of origin in the intertidal zone to distances of over a hundred miles seaward.

It is interesting that somewhat greater numbers of larvae were taken at stations relatively far from the coast, and the frequency of separate stations yielding one or more larvae is about the same here as in the

inshore area. These offshore stations fall in an extended area paralleling the coast as indicated by the line in Figure 1 enclosing locations where a total of six or more larvae have been taken. This is particularly striking since it is contrary to expectations in view of the coastal origin of the larvae and the diminution of numbers expected to accompany offshore dispersal. In seeking an explanation, it should be pointed out that it has been shown (Sverdrup and Fleming, 1941) that during

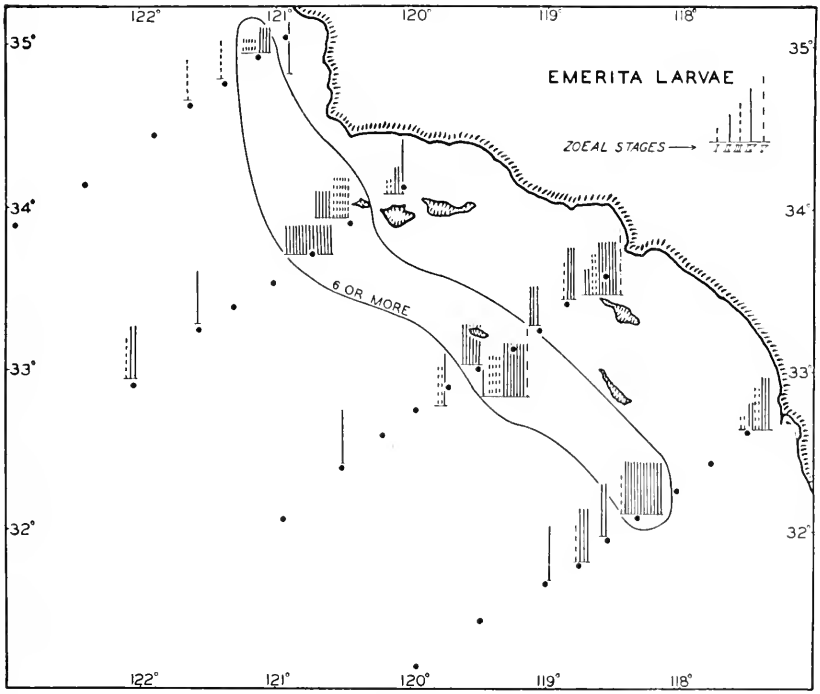

FIGURE 1

this period there exists a body of upwelled and mixed water streaking southward off-shore corresponding roughly to the area abundant in Emerita larvae. These waters are also notably productive in phytoplankton (Sverdrup and Allen, 1939) and in zoöplankton (Johnson, unpublished). Certain marine birds, e.g., the black-footed albatross, occur in greatest numbers in a long narrow band corresponding roughly to this area of increased organic production (Miller, 1940). It may therefore be suggested that, perhaps, the abundant plankton food supply makes this also a better region for survival of larvae than is true of the waters between this region and the immediate coast.

Studies of the ecology and offshore distribution of Emerita larvae in other areas are not available for comparison.

Methods

In the preliminary study, oviferous females were collected on the beach and kept in aquaria until the eggs were about to hatch, which is indicated by a change of color in the eggs from a bright coral red to a dull grayish-brown. At this stage an entire appendage with its egg mass was removed and placed in a glass culture chamber constructed from a 60 by 19 mm. shell vial. The bottoms of the vials were removed and replaced by a piece of number 8 × (average mesh aperture of 0.21 mm.) bolting cloth held in place by a rubber band. The tops were closed in a similar manner. Several culture chambers stocked with eggs were then placed end on end in a vertical glass tube approximately 45 centimeters long and 22 millimeters in diameter, or just sufficient to allow the shell vials to slip into place. The tube was held in a vertical position by a clamp and ringstand. Aerated sea water, pumped from the sea, was allowed to pass upward through the tube and culture chambers and overflow from the top. The rate of flow was regulated by screw clamps such that the eggs were almost held in suspension by its force. In this manner hatching usually occurred within a period of 24 hours and approximately half of the larvae hatched by this method were already free-swimming by this time. The young zoea are very helpless until their maxillipeds and their posterior setae are fully extended, for it is by means of these that the larvae are enabled to swim. Zoea which were hatched in the above manner lived without attention from six to eight days in the same culture chamber.

Feeding the larvae diatoms, ground Ulva or fresh plankton in the tubes failed to lengthen the period of life. Thus it was not possible to rear the larvae in the tubes sufficiently long to enable them to pass into the second zoeal stage; nevertheless, the method was the most satisfactory in caring for the eggs and bringing about a high percentage of hatching.

The best success in rearing the larvae beyond this age was realized in cultures consisting of a small number of individuals. Two free-swimming zoea were kept alive in a Syracuse watch glass for 34 days. The water was changed daily by means of a pipette and a portion of a fresh plankton haul, which had been strained through number 8 × bolting cloth to remove large and possibly harmful organisms, was fed daily. Similar success was also obtained with two zoea placed in a finger bowl and kept at approximately 18° C. in a constant temperature room which was totally dark. The water was kept in motion by a mechanical rocking

table, which was in constant operation throughout the period. One of the two larvae died on the twenty-fourth day without further change. The remaining one lived an additional ten days in which time it entered the second zoeal stage. Two long spines had developed from the posterior lateral margins of the carapace. These extended obliquely downward at an angle of about 45 degrees from the dorsoventral line and were approximately as long as the carapace was wide. The rostral spine had become greatly elongated. This specimen, unfortunately, soon died, but it showed the distinguishing features of the second zoeal stage and provided a key to following through the subsequent stages from specimens collected from the plankton as indicated below.

Some zoea of stages I and II were obtained from routine plankton hauls made with small nets at the Institution's pier and also from occasional samples collected at stations five to ten miles off Point Loma. Stages IV and V were, however, absent from all of these near-shore collections. The later stages and also most of stages II and III were collected off-shore, mainly in vertical plankton hauls taken from 200–0 meters with a 70 cm. net aboard the research vessel, "E. W. Scripps," at four lines of stations extending seaward to distances of 150 to 180 miles off the coast from San Luis Obispo to San Diego (Fig. 1).

Description of Zoeal Stages

stage i (Plate I, Figure 1)

Average sizes:

Maximum width of carapace	.53 mm.
Maximum length of carapace	.70 mm.
Length of abdomen including telson	.72 mm.
Length of rostral spine	.20 mm.

The average sizes given for this and all subsequent stages except Stage V are based on measurements of ten or more specimens. In Stage V only five specimens were available. The carapace measurements are used in preference to total length because the inclusion of the abdomen in the latter is not ordinarily practicable owing to its being strongly flexed in a position making exact measurement difficult.

Stage I is essentially similar to the first stage in the development of *E. talpoida* as given by Faxon and also that of *E. asiatica* as described by Menon. It is characterized by a short rostral spine and by the absence of lateral spines on the carapace. Slight thickenings mark the region where the latter will appear in the second zoeal stage. The eyes are conspicuously stalked and project laterally at right angles, slightly beyond the margin of the carapace. The carapace in this and subsequent stages is translucent, colorless and of uniformly smooth texture.

First Antennae (Plate II, Figure 1).—These are thick, short, unjointed processes bearing three aesthetes at the distal ends. No rudiments of the secondary flagellum were observed until the post-zoeal stage as was found also by Smith and Menon in *E. talpoida* and *E. asiatica* respectively.

Second Antennae (Plate II, Figure 6).—The antennae are each terminated by an outer spine-like process with a thickened base that is continuous with the main body of the antenna. There is also a smaller dentiform process and from near its base there issues a small setose spine which remains essentially unchanged throughout the following zoeal stages.

Mandibles (Plate II, Figure 11).—The mandibular blades bear from six to ten sharp teeth arranged in a row, the ventral tooth being always much heavier and longer than the others. These appendages remain practically unchanged except for general growth throughout the zoeal stages.

During metamorphosis to the adult state, the mandibular blades are lost. This is doubtless correlated with the radical change that occurs in feeding habit at this time, for in the adult state the animal has secondarily adopted the habit of remaining quiescent in the sand while the plumose antennae screen microscopic food from the outgoing waves.

First Maxillae (Plate II, Figure 13).—These are similar to the first maxilla in *E. asiatica,* but have one less seta on the endopod than in *E. talpoida.* The exopod is terminally cleft and each branch bears a stout setose spine. On the newly hatched zoea these spines are deeply imbedded. Branching off about half way down the outer margin of the exopod is a small lobe armed with a single long seta. The endopod bears a group of three partially imbedded setae. A single small seta is found proximal to this group and remains unchanged throughout the zoeal development. This was also found to be true of *E. asiatica* by Menon.

Second Maxillae (Plate II, Figure 16).—The second maxillae are similar in shape and arrangement to *E. asiatica* and *E. talpoida.* However, the number of setae on the scaphognathite differs from both of these species. Faxon shows a different arrangement of setae on the

PLATE I

Emerita analoga—zoeal stages I–V

FIGURE 1. Stage I (enlarged scale).
FIGURE 2. Stage I (same scale as following stages).
FIGURE 3. Stage II.
FIGURE 4. Stage III.
FIGURE 5. Stage IV.
FIGURE 6. Stage V.

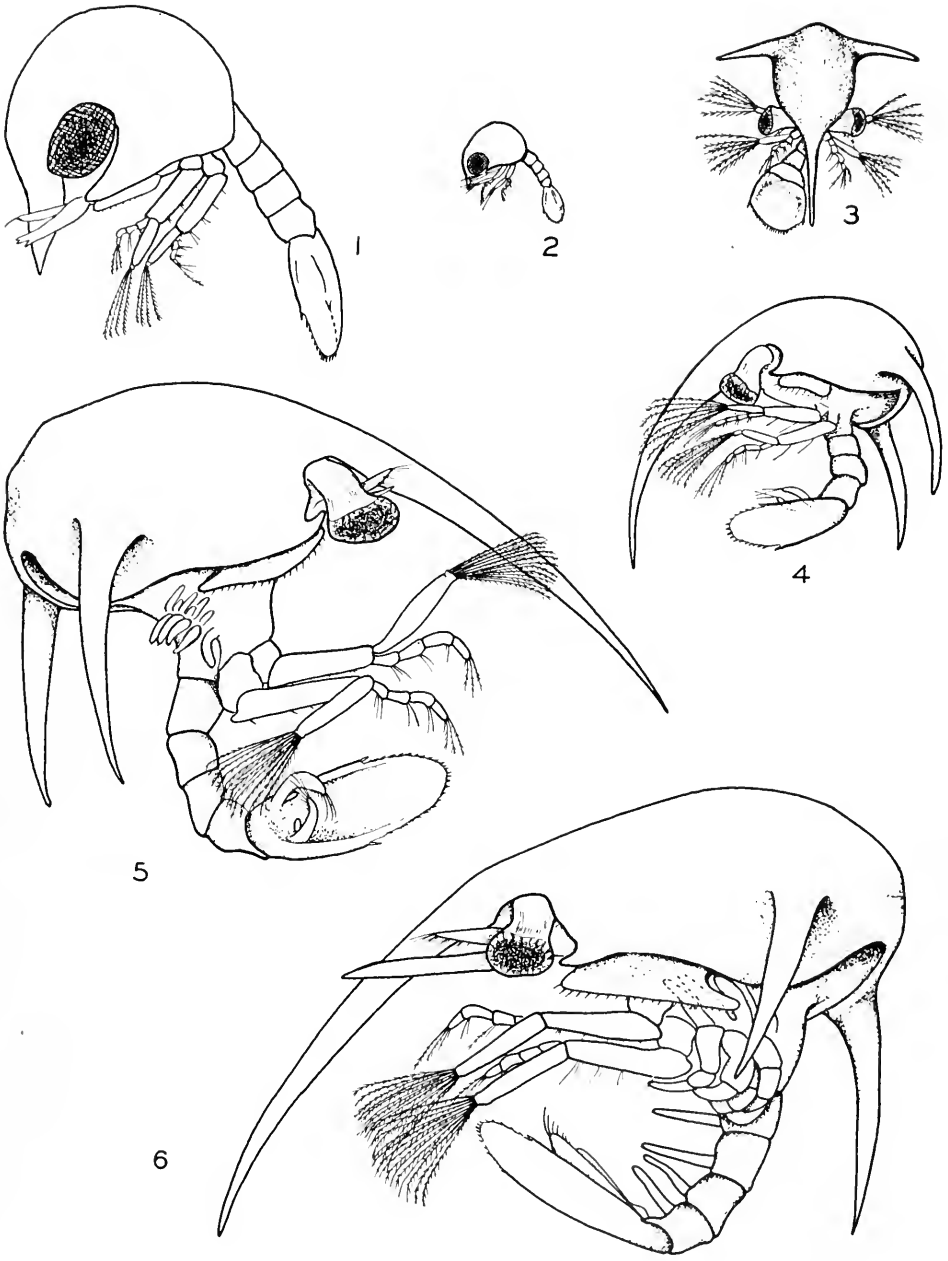

PLATE I

protopod for *E. talpoida*. The appendage of *E. analoga* is divided into two parts; the protopod bears a cluster of three short rudimentary setae accompanied by a fourth seta somewhat removed down the median margin. This isolated seta remains unchanged throughout the remainder of the developmental stages as is also the case in *E. asiatica*. The scaphognathite bears nine to ten setae along its anterior-outer margin. The posterior and inner margins are naked, differing in this respect from *E. talpoida*.

First Maxillipeds (Plate II, Figure 18).—These are essentially similar to those figured for *E. asiatica*. They are composed of a very short coxopod with a long basipod about equal in length to that of the endopod, including the three distal setae. The basipod bears six setae along the inner margin. The endopod consists of four segments each bearing setae. The first segment has three setae just below the joint, each of the next two has two in the same position. The distal segment has four setae of unequal length, the outermost two being the longest. No spinules as mentioned by Menon for *E. asiatica* were observed. The exopod consists of an elongated segment slightly longer than the endopod and a very short terminal segment bearing four long plumose setae.

Second Maxillipeds.—These are very similar to the first maxillipeds except that the distal segment of the endopod is relatively longer. The basipod is more slender and has less setae on its inner margin. The segmentation in both of these appendages remains the same throughout the later stages, the only point of difference, other than general growth, being in the number of setae on the distal segment of the exopod as

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PLATE II

Emerita analoga—zoeal appendages

FIGURES 1 to 5. First antenna, Stages I to V.
FIGURES 6 to 10. Second antenna, Stages I to V.
FIGURE 11. Mandible, Stage I.
FIGURE 12. Mandible, Stage V (reduced scale).
FIGURE 13. First maxilla, Stage I.
FIGURE 14. First maxilla, Stage III.
FIGURE 15. First maxilla, Stage IV.
FIGURE 16. Second maxilla, Stage I.
FIGURE 17. Second maxilla, Stage V.
FIGURE 18. Maxilliped, Stage I.
FIGURE 19. Maxilliped, Stage V.
FIGURE 20. Telson, Stage I.
FIGURE 21. Uropod, Stage V.
FIGURE 22. Uropod, Stage IV.
FIGURE 23. Uropod, Stage III.
FIGURE 24. Arrangement of mouth parts, Stage V.

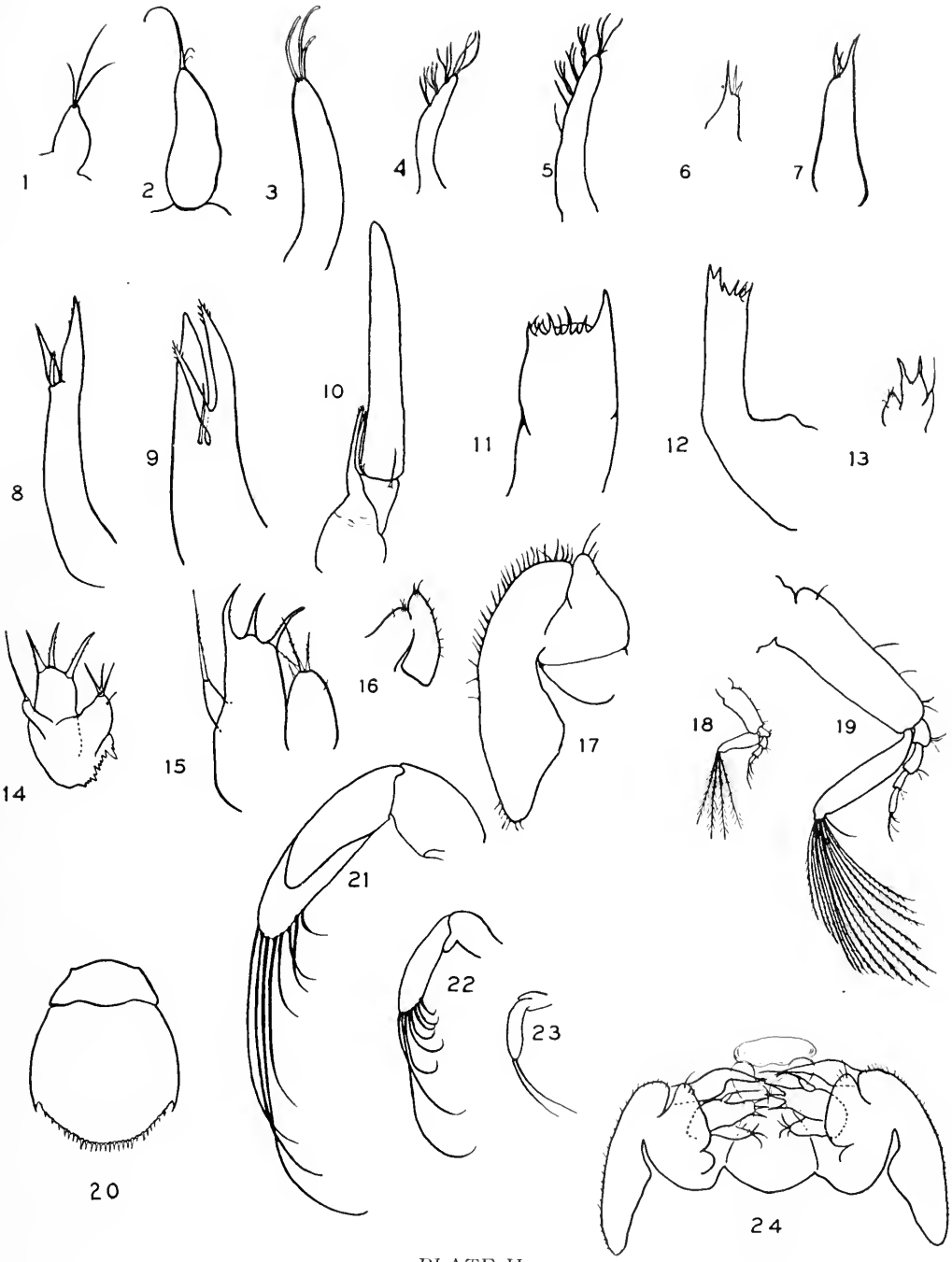

PLATE II

mentioned later. No rudiments of other thoracic appendages were found posterior to the second maxillipeds.

Abdomen.—The abdomen is composed of four free segments and the telson; the fifth and sixth segments are consolidated in the telson according to Smith. Proceeding from anterior to posterior the individual segments become shorter and markedly broader. No appendage rudiments appear either on the telson or on the abdominal segments until a later stage.

The telson (Plate II, Figure 20) agrees with *E. talpoida* but differs from the Asiatic species in that its width is slightly in excess of its length. However, there is a good deal of individual variation in this respect and in some it is fully as long as wide. The number of spines on the posterior margin is the same as that given for both *E. asiatica* and *E. talpoida.* This number, twenty-six, remains constant throughout the later stages.

STAGE II (Plate I, Figure 3)

Average sizes:

Maximum width of carapace	0.8 mm.
Maximum length of carapace	1.0 mm.
Length of rostral spine	1.2 mm.
Length of lateral spine	1.0 mm.

Stage II is characterized by the presence of two long, stout, posteriolateral spines nearly as long as the carapace is wide. These spines together with the rostral spine, which has increased to over three times the length attained in the preceding stage, form a tripod upon which the zoea is supported when resting on its ventral side. No additional appendages make their appearance in this stage. The carapace is no longer circular when viewed dorsally, but is now considerably longer than wide, and the eye stalks are projected more forward than in the previous stage.

First Antennae (Plate II, Figure 2).—Instead of three setae of about the same size, as found in Stage I, there are now two very much reduced slender setae and one long stout aesthete. *E. analoga* differs in this respect from both *E. asiatica* and *E. talpoida.*

Second Antennae (Plate II, Figure 7).—As in Stage I.

Mandibles.—As in Stage I.

First Maxillae.—The first maxillae now have six plumose setae issuing from the tips of the exopods.

Second Maxillae.—One additional seta is present on the margin of the scaphognathite.

First and Second Maxillipeds.—Each of these appendages now has six plumose setae on the tips of the exopods. No rudimentary appendages were present posterior to the maxillipeds.

Telson.—The telson is longer than wide. The number of spines on the proximal margin remains as in Stage I, but the number of denticles between the spines has increased.

STAGE III (Plate I, Figure 4)

Average sizes:

Maximum width of carapace	1.3 mm.
Maximum length of carapace	1.6 mm.
Length of rostral spine	2.1 mm.
Length of lateral spine	1.4 mm.

Stage III differs from the preceding stage mainly in size and in the presence of uropods on the telson. Two more setae make their appearance on the distal ends of the exopods of the first and second maxillipeds. The eyestalks have enlarged and now extend at an oblique angle, downward and forward. This is a continuation of the shift from a lateral position in the early stages to a position parallel to the median line as in the adult. In agreement with Menon, it appears that Smith's description and figures of what he (Smith) calls Stage two in the development of *E. talpoida,* correspond more closely to Stage III.

First Antennae (Plate II, Figure 3).—The first antennae have grown slightly longer and more slender, and bear three terminal aesthetes of unequal length.

Second Antennae (Plate II, Figure 8).—These are now slightly longer than the first antennae.

Mandibles.—As in preceding stage.

First Maxillae (Plate II, Figure 14).—As in preceding stage.

Second maxillae have eleven setae on the outer margin of the scaphognathite, being an increase of one over the previous stage.

First and second maxillipeds now bear eight plumose setae on the tips of the exopods, but there is no other change except general growth.

No other thoracic appendages appear posterior to the maxillipeds. Both Smith and Menon found rudiments of such appendages in *E. talpoida* and *E. asiatica* respectively. These rudiments may occur just prior to the molt to the fourth stage.

The *abdomen* is still without pleopods.

The *telson* is unchanged except for development of uniramous rudiments of uropods on its anterior ventral side (Plate II, Figure 23). These are each composed of a short unjointed basal segment and a long, curved, flattened lobe extending from it. This lobe becomes the exopod of the appendage in the later stages and is tipped with two long and one short setae.

STAGE IV (Plate I, Figure 5)

Average sizes:

Maximum width of carapace	2.0 mm.
Maximum length of carapace	2.4 mm.
Length of rostral spine	3.9 mm.
Length of lateral spine	2.2 mm.

This stage shows the first evidence of additional thoracic appendages and rudimentary pleopods on the abdomen. The number of plumose setae on the exopods of the first and second maxillipeds has increased to 16.

First Antennae (Plate II, Figure 4).—In addition to the three terminal aesthetes found in stage three, there are now eight more, distributed in two groups of three each and one group of two located on the inner side. Smith found only five or six in *E. talpoida* while Menon shows five for *E. asiatica.*

Second Antennae (Plate II, Figure 9).—These have grown considerably and the flagellum is now nearly as long as the dentiform processes. A small setose spine is situated at the base of the inner dentiform process. This appendage agrees generally with its equivalent in *E. talpoida* and *E. asiatica,* the difference being in the growth of the flagellum and in the number of spines on the dentiform processes.

Mandibles as in preceding stage.

First Maxillae (Plate II, Figure 15).—The inner lobe has elongated somewhat and bears one more small spine slightly proximal to the group of three on the distal end.

Second maxillae have now each 29 setae on the scaphognathite; an increase of 18 over the number in Stage III.

First and second maxillipeds differ from Stage III only in that the number of long plumose setae on the tips of the exopods have increased to 16. *E. asiatica* and *E. talpoida* each possess a total of only ten setae on these parts, which corresponds to lower Stage IV (see below) in *E. analoga.*

The additional thoracic appendages that are more fully developed in later stages appear as rudiments just posterior to the second maxillipeds. These consist of a third pair of maxillipeds and four pairs of legs which develop into walking legs in the first post-zoeal stage. Rudimentary gills appear above each pair of legs and are partially hidden by the margin of the carapace.

The *abdomen* is unchanged except for the presence of slight projections on the first four segments which were taken to be rudiments of the pleopods.

Uropods (Plate II, Figure 22).—The uropods have developed considerably over the preceding stage. The setae on the ends of each exopod have increased to eight which is three more than found in *E. asiatica* and four more than were reported in *E. talpoida*. These setae are of unequal length as was also reported for the other two species. Rudimentary endopods have appeared.

The *telson*, now narrower and longer, has no increase in number of marginal spines but the number of denticles between these has increased slightly. Three to six denticles were found between the spines near the median line and 25 between the last spine on the ventral margin and the large spine at the angle.

It should be mentioned here that a number of specimens were examined that appear to be intermediate between Stage III and Stage IV, being somewhat nearer the latter and may therefore, for convenience, be called "Lower Stage IV." It is not clear whether they represent a distinct instar between the above stages or are simply variables in Stage IV. In size they are comparable to the smaller specimens of that stage. The distinguishing features of Lower Stage IV are: ten setae on the tips of the exopods of the maxillipeds, a small precursory rudiment of the second antennal flagellum of later stages, absence of rudimentary thoracic appendages back of the second maxillipeds and no rudimentary endopod buds on the uropods.

STAGE V (Plate I, Figure 6)

Average sizes:

Maximum width of carapace	2.6 mm.
Maximum length of carapace	3.5 mm.
Length of rostral spine	4.2 mm.
Length of lateral spine	2.1 mm.
Length of abdomen, including telson	3.6 mm.
Width of telson	1.9 mm.
Length of telson	2.8 mm.

Stage V, which is the last zoeal stage, shows its greatest advancement over preceding stages mainly in the development of pleopods on all four segments of the abdomen and in the increased development of the thoracic legs. The flagellum of the second antenna is now conspicuous from a dorsal view of the animal.

First Antennae (Plate II, Figure 5).—The aesthetes have increased to fifteen, quite clearly arranged in groups: a terminal group of three long and one short, followed proximally by three successive groups consisting of three long aesthetes in each group. These in turn are followed by two solitary aesthetes. This arrangement is similar to that

found in the descriptions of Smith and of Menon for their species, but the numbers are greater by five in *E. analoga*.

Second Antennae (Plate II, Figure 10).—The flagellum has elongated to about three times the length of the dentiform processes. The distinct segmentation of the flagellum and of the first antenna as shown for *E. asiatica* was not in evidence but this condition becomes obvious only shortly before the zoea moults to the first post-zoeal stage.

Mandibles (Plate II, Figure 12).—No change.

First maxillae as in preceding stage.

Second maxillae (Plate II, Figure 17).—These now have 33 setae along the outer margin of the scaphognathite which agrees with the asiatic species. There are no setae on the inner margin.

First and second maxillipeds (Plate II, Figure 19).—These appendages show no increase in the numbers of setae on the exopods, the number remaining 16. The asiatic species has an increase from 10 in Stage IV to 12 in Stage V.

The other thoracic appendages are imperfectly segmented; the third pair of maxillipeds having three segments, and the legs each having a three-segmented basis and a one-segmented exopod, and a very rudimentary endopod.

The pleopods show no evidence of segmentation or other differentiations. They are still uniramous.

Uropods (Plate II, Figure 21).—The endopod is now approximately three-fourths the length of the exopod. The latter bears eight curved setae of unequal lengths. The endopod is unarmed. These appendages agree with the descriptions *E. talpoida* and *E. asiatica* except for the number of setae, the former having six present in this stage and the latter seven.

No attempt was made to rear the first post-zoeal stage from the fifth zoeal larva, and the numerous small specimens collected on the beaches were not studied sufficiently to warrant a detailed report. In view of the few fifth zoeal stages found in the plankton, and none of these being

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PLATE III

Blepharipoda occidentalis—first zoea

FIGURE 1. Mandible.
FIGURE 2. First maxilla.
FIGURE 3. Second maxilla.
FIGURE 4. Larva, lateral.
FIGURE 5. Larva, dorsal.
FIGURE 6. Second antenna.
FIGURE 7. First antenna.
FIGURE 8. Second maxilliped.

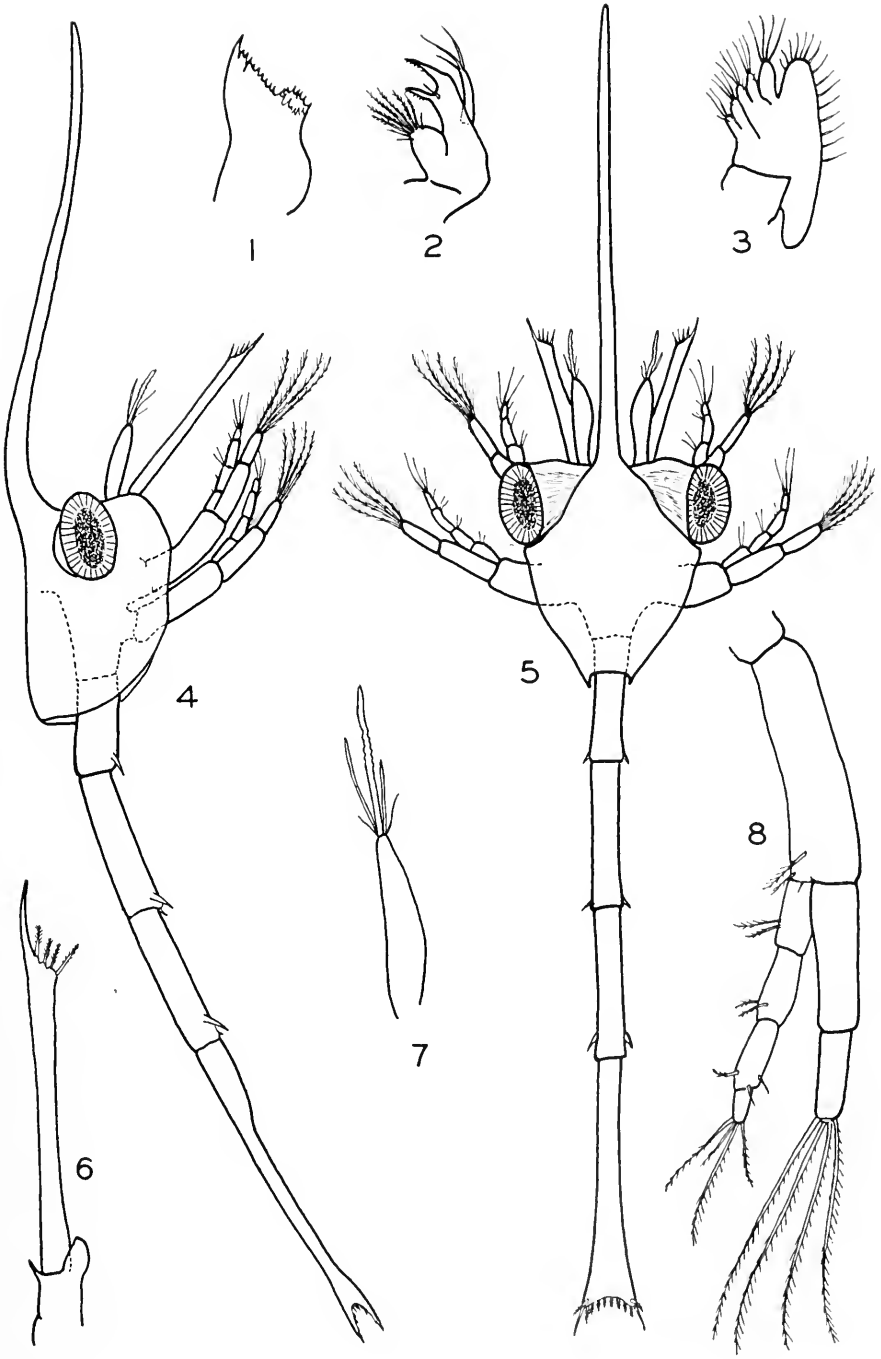

PLATE III

taken in the immediate vicinity of the beaches, it is a most profound mystery how these scattered planktonic stages or post-zoeal stages become sufficiently aggregated to account for the thousands of early post-zoeal stages constituting the large swarms found buried in the intertidal sands.

Blepharipoda occidentalis and Lepidopa myops

The adults of these two species occur in small numbers on sandy bottoms along the coast of southern and lower California. Though this study of their pelagic larval stages is incomplete, it is sufficient to enable distinguishing them from those of Emerita, and to make possible their identification for future studies on larval dispersal through water movements off the west coast.

The first zoeal larvae of Blepharipoda and Lepidopa were readily obtained by confining gravid females in small aquaria with sand and supplied with running sea water until the larvae hatched from the eggs which they carried. These stages are found sparingly also in the inshore plankton. Additional verification of the identity of the first stage of the former was also made from larvae reared by Mrs. S. Davis. No later larval stages have been obtained through rearing, but a plankton sample taken from 200 to 0 meters 30 miles off San Diego yielded a late zoeal stage of Blepharipoda.

Contrasted with the first zoea of Emerita, the corresponding stages of these sand crabs are strikingly distinctive. In Blepharipoda this is clearly reflected in the late stage as shown below, and the same is doubtless true also of Lepidopa. In Emerita it was necessary to know the anatomy of the second zoeal stage before the distinguishing features common to the later stages were recognizable. In that species the structure of the telson is the only striking characteristic carried forward to the successively older stages.

Blepharipoda occidentalis—first zoea (Plate III, Figures 1–8)

Carapace length not including spines	1.3 mm.
Carapace width	1.0 mm.
Rostrum	2.5 mm.
Abdomen including telson	3.5 mm.

Plate IV

Blepharipoda occidentalis—late zoea

Figure 1. Larva, dorsal (appendages omitted).
Figure 2. First antenna.
Figure 3. Second antenna.
Figure 4. Larva, lateral.
Figure 5. Urosome with uropod.
Figure 6. Third maxilliped.

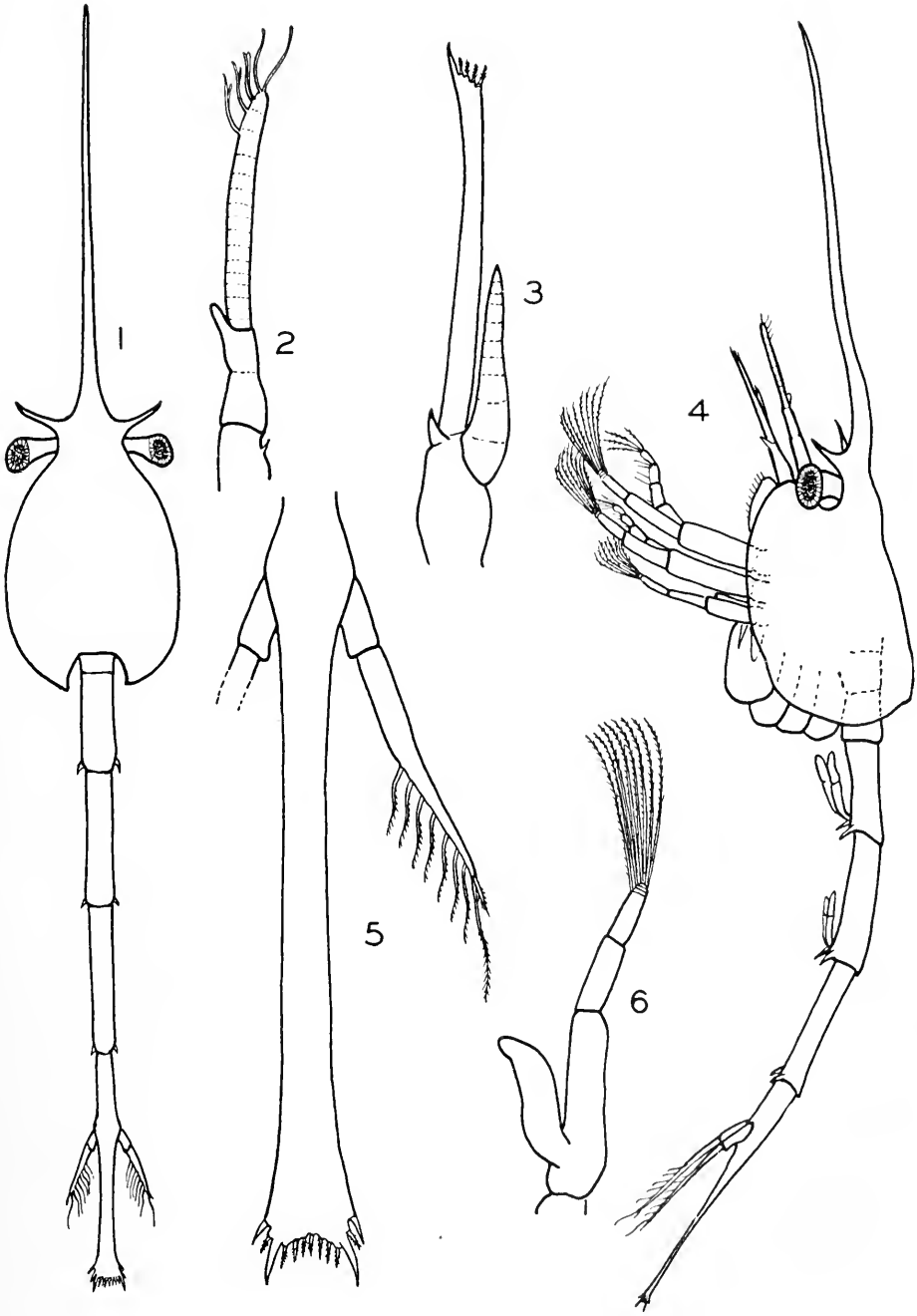

PLATE IV

The general features which sharply distinguish the larvae of this species from the corresponding stage in Emerita are the long rostral spine, the long, slender abdomen, and the narrow telson. It is separated from Lepidopa by the absence of lateral spines and by the character of the telson. Other details of the appendages as given below are also distinctive.

First Antennae (Plate III, Figure 7).—These appendages each bear a group of three terminal aesthetes and two small setae. They are relatively indistinctive in the three species discussed.

Second Antennae (Plate III, Figure 6).—These are very characteristic, each with a long distal segment, i.e. the scale or exopod, terminating in a curved spine-like process and four shorter setose spines on the inner side. The flagellum or endopod appears as a short rounded knob.

The *mandibles, first maxillae* and *second maxillae* (Plate III, Figures 1, 2 and 3) are in general not distinctive from the other species, though all are less rudimentary than in Emerita.

First and *second maxillipeds* are similar (Plate III, Figure 8). The exopod consists of two long segments and a very short terminal segment. Thus the segmentation differs from the other two species in that Emerita has but one distinguishable segment while Lepidopa has one long and two short segments. In each species however there are four long terminal setae. The endopod consists of four segments as in Emerita.

The *telson* (Plate III, Figure 5) is very narrow, with a thin concave posterior margin armed with two lateral smooth spines between which is a row of eight shorter setose spines. On the dorsal surface of the telson a short setose spine is situated at the base of each of the smooth spines.

Late zoea (Plate IV, Figures 1–6)

Carapace length not including rostrum	4.5 mm.
Carapace width	3.0 mm.
Rostrum	6.5 mm.
Abdomen including telson	10.0 mm.

PLATE V

Lepidopa myops—first zoea

FIGURE 1. Mandible.
FIGURE 2. First maxilla.
FIGURE 3. Second maxilla.
FIGURE 4. Larva, lateral.
FIGURE 5. Larva, dorsal.
FIGURE 6. First antenna.
FIGURE 7. Second antenna.
FIGURE 8. Second maxilliped.

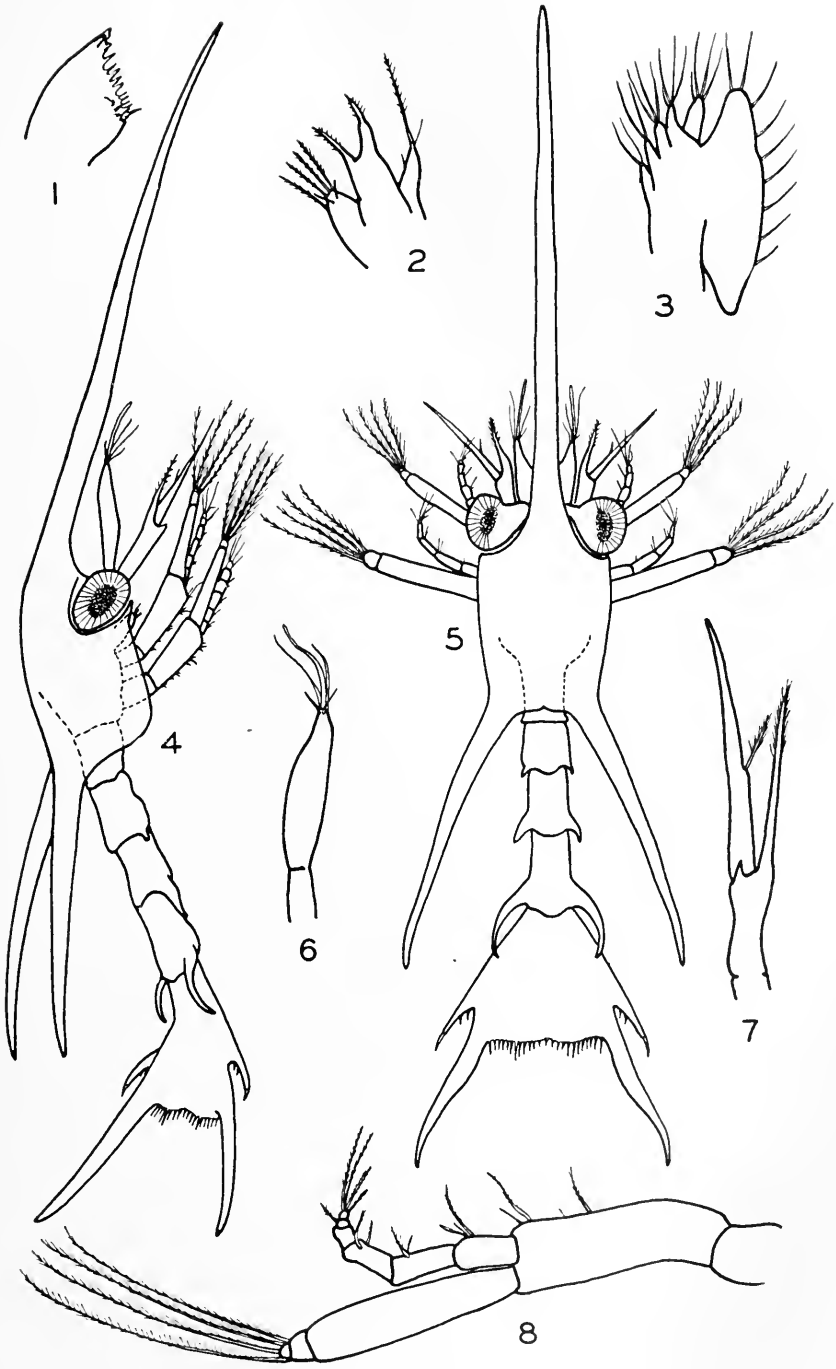

PLATE V

The most pronounced differences between this and the first zoeal stage are found in the development of (1), a pair of strong anteriorly directed spines on the carapace at the base of the rostrum; (2), a pair of slender uropods on the telson; and (3), third maxillipeds and rudiments of other thoracic and abdominal appendages as shown in Plate IV, Figure 4. The number of terminal setae on the exopod of the first, second, and third maxillipeds is eight. The scale or exopod of the second antenna has not changed but the flagellum has increased in length and shows segmentation under the skin. The telson armature has changed only by addition of a spine at each side of the posterior lateral angles. The eyes have become relatively much smaller and have very slender stalks, presaging the adult condition which perhaps will follow the next moult.

Lepidopa myops—first zoea (Plate V, Figures 1–8)

Carapace length not including rostral or lateral spines	1.2 mm.
Carapace width	0.8 mm.
Rostrum	2.8 mm.
Abdomen including telson	2.2 mm.

Distinguishing features characterizing this stage are: (1), the long rostrum; (2), carapace with long posterior lateral spines directed backwards and reaching well beyond the last abdominal segment; (3), relatively broad abdomen with conspicuous lateral spines which on the last abdominal segment are especially strongly developed; and (4), the very broad triangular telson provided with two pairs of heavy lateral projections between the larger of which is a series of 24 small spines. Smaller but distinctive features are found in the second antenna (Plate V, Figure 7) and the first and second maxillipeds (Plate V, Figure 8) the endopod of which consists of six distinct segments.

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PRECIPITIN REACTIONS AND SPECIES SPECIFICITY OF MONEZIA EXPANSA AND MONEZIA BENEDENI

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Introduction

Speciation has long been one of the difficult problems of biology. It is the purpose of this experiment to apply the precipitin test to the study of speciation. The value of the precipitin test in studies of this type lies in the fact that it is an objective test, reducing the subjective interpretations of the investigator to a minimum.

This study is a continuation of those done by Doctor R. W. Wilhelmi (1940). The author wishes to express his sincere appreciation to Doctor H. W. Stunkard under whose direction the work was done.

Material and Methods

The antigen was prepared by the same method as that of Wilhelmi (1940). The worms were washed through copious amounts of Ringer's solution. Following this treatment, they were washed through sterile physiological salt solution to reduce bacterial contamination, and finally through sterile distilled water to remove excess salts. The worms were frozen rapidly with dry ice, and then placed in a vacuum desiccator until dehydrated. The worm material was then removed from the desiccator and ground to a fine powder with an agate mortar and pestle. It was again placed in the vacuum desiccator to insure complete dehydration.

The lipids were removed from the powdered material by extraction for twenty-four hours with Bloor's solution at room temperatures. After the lipids had been removed, the saline-soluble material was extracted with a buffered physiological salt solution (pH 7.2 to 7.4). This was filtered through sterile fritted Jena glass filters. The clear, sterile filtrate contained the soluble proteins and carbohydrates of the worm material. The substances that did not go into solution, of course, constitute the saline-insoluble worm material. The concentration of soluble materials in the clear solution was determined by subtracting the weight of the insoluble residue from the weight of the lipid-free material.

The saline-soluble material was stored in the refrigerator until needed for injections.

For the production of antisera, injections were made into the lateral ear veins of healthy rabbits of the same age and weight. The antigen was injected in four doses, at three-day intervals. With each successive injection the dosage was increased, viz., 4 mg., 8 mg., 12 mg., and 16 mg., making a total of 40 mg. of antigen injected into each rabbit.

Two weeks after the last injection, blood was drawn by heart puncture, using a sterile syringe fitted with a large sterile hypodermic needle. The blood was allowed to clot and the serum, after centrifugation, was kept in the refrigerator in sterile flasks until needed for precipitin tests.

The precipitin reaction was performed using the ring technique. For each test 0.3 cc. of antiserum was carefully overlaid by 0.5 cc. of antigen. The dilution of antigen is expressed as grams per cubic centimeter.

Observations and Results

Homologous and Heterologous Titers*

Antiserum \ Antigen	*Monezia expansa*	*Monezia benedeni*
Monezia expansa	1 : 4000	1 : 1000
Monezia benedeni	1 : 1000	1 : 4000

* Tests were performed in triplicate. The antigens were prepared from several specimens of *Monezia expansa* and *Monezia benedeni*, respectively. In each case mature proglottids were removed from each of the strobila, fixed and stained in order to be certain of the specific identity.

The homologous titer of antisera developed against lipid-free, saline-soluble antigenic material for both *M. expansa* and *M. benedeni* was 1 : 4000. The heterologous titers for both species were 1 : 1000. The latter titers were confirmed by reciprocal tests.

Discussion

Wilhelmi (1940) reported: "'Species' of helminths may be defined tentatively as a group of organisms the lipid-free antigen of which, when diluted to 1 : 4000 or more, yields a positive precipitin test within one hour with a rabbit antiserum produced by injecting 40 mg. of dry-weight, lipid-free antigenic material and withdrawn ten to twelve days after the last of four intravenous injections administered every third day."

He tested nine species of helminths from nine different genera. The homologous reactions all took place at dilutions of 1 : 4000 or greater and none of the heterologous reactions would take place at a dilution as high as 1 : 4000. These experimental facts were the bases for his tenta-

tive definition of a species. However, he did not determine the heterologous titer of two species in the same genus.

In the present work the homologous and heterologous titers were determined for *M. expansa* and *M. benedeni*, two species in the same genus. The homologous titers for both species were 1:4000. The heterologous titers were 1:1000, confirmed by reciprocal tests. Therefore, the results of this experiment confirm the definition of a species advanced by Wilhelmi (1940) and demonstrate that the precipitin test is sensitive enough to differentiate between two species in the genus *Moniezia*.

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PARAFOLLICULINA VIOLACEA (GIARD) AT WOODS HOLE

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The purpose of this communication is to show that the ciliated infusorian, *Parafolliculina violacea* (Giard), is a continuing member of the marine fauna at Woods Hole, Massachusetts.

The family, Folliculinidae, was formed to separate from the stentors these one-celled animals that have greater perfection of feeding apparatus through development of right and left lobes giving bilateral symmetry to a creature generally sedentary. In the life cycle there are frequent transformations from sedentary to motile phases and back again, and it is notable that the complex feeding apparatus of the sedentary phase undergoes complete involution before the motile phase is formed. The motile phase may be the whole body or only half of the body of the sedentary phase. The greatly simplified motile phase is like a larva, but, free swimming, it distributes the species and soon regrows the needed feeding apparatus before it functions as the next sedentary form.

While the sedentary form is highly contractile and irritable, it is the swimming form that shows special responses in selecting its site for building its follicle. In some species such responses lead to the sedentary form being found only in very restricted habitats, and in others lead to association of individuals in colonies.

The number of species in the Folliculinidae was judged to be 25 by Kahl in his review of the ciliated protozoa, in 1930–35. Most of these were described from the coasts of Norway and France, and from the Adriatic.

The Folliculinidae known at Woods Hole, Massachusetts, are all species previously described from Europe, and they have been spoken of under the following eight names: *Semifolliculina boecki* (Clap. Lach.) and *Parafolliculina violacea* (Giard) (Andrews, 1921); *Folliculinopsis producta, Parafolliculina amphora, Folliculina elegans,* and *Folliculina simplex* (Fauré-Fremiet, 1936); *Folliculina elegans, F. aculeata,* and *F. viridis* (Dewey, 1937). Moreover, in 1915, Dons stated that his *Semifolliculina spirorbis* of Norway was also found on materials from the east coast of North America and this may well have been Woods

Hole. But granting nine names so far accredited to Woods Hole, some are synonyms, and four or five species may be all known there.

One of these is quite different from the rest and unusually well characterized, *Parafolliculina violacea* (Giard), but as yet credited to Woods Hole merely on observation of one specimen, by the present writer in 1921. Renewed examination of that specimen occurring with several *Semifolliculina Boecki* Dons attached to Obelia mounted early in this century, along with study of four like ones mounted about the same period, has been supplemented with examination of two specimens on Obelia attached to laminaria from Woods Hole in late summer of 1941.

Students of this group agree that the very resistant, horny or chitinous follicles secreted by the swimming form furnish in their shapes and dimensions good specific and even generic characters.

The illustration (Figure I) represents two views of one follicle with enclosed sedentary animal as preserved with the Obelia in late summer of 1941, and so subjected to action of epsom salts followed by formalin with distortion of the form of the animal.

The full face and the profile views differ so much that older observers thought there were two forms of follicle but Fremiet saw that these were but the two aspects of any one follicle.

Standing erect, firmly attached by a rounded disk, the follicle resembles a bottle with body, or sac, much darker than the tube or neck which swells out right and left where it joins onto the body. This swelling has been called annular, but profile views show little of it. The body of the bottle is much flattened toward its top, and broad from side to side.

The cavity of the neck expands as the vestibule where it joins the body of the bottle and partakes of its flattening so that in two instances the vestibule was 50 micra wide but only 30 or 37 micra deep. In this region are the characteristic and unique valves formed by a membrane rising up from the edges of the body inside the vestibule, and with the same dark tint.

This membrane projecting into the vestibule cavity has a slit along its middle from right to left which is commonly held nearly closed by the elastic membrane but can be opened by the emerging animal and in this illustration one rather plasmolized arm of the animal is seen sticking up through the slit in the membrane. When the animal withdraws into the body of the follicle, the collapsing dorsal and ventral halves of the membrane act as valves to check intrusion from above but readily yield to pressure of the elongating animal that is to reach up its arms into the outside water to collect food.

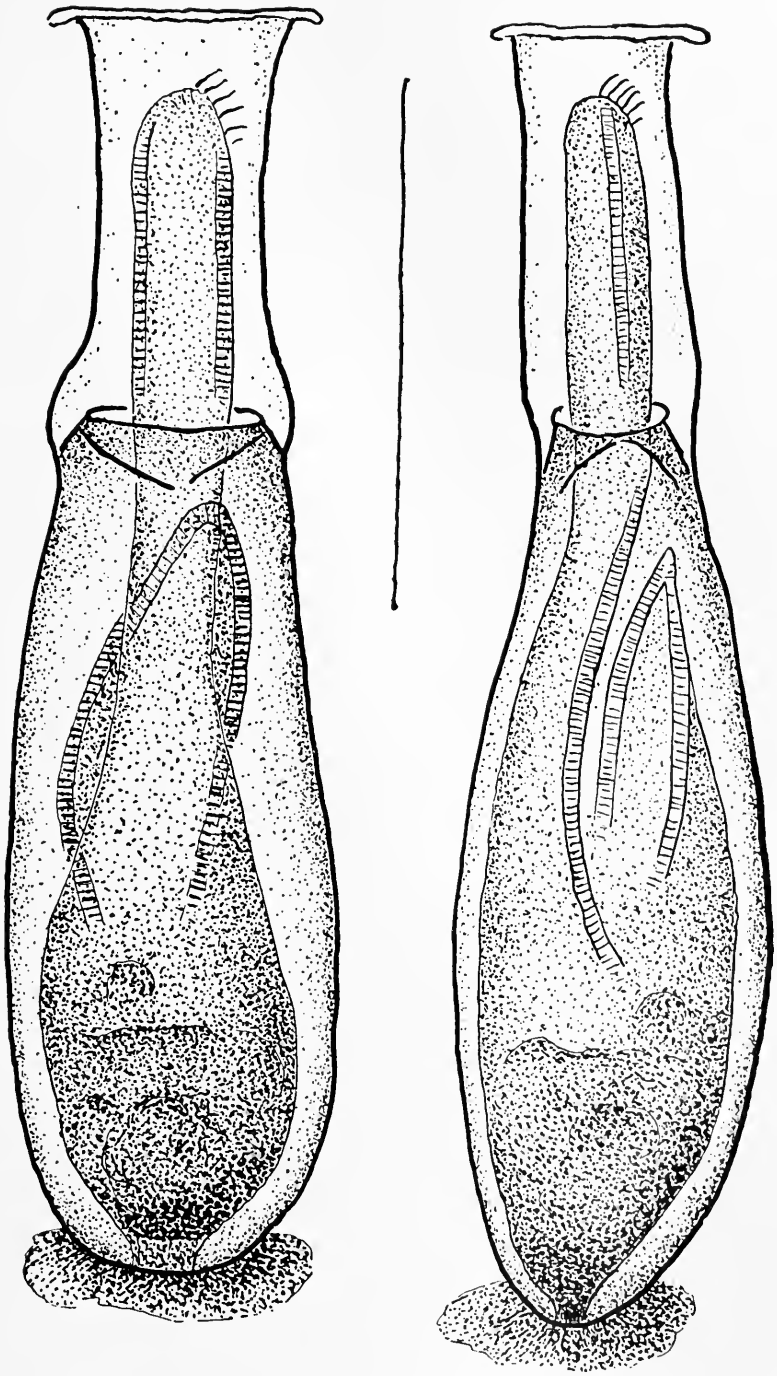

FIGURE I. Full-face and profile views of one follicle with preserved animal taken on Obelia and laminaria at Woods Hole, Massachusetts, in late summer of 1941. The line represents 100 micra.

Fremiet observed that the valve membrane was made as an afterthought by the swimmer that first secreted the body, then the neck, then the rounded collar or lip and, finally withdrawn down into the body, secondarily secreted an inner continuation of the body wall as the above membrane, which might be likened to an inner collar and was made by the collar-making organ of the swimmer.

Typically, the color of this species is not the usual green but a rare reddish-blue or violet. In these Woods Hole specimens, after preservation, the color contains a red element that makes the follicle brown or olive, while the attaching disk is darker and sometimes violet.

Both Hans Laachmann and Dons found a duplication of the upper half of the wall of the sac as an inner mantle that continues the valve membrane downward. Two of the Woods Hole specimens show this mantle in full face view but in profile only as creases. It was not observed in the specimen here illustrated, however, and may not be a constant character.

Another variable is the form of the lower end of the sac which in these few specimens is rounded, as in our illustration, and in most of the follicles figured by Dons, though Fremiet found flat bases in France and Hadzi shows one with a very flat base. He even found follicles reclining and fastened all along the side face of the body or sac.

The neck and vestibule of the follicle we illustrate was closely entwined by three turns of a clear spiral; although this suggested the spiral ridge known in some other species, we interpret it as accidental investment by a filamentous plant.

Of the nine specimens from Woods Hole, three were empty follicles and the others showed but remnants of the animal, yet some of these, as in the figure, show the chief anatomical features. Some of them revealed the usual spheroidal nucleus not seen in the one illustrated. Most all these were found associated with Obelia on laminaria from 5–10 feet depths and were in company with *Semifolliculina boecki* of Dons. Elsewhere this species occurs on various substrates but it may be noted that Dons found several dozens widely scattered on laminaria on the coasts of Norway, and Giard found them on laminaria on the coast of France. Failure to find this species on large collections of laminaria with Obelia and other hydroids and bryozoa collected at Woods Hole in January and in March 1942 adds to the suspicion that this species vanishes from shallow waters in winter.

The general uniformity in size and proportion of these animals is shown in the following measurements [1] of seven follicles, of which the first is that mentioned in 1921:

[1] All dimensions given are in micra.

Total length: 250, 250, 225, 230, 288, 240, 245.
Length of sac with valves: 168, 165, 150, 155, 168, 157, 152.
Width of mouth and collar: 42, 37, 32, 42, 45, 40.
Width of neck: 30, 32, 25, 42, 33, 35, 33.
Width of vestibule: 51, 42, 50, 52, 50, 50, 48.
Greatest width of sac: 63, 58, 62, 63, 60, 60, 65.
Greatest depth of sac: 50, 63, 58, 50, 63, 63, 50.
Least depth of sac: 25, 25, 32, 25, 33, 30, 25.

Also, the diameter of the nucleus was 18 and 25 in specimens third and fifth, and the diameter of the attachment disk was 35, 50, 37, 23, in the last four. The height of the valves was 12 and 13 in the fifth and the seventh.

While previous measurements made by authors do not include so many dimensions, it is evident that these Woods Hole specimens fall well in line with the others of this species. Thus Dons in Norway found that the total lengths ran from 260–310; Fremiet in France 230–260; Laachmann in Australia 200–260. The sac length given by Fremiet was 155–180. Its greatest width 75 in France and 60–70 in Australia, 55–90 in Norway. The only other measurements were greatest depth of sac in France 60; greatest width of mouth in Australia also 60. Laachmann states the nucleus measured 15.

Parafolliculina violacea (Giard) has been found on the north and the west coasts of France, on the Norwegian coast, in the Adriatic and on the west coast of Australia, as well as Sumatra. On this side of the Atlantic it occurs not only at Woods Hole but at Beaufort, North Carolina, in Chincoteague Bay, near the mouth of York River and in Tangier Sound of the Chesapeake Bay, and on the north coast of Long Island; as will be shown in other papers.

Summary

Parafolliculina violacea (Giard) was living associated with other Folliculinids and Obelia at Woods Hole, Massachusetts, early in this century in the summer season in different years. It was again found in small numbers on Obelia and laminaria in late summer of 1941.

In all essential characters and measurements these specimens agree well with those previously known from Europe. Failure to find any on material collected at Woods Hole in January and March 1942 may mean that this species, like some others of this family, has a winter season of scarcity.

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A NEW SPECIES OF ECTOCHAETE (HUBER) WILLE, FROM WOODS HOLE, MASSACHUSETTS

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Nomenclature of the Genus

In 1892 Huber described two new species of algae under the names *Endoderma leptochaete* and *Endoderma Jadinianum* (b, pp. 319–25). In recognition of the fact that these species have setae, he placed them in the section Ectochaete, along with *Endoderma endophytum* (Möbius) Huber, (*Bolbocoleon endophytum* Möbius, 1891, p. 192). Wille (1909, p. 79) raised the section Ectochaete Huber to the position of a genus. He held the view that *E. Jadinianum* Huber was synonymous with *E. endophytum* (Möbius) Huber. A complete description of the genus may be framed as follows:

Ectochaete (Huber) Wille. (Bolbocoleon Möbius *pro parte*, Endoderma Lagerheim *pro parte*). Thallus microscopic, disklike or consisting of interwoven filaments, endophytic in the cell wall of larger algae or imbedded in the slime between the loose cortical filaments of brown or red algae; branching monopodial, but sometimes pseudodichotomous; branches free peripherally and more or less pseudoparenchymatous in the center of either the disk or mass of interwoven filaments; cells uninucleate, cylindrical to round, diameter 5–30 μ, length 1–5 times; many, or at least some, of the cells each provided with a long straight seta; seta not separated by a cross wall from the subtending cell; chloroplast parietal, platelike forming an incomplete girdle, and showing a few perforations occasionally; pyrenoids 1 to about 8; reproduction taking place by biflagellate zoospores or gametes; zoosporangium or gametangium usually saclike, lacking a seta but having a wide neck of varying length; gametes syngamous or parthenogenetic; zoospore, gamete, and zygote attaching themselves to the substratum and germinating directly; in the species growing within the cell wall, germination taking place from the anterior end of the zoöid along the long axis, but occurring at the posterior end at an angle to the long axis of the zoöid in the species found between the free cortical filaments of the host.

A KEY TO THE SPECIES OF ECTOCHAETE (HUBER) WILLE

1. Marine 2
1. Fresh water 3
2. Endophytic inside cell wall of certain green, brown, and red algae; setae few, delicate, with a constriction at the base; cells 5–15 μ diam. by 1–3 times; pyrenoids 1–3 ..... *E. leptochaete*
2. Endophytic in slime between cortical filaments of certain brown and red algae; setae numerous, firm, without a constriction at the base; cells 8–18 μ diam. by 1–5 times; pyrenoids 1–8 ..... *E. Taylori*
3. Endophytic inside cell wall of certain green algae; setae usually few, lacking a constriction at the base; cells 8–30 μ diam. by 1–5 times; pyrenoids 1–6 ..... *E. endophytum*

Localities

E. leptochaete (Huber) Wille. Croisic, Bretagne, Huber in Sept. on a species of *Chaetomorpha*. L'Étang de Thau, Huber in Apr. on Cladophora spp., Chaetomorpha and *Ceramium diaphanum* (Lightf.) Roth. Banyuls, île Grosse, cap du Troc, Feldman, May to June on *Dictyota dichotoma* and *Dilophus fasciola*. Tatihou, Hariot on *Cladophora tenerrima*. Devon, England, Newton, on *Ectocarpus penicilliformis, Ceramium diaphanum* and *Cladophora*.

Geographical distribution: W. Meriterranean: Atlantic coasts of England and France.

E. endophytum (Möbius) Wille. Heidelberg Bot. Gardens, Germany, Möbius on *Cladophora fracta* Kütz. growing in fish ponds. Albères, Pyr.-Or., Jadin and Huber in April and May on *Cladophora*.

Geographical distribution: Europe.

E. Taylori n. sp. Woods Hole, Mass., July to Sept. on *Mesogloia divaricata* (Ag.) Kütz., *Leathesia difformis* (L.) Aresch. and *Nemalion multifidum* (Weber and Mohr) J. Ag.

Ectochaete Taylori sp. nov. Punctiformis, 0.3–0.65 mm. diametiens vel annuliformis et minus quam 7 mm. lata, endophytica, inter filamenta algarum hospitalum penetrans; filamentis ramosis saepe inter se conjunctis pseudoparenchyma formantibus; cellulis cylindricis vel sphaericis, 8–18 micra crassis, 1–5 longioribus quam crassis, uninucleatis; chromatophoro singulo, planato, parietali, pyrenoideis 1–8 praedito atque seto longissimo nec basi septato non constricto; gametangio sacciformi vel irregulari, 8–18 micra crasso, longitudine usque ad quintuplo longiore quam crassiore, ostiolo tubuliformi lato praedito et numerosos zoosporos sexuales biflagellatos emittente, eosdem germinatione epiphyticos.

Habitat in algis variis Phaeophyceis et Rhodophyceis, videlicet Mesogloia divaricata, Leathesia difformi et Nemalio multifido.

Habit endophytic; thallus punctiform, 0.3–0.65 mm. in diameter, or annulate, 7 mm. or less in width, filamentous, embedded in the jelly

present between the cortical filaments of the host; filaments laterally branched, interwoven, partly fused; cells isodiametric to cylindrical, 8–18 microns in diameter, usually 1–5 times as long, uninucleate, pyrenoids 1–8; chloroplast parietal, forming an incomplete ring, platelike; most of the cells bearing a dorsal seta; setae conspicuous, long, straight, not constricted at the base and not separated by a septum from the subtending cell; usually 2.66 microns in diameter, up to 0.8 mm. in length; gametangium saclike or variously shaped, agreeing in diameter with vegetative cells, 1–3 diameters in length, provided with a wide neck about 8–18 microns in diameter and ⅓–5 times as long, with numerous biflagellate, syngamous or parthenogenetic gametes; germination epiphytic in type.

The author has taken the privilege of naming the new species described above after Dr. Wm. R. Taylor, who is closely associated with the phycology of the region of Woods Hole.

Occurrence

The endophyte was restricted to calm bays and grew near low-water mark, appearing when the hosts are mature and taking about four weeks to reach its fruiting stage. The germlings were seen in the last week of July, in 1939 and 1940, in *Mesogloia divaricata* (C. Ag.) Kütz., and fruiting commenced in the last week of August; in September there was evidence that the new crop fruited in a shorter time, namely from two to three weeks.

In *Nemalion multifidum* (Weber and Mohr) J. Ag., fruiting and germinating stages of the endophyte were seen together in 1940, from the middle of August up to the disappearance of the host in the beginning of September. In *Leathesia difformis* (L.) Aresch., the endophyte was in fruiting condition at the beginning of August, 1940 and 1941, and it was still present when the host disintegrated in the middle of the month.

In the summer of 1941 Mesogloia and Nemalion did not appear on Rocky Beach. Early in the summer the rocks were found covered by *Mytilus edulis* L., which perhaps had a harmful effect on the microscopic phases of these hosts.

Winter Phase of the Endophyte

The above hosts are summer annuals that pass through the winter in the form of microscopic plantlets. In *Leathesia difformis* (L.) Aresch. the zoöids of the plurilocular sporangia were found by Sauvageau (1925, pp. 1633–35) to grow into microscopic thalli, and their recapitulation to the third generation by means of apparently asexual plurilocular spo-

rangia was noticed. Kylin (1933, pp. 64–68) obtained microscopic phases from both kinds of zoöids. The plantlets in his cultures coming from unilocular sporangia were sterile, while those from plurilocular sporangia grew into disks, cushions, or plantlets apparently in the early macroscopic stage, all three bearing asexual plurilocular sporangia. Dammann (1930, p. 11) proved that the microscopic plantlets produced by zoöids from the unilocular sporangia bear plurilocular sporangia which are apparently asexual, and he obtained five successive generations from them, all of which in turn showed similar plurilocular sporangia. Thus, though alteration of generations has not been demonstrated in *Leathesia difformis* (L.) Aresch., the development of microscopic phases by both types of zoöids has been observed.

In *Mesogloia divaricata* (C. Ag.) Kütz., Hygen (1934, pp. 258–59) concludes from results obtained in cultures that zoöids from unilocular sporangia give rise to filamentous or disklike gametophytes, that reduplication of the latter occurs by macrogamete parthenogenesis, and that the union of macro- and microgametes starts the large sporophyte.

Nemalion multifidum (Weber and Mohr) J. Ag., according to Knight and Parke (1931, pp. 14, 23, and 24), is a "summer annual" that hibernates in a microscopic juvenile state. The earliest stage of the species is a horizontal branched or unbranched filament or a cell expanse (Chester 1896, p. 342, Figures 1–10; Lewis 1912a, p. 154; Cleland 1919, pp. 342–43, Plate XXIV, Figures 66–94).

It is not likely that the endophyte could be sufficiently protected by growing associated with the above microscopic winter phases consisting of filaments, disks, cushions or, at most, tuftlike plantlets. Also, the circumstance that the zoöids or carpospores are shed at about the same time as the zoöids of the endophyte further reduces the capacity of the microscopic phase of the host for sheltering the endophyte. It is found that the endophytic alga can go through its life history *in vitro,* and is able to exist independent of the host. No resting spores are found either in nature or in cultures. From these facts it appears probable that the endophyte hibernates in the form of juvenile plants which grow protected in minute crevices in rocks.

So far as is known, the summer phase of the endophyte is found only in a few hosts, and these have a cortex of radial assimilatory filaments embedded in a jelly. The compactness of the filaments and the firmness of the jelly may be determining factors in invasion, as would appear from the absence of the endophyte in *Chordaria flagelliformis* (Müller) J. Ag., which was common at Rocky Beach. Here the cortical filaments are more closely arranged and the gelatinous substance is firmer than in Mesogloia and Leathesia (Taylor 1937, pp. 139–40 and 143) or in

Nemalion. *Aegira virescens* (Carmichael) Setch. and Gard. was not found to be invaded, regardless of its very loose cortex and soft jelly, but it should be noted that the latter grows in exposed situations where the sea is quite rough.

Habit

The endophyte is hardly visible to the eye, but when the host becomes discolored and is dying, the cushions of the former appear as recognizable green specks or bands. Viewed with a hand lens, the areas occupied by the endophyte have a fuzzy appearance owing to the presence of setae. The interwoven filaments form a cushionlike thallus, which in Leathesia and Nemalion is punctiform with a diameter of 0.3–0.65 mm. In the strands of Mesogloia it extends into a complete or incomplete ring 0.7 mm. or less in width. In either case the thallus is firmly embedded in the gelatinous matrix present in the cortex of the hosts. Because fusions are common between filaments, the cushion is fairly compact, especially as growing in Leathesia (Plate I, Figures 7 and 10), and in Nemalion (Plate I, Figures 8 and 13), but in Mesogloia fusion takes place to a less extent. In cultures of the endophytes from all three hosts grown from the isolated endophytes, the degree of fusion is the same; the early stage consists of a regular or irregular disk of fused cells (Plate II, Figures 1–6), and later the mature thallus shows entangled filaments with considerable fusions (Plate I, Figure 3).

The tendency of branches to fuse is common to the three species of Ectochaete, though their habit is essentially filamentous. In *E. leptocaete* (Huber *l. c.*, p. 320, Plate XV, Figure 2) the disk is monostromatic with fused cells in the center and free filaments at the margins. *E. endophytum* develops into a polystromatic cushion with colorless cells in the interior (*l. c.*, pp. 322–24, Plate XV, Figures 12 and 13), thus the latter species exhibits greater pseudoparenchyma development than *E. Taylori* or *E. leptochaete* (*l. c.*, p. 325). In host relation *E. leptochaete* and *E. endophytum* differ from the species under discussion in that they enter the cell wall and live within it. In the later stages of *E. endophytum*, however, the cushion may break through and become free.

Setae

Hyaline setae not separated by a wall from the cell below and typically not twisted nor having undulating walls are characteristic of Ectochaete. In the present species they are always numerous when it is growing in its natural habitat, and they are firm and conspicuous, reach-

ing a length within 0.8 mm. and a diameter usually of 2.66 microns. *E. endophytum* is described as bearing similar setae, but they are not numerous; they have been found to develop in large numbers, however, in cultures (l. c., pp. 322 and 324). In *E. leptochaete* the setae are extremely delicate. Feldman (1937, p. 181) used iodized hydriodic acid to detect them and mentioned that they are seen with difficulty when examined directly; in the latter species the seta exhibits a constriction at the point where it emerges from the host. The seta has colorless transparent cytoplasm but has no nucleus. In *E. Taylori* it may break near the tapering apex, and if so it remains open and devoid of contents. When a young seta breaks, proliferation may occur, and if it does, the old part looks like a sheath (Plate I, Figure 12). Similar false sheaths have been demonstrated in *Acrochaete repens* Pringsh. and *Bolbocoleon piliferum* Pringsh. (Huber 1892a, p. 329).

Cell size and structure

The cell diameter varies from 8 to 15 microns and may reach 18 microns. The length is 1 to 3 times the diameter and occasionally 5 times. In *E. endophytum* the diameter is 8–20 microns and may be as great as 30 microns, the length being 1–3 times or as great as 5 times. *E. leptochaete* has a cell diameter of 5–10 microns, sometimes attaining 15 microns, and the length is 1–3 times. The chloroplasts of all three species are platelike or occasionally perforate; their form is that of an incomplete girdle; they are either homogeneous or, owing to the presence of starch grains, granular. *E. leptochaete* has fewer pyrenoids than the other two. In *E. Taylori* the single nucleus in a cell becomes visible after having been stained with acetocarmine.

Gametangia

A large number of nonsetigerous cells are transformed into gametangia. They are usually sac-shaped, because of the presence of a wide dorsal neck. When the gametangia are more or less deeply embedded

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PLATE I

E. Taylori. 1, 2, 3, 4, 5, 12 from Hesogloia. 6, 7, 9, 10 from Leathesia. 8, 11, 13 from Nemalion. (1) Germling showing chloroplasts, 1000 ×. (2) Branch of horizontal system with terminal seta, 450 ×. (3) Gametangia and fusion of filaments (culture), 528 ×. (4) Erect branches in the place of setae (culture), 400 ×. (5) Germling attached to Mesogloia filament and showing growth at an angle to the long axis of zoöid, 450 ×. (6) Gametangia with short necks, 875 ×. (7 and 10) Fused filaments among host filaments, 450 ×. (8) Disco-like germling on surface of host, 400 ×. (9) Gametangia with long necks among host filaments, 450 ×. (11) Germlings, note gametangium, 400 ×. (12) Cell bearing seta with false-sheath, 750 ×. (13) Germling on surface of host, 400 ×.

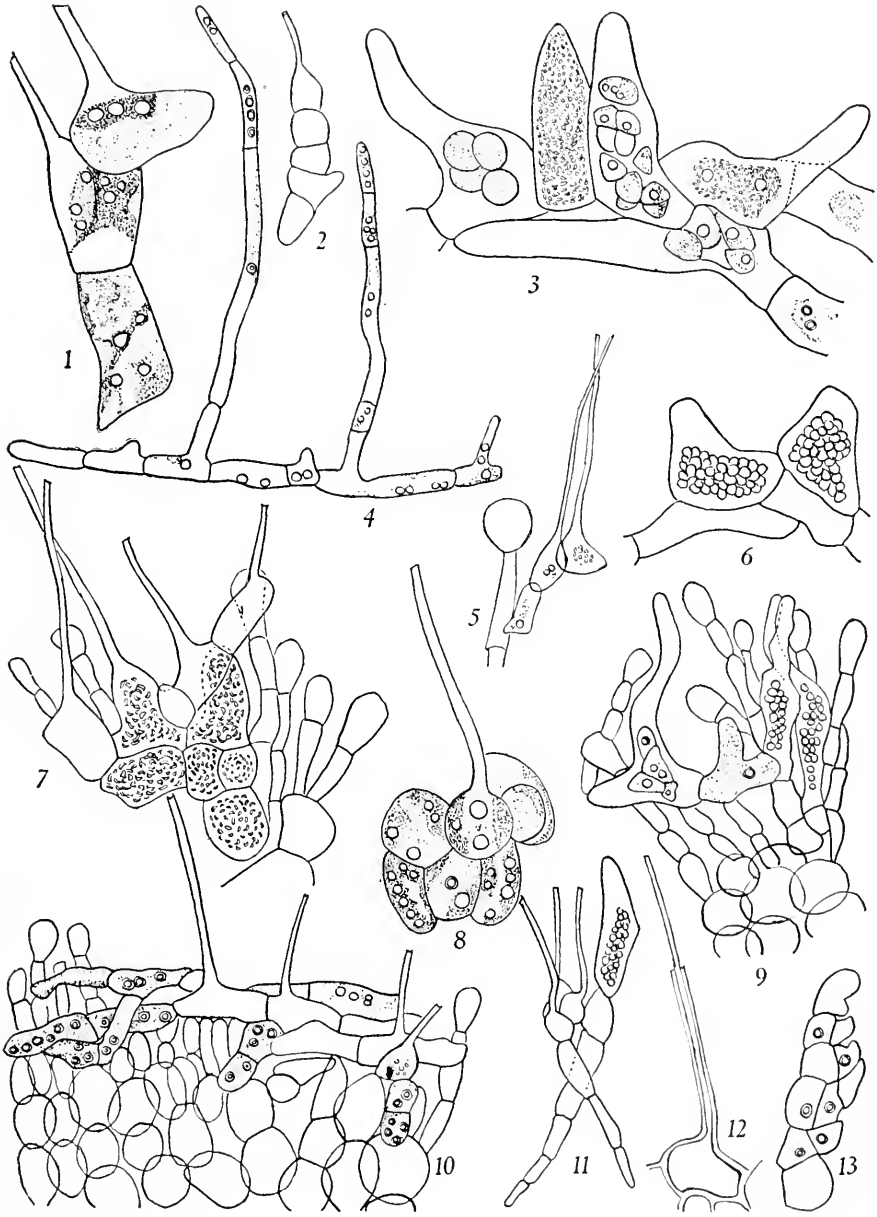

PLATE I

the neck may be two to five times the width of the cell proper, and, further, it may be sinuous (Plate I, Figure 9), but if the gametangia are near the surface of the host the neck is straight and short, being ⅓–2 times the diameter of the cell (Plate I, Figure 6). Without regard to the length the base of the neck agrees in width with that of the cell; it tapers slightly towards the apex, where it is quite rounded and is 2.66–12 microns across. The gametes are numerous, and usually about 30–60; they are biflagellate, ovoid or, sometimes, elliptic; their diameter varies from 1.76 microns to 4.4 microns and may at times be 6.2 microns; in length they are 3.52 microns to 6.2 microns and occasionally reach 8.8 microns. The flagella are 1½ to 2 times the length of the gamete. There is a choroplast with a pyrenoid; an eye spot is present (Plate II, Figures 8*c* and 9*a*). The gametes are expelled suddenly and with considerable force, and in this respect the present alga resembles *Ochlochaete ferox* Huber (1892b, p. 293). Emission of gametes may be easily observed upon exposing sections of the host with fruiting thalli of the endophyte in the illuminated field of the microscope for a few minutes. The contents of the gametangia are seen to stream quickly upwards through the neck and are expelled in a mass to a distance of about 0.4–0.8 mm. beyond the surface of the host, but not beyond the projecting setae. After this they either instantaneously swim, with a rapid motion, in the direction in which they were shot out, or they are motionless for some seconds and gradually begin to swim severally in various directions—avoiding the host, however. At times the swarmers under the cover glass rotated rapidly (Plate II, 9*b*), remaining in one spot as though pivoted on the beak.

The gametes are either parthenogenetic or they unite in pairs. The fusing gametes were found to be equal in size. At times, a pair after joining by their beaks spin rapidly in one spot just as single gametes may do. At the end of a few minutes the spinning stops and the zygote, which is visibly larger than a gamete, moves away with a slow motion. Gametes are more or less positively phototactic, while the zygote is nega-

PLATE II

Ectochaete Taylori. 1, 2, 3, 4, 5, 6 (cultures in 0.375 Detmer sol.), from Mesogloia. 7 from Mesogloia kept in specially aerated sea water. 8 from Nemalion. 9, 10 from Mesogloia. (1) Germination at an angle to the long axis of zoöid, 715 ×. (2, 3, 4) Stages in growth of irregular disklike germlings, 715 ×. (5) Regular disklike germling, 715 ×. (6) Later stage than 5, but prior to development of erect branches, 275 ×. (7) Germling with abnormal setigerous cell, 450 ×. (8) 1000 ×: (*a*) fusing gametes. (*b*) Zygote showing two stigmata. (*c*) gamete. (9) 1000 ×: (*a*) gamete. (*b*) Spinning gamete. (*c*) Fusing gametes. (10) Zygotes. (*a*) 1200 ×. (*b*) Shows two stigmata, 1500 ×. (*c, d*) Show two plastids each, 1000 ×. 8, 9, 10, except 9 *b*, fixed in iodine.

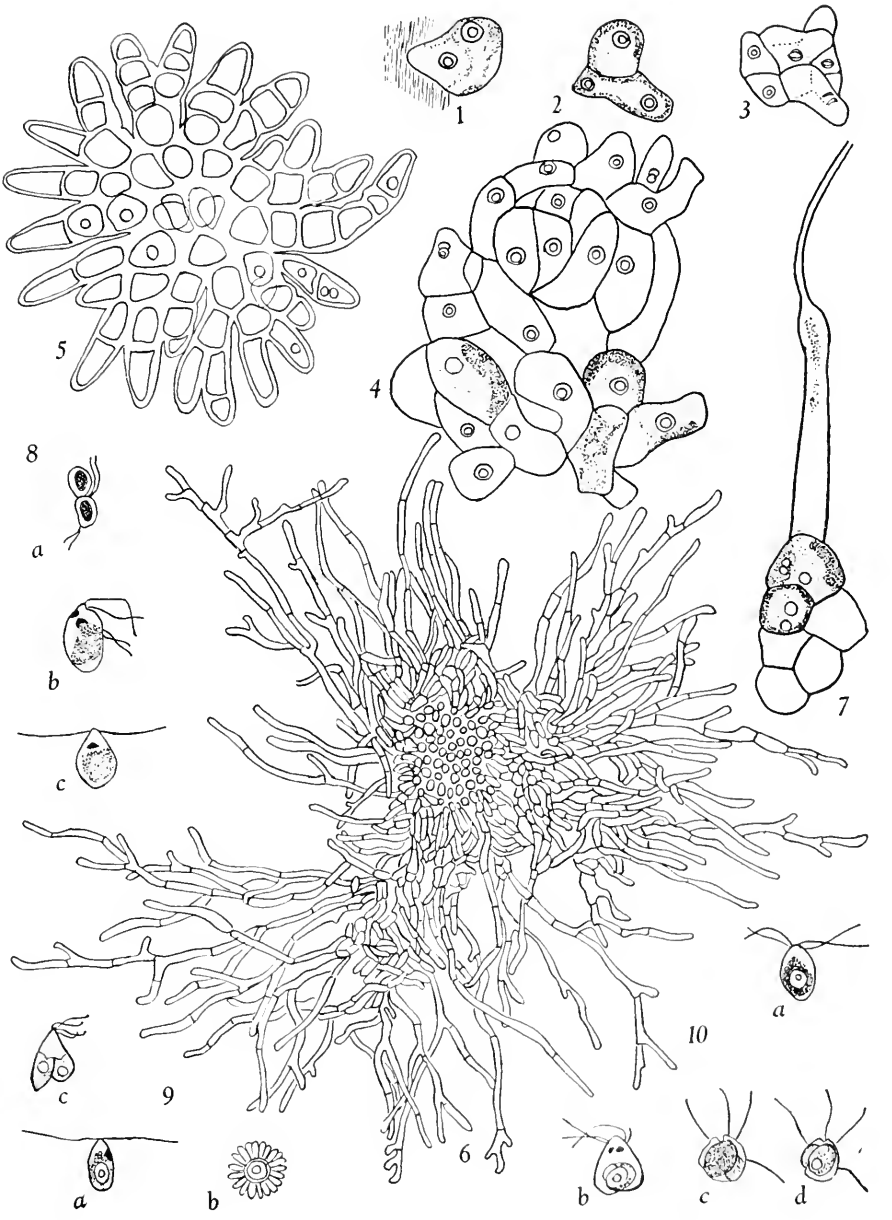

PLATE II

tively phototactic and is soon lost to sight, as it becomes hidden in the host tissue. In some instances fusing pairs of gametes stopped spinning for a time and it was possible to observe that fusion proceeded from the beak to the posterior end. Zygotes were fixed with a 2 per cent solution of I_2. Fusion appears to take place rapidly as a rule, for it is difficult to find examples of intermediate stages of the process (Plate II, Figures 8*a* and 9*c*). Zygotes seen were invariably near the host tissue and about to enter it. In some cases paired chloroplasts, eye spots, and pyrenoids could be recognized in the zygotes (Plate II, Figures 8*b*, 10*b*, 10*c*, and 10*d*).

Sexual reproduction has not been observed in *E. leptochaete* and *E. endophytum,* but germination of biciliate zoöids is known for the former, while in the latter, zoöids have not been seen after liberation, though germination stages were obtained. Huber (1892b, Plate VI, Figure 6) states that the zoospores of *E. leptochaete* are 4–5 microns in diameter and ovoid or round in shape; the flagella appear from the figure to be nearly 3 times the length of the zoospore. This species lacks an eye spot (l. c., p. 325).

The sporangia of *E. leptochaete* and *E. endophytum* are short-necked and resemble the short necked type of gametangium found in *E. Taylori.* In *E. leptochaete* the length of the neck is one-third of the width of the cell (l. c., Plate XIV, Figure 5) and in *E. endophytum* (Möbius 1891, Figures 5 and 7) it is the same as the cell width. Huber observed the liberation of zoospores in *E. leptochaete* but did not relate anything special about the process.

Germination

The gamete or zygote settles on its beak, surrounds itself with a cell wall, and begins to elongate at the posterior end, at an angle to the principle axis, and goes on to develop a filament (Plate I, Figures 1, 5, and 11). In cultures, in the absence of the host, as well as when the alga in nature germinates superficially on its host, the zoöid grows in the same way but usually in several directions perpendicular to the long axis, and thereby forms a disk or irregular expanse of cells (Plate I, Figures 8 and 13; Plate II, Figures 1–6). This type of germination is characteristic of the epiphytic series of prostrate Chaetophoraceae (l. c., p. 350), and it is interesting to find that this species of Ectochaete, though endophytic, still retains a relatively simple form of germination.

In *E. leptochaete* and *E. endophytum* the zoöid, after coming to rest and forming a wall around itself, sends a tube from its anterior end into the host wall. Once within, the germination tube expands in a plane

perpendicular to itself and gives rise to an elongate cell into which the contents of the zoöid pass. *E. endophytum* may sometimes vary slightly from the above process, and its zoöid then grows into a large cell provided with a seta; but it is later that the germination tube grows out from the anterior end.

Germination in the Chaetophoraceae appears to be a somewhat variable character. On the one hand it permits flexibility in the relation of algal habit to host or substratum and on the other it probably helps in bringing about speciation.

The variation in germination within the genus Ectochaete may be compared to that in Stigeoclonium which has two kinds of epiphytic germination. Species like *S. flabelliferum* and *S. variabile* (Berthold 1878, pp. 199–200, Taf. I, Figures 16 and 18) germinate by growth along the principal axis, from the posterior end of the zoöid, that is, in a direction opposite to that obtaining in typical endophytic germination. An erect filament thus is developed, and from its basal cell the horizontal system of branches takes its origin. *S. lubricum* is representative of the second kind of epiphytic germination found here, and its zoöids grow in several directions perpendicular to the principal axis, thus forming the creeping system from which the erect system arises (l. c., pp. 200–201, Taf. I, Figure 9).

Differences in germination between the species of a genus is present also in *Endoderma* Lagerheim. *E. perforans* Huber is one that produces a germination tube at the anterior end of the zoöid. *E. majus* Feldman (1937, pp. 182–83) and *E. testarum* Kylin (1935a, p. 199) are among species that are epiphytic in germination while they are in habit endophytic and shell inhabiting respectively.

Proof of similar variation within a species is afforded by *Phaeophila dendroides* (Crouan) Batters. On certain hosts it is endophytic and on others epiphytic in germination (Huber 1892b, p. 330, Plate XVI, Figures 4 and 7) and in habit.

The above account and comparison go to show that on the grounds of habit, structure, and host relationship *Ectochaete Taylori* appears to be a well-circumscribed species and is distinct from the two previously known species of the genus.

Homology and Function of Setae

In cultures of the alga grown in 0.375 Detmer's solution, it was noticed that the swarmers developed into minute, more or less regular disks (Plate II, Figures 1–5), the cells of which lacked hairs. As the disks grew older they produced horizontal branches in many directions

(Plate II, Figure 6) and still later erect filaments arose from the creeping filaments (Plate I, Figure 4). The former differ from the latter in being slightly narrower, in consisting of only a few very long cells, the chloroplasts of which are rather poorly developed, and in usually lacking a cross wall between the basal cell and the parent filament. When these cultures were transferred back to sea water without additional nutrients, it was found that setae arose from the horizontal system of filaments and also occasionally from the erect filaments formed earlier in these cultures, but it was noteworthy that new erect filaments did not arise. The erect filaments seen in cultures of *E. Taylori* which are comparable to those obtained by Huber (1892a, p. 333, Figure 7) in *Phaeophila dendroides* (Crouan) Batters (*Phaeophila floridearum* Hauck) bear out further the theory (Huber 1892a, p. 222; 1892b, pp. 344–45) that hairs and setae in the procumbent Chaetophoraceae are reduced erect branches of the heterotrichous habit.

Another fact regarding the development of setae in the group is observable in the alga under discussion. It is noticed that both in nature and in cultures the apex of a horizontal filament may sometimes end in a seta which is thus a continuation of the long axis of the filament concerned (Plate I, Figure 2). Attention has been directed to a similar origin of the solid setae of *Gonatoblaste rostrata* Huber (Fritsch 1929, p. 258, Figures G and H). It therefore appears that in the procumbent Chaetophoraceae the reduction of branches and development of setae may occur in the horizontal system as well.

That intact setae with living cytoplasm may possibly function in absorption is suggested by their reappearance on bringing cultures from 0.375 Detmer solution in sea water to plain sea water. In this connection it may be mentioned that Hygen (1934, p. 248) found that, under unfavorable conditions, especially those caused by nutrient deficiency, hair development increased in the microscopic phases of *Nemacystus divaricatus* (Ag) Kuck., and Huber described greater hair development under similar conditions for *Stigeoclonium* (1892a, p. 323–24).

Taxonomic Position

Since *Ectochaete* (Huber) Wille has been segregated from *Endoderma* Lagerheim it requires to be given a place with reference to the genera of procumbent Chaetophoraceae (Huber 1892b, pp. 352–3). Its natural position would seem to be between *Bolbocoleon* Pringsh. and *Endoderma* Lagerheim. The former is characterized by small, bulbous, setigerous cells that represent erect branches, whereas in *Ectochaete* (Huber) Wille the erect system of branches is further modified in that

the setae arise directly from the horizontal filaments. In *Endoderma* Lagerheim the reduction is almost complete and setae are absent.

The reduced number of setae in *Ectochaete endophytum* and *E. leptochaete* and their typical endophytic germination signify that both species are probably nearer to Endoderma than to Bolbocoleon. The simple monostromatic thallus of *Ectochaete leptochaete* would seem to indicate that the latter is the species nearest the level of Endoderma. In contrast, the presence of conspicuous setae, the method of germination, and the general habit show that *Ectochaete Taylori* is the least specialized of the related species and is on the whole more representative of the Bolbocoleon level of development in the group. *Phaeophila* Hauck and *Ochlochaete* Thwaites have a degree of reduction of the erect system of branches similar to Ectochaete, but they differ from the latter in other important respects. Ochlochaete forms definite disks of closely fused cells and is thus more strongly dorsiventral than Ectochaete; in detail, the former differs from the latter in having but one to two pyrenoids in the cell and in its zoospores, each of which is provided with four flagella. Unlike Ectochaete, Phaeophila ordinarily lacks fusions between filaments, and a cell may frequently bear two setae. Phaeophila may be readily distinguished from Ectochaete: its setae are twisted or have undulating walls, the emission tube of its sporangium is narrow, and its zoospores are four-flagellate. The differentiation of the chloroplast into thicker minute disklike areas, referred to by Huber (1892b, p. 328) was not evident in *Phaeophila dendroides* obtained at Woods Hole; nevertheless, its identity was clear when based on the above characters.

The writer wishes to express her thanks to Dr. Wm. R. Taylor for the guidance received in the course of this study, to Dr. Alma G. Stokey for her invaluable suggestions, and to Professor H. H. Bartlett for extending his help in writing the Latin description of the species.

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THE EFFECT OF ENVIRONMENTAL FACTORS ON THE SPERM CYCLE OF TRITURUS VIRIDESCENS[1]

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Introduction

The problem of vertebrate cyclic gametogenesis and its relation to the environment has been the subject of considerable experimental investigation (for reviews, see Bissonnette, 1936, 1937; Marshall, 1936, Rowan, 1938a, b). However, the reproductive cycles of relatively few species of Amphibia have been experimentally studied.

In South African clawed toads, *Xenopus laevus,* Hogben, Charles, and Slome (1931) reported the ovaries of blinded animals to be in a subnormal condition while ovarian regression did not occur in animals in constant light. The so-called "captivity effect," resulting in the loss of sexual activity in Xenopus, was noted by Shapiro and Zwarenstein (1933) and Shapiro (1936) when the animals were kept in the laboratory. Alexander and Bellerby (1935) concluded that the gonads did not degenerate in the laboratory if sufficient food was available and the normal periodicity occurring in nature disappeared. Later (1938) these authors found ovarian regression was induced by decreasing the water volume of the tanks and concluded the seasonal decrease of water volume in Xenopus' ponds was a contributory cause of the ovarian cycle. Bles (1905) had considered this phenomenon earlier. Light was not found essential for reproductive activity (Bellerby, 1938). Hypophysectomy led to gonad regression in both sexes (Bellerby and Hogben, 1938).

Galgano (1931, 1932, 1934, 1936) in a series of studies on *Rana esculenta* found that animals in the laboratory at 18°–24° C. in December or January, without additional light, resumed spermatogenesis when normally there is no gonadal activity at that time. He concluded (1936), "Dalle osservazioni eseguite e sopra succintamente esposte, trassi la convinzione che la spermatogenesi in Rana esculenta fosse potenzialmente per fattori intrinseci di tipo continuo e che soltanto la causa occasionale esterna della temperatura insufficiente determinasse la sua interruzione nella cattiva stagione." Galgano observed that the phase of the

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sperm cycle of animals collected from different localities in Europe and Africa could be correlated directly with the varying temperatures of the diverse localities; Witschi (1924), however, found that in certain races of *R. temporaria* the spermatogenic cycle had become hereditary and was not modified by climatic changes. Shapiro (1937) states that constant additional light of 300 candle power from February 26 to April 12 did not show whether light had any effect on mating and oviposition in *R. temporaria*. Rowan (1938b) and Bullough (1939) reported a personal communication from Spaul and Gladwell that *R. temporaria* reacted by gonad growth, gametogenesis and spawning when subjected to fourteen hours of electric light per day during the winter with a temperature of 20° C. The newt, *Triton cristatus,* also bred under these conditions, while gravid females of the viviparous salamander, *Salamandra maculosa,* gave birth to young when exposed to lengthy illumination, but not otherwise.

In view of the relative incompleteness of the knowledge of this problem in Amphibia, the present study was undertaken to discover if the external environment influenced the sperm cycle of the salamander, *Triturus viridescens viridescens.*

The author wishes to thank Professors J. S. Nicholas and Daniel Merriman under whose direction the study was made, Mr. D. J. Zinn for assistance in collecting animals, and the Choate School where part of the work was done while the author held the Choate Research Fellowship in Biology.

Materials and Methods

Adult male *Triturus viridescens viridescens* of approximately the same size and from the same pond in Hamden, Connecticut, were used in all the experiments.

Conditions in the experimental tanks were kept as uniform as possible except for the variable being tested; the water volume was the same for each animal and all animals were fed a similar diet of ground beef. In most of the lighting experiments the foot candles of illumination were measured five inches below the water surface by a submarine photometer (Zinn and Ifft, 1941); the illumination for each specific group is given in the section on the experiments. Animals kept at low temperatures were kept in an electric refrigerator with a recording thermometer to show the temperature variations.

The effects of the experimental conditions on the spermatogenic cycle were determined by the following methods:

(1) The most anterior lobe of the testis of the right side was removed, fixed in Bouin's, sectioned at 25 microns and stained with Delafield's haemotoxylin. Sections of 25 microns in thickness were suitable for the study, since the long axes of the various cysts range approximately from 100 microns to 500 microns. Planimeter measurements were made from microprojection drawings ($\times$ 10) of every tenth section; primary and secondary spermatogonia were measured together as were the primary and secondary spermatocytes. The percentage of the total area occupied by each spermatogenic stage was then determined. Care was taken to check each measured area with higher magnification ($\times$ 880) to make certain of the spermatogenic stage.

(2) A second method was to make planimeter measurements of the center section of each testis.

(3) A third method was to separate by dissection the sperm areas from the non-sperm areas and weigh each. This separation can be easily made at certain stages of the cycle as the gross appearance (Adams, 1940) of the two areas is distinct and a definite plane exists between them.

Normal Sperm Cycle of Triturus viridescens

The spermatogenetic cycle of *Triturus viridescens* has been described by Hilsman (1932a, b) and Adams (1940). The normal cycle studied in the course of this investigation agrees with that reported by Adams. At the time of the spring breeding season in late April the testes consist of spermatogonial vesicles clearly divided from the region of mature sperm cysts and a varying number of evacuated cysts from the "false" breeding season of the previous autumn. The discharge of sperm continues into June. During the summer active gametogenesis is in progress, and all stages of germ cells may be found until September, when the testes consist chiefly of spermatogonia and mature sperm. At this time the "false" breeding season begins, and varying amounts of sperm are discharged, leaving the evacuated cysts which remain until spring. The females do not ovulate at this time. During the winter months the testes are dormant with the same appearance as that described for the beginning of the spring breeding season. Adams concludes that normally there is one annual cycle of spermatogenesis in *Triturus viridescens* and this is confirmed by the present investigation, but does not agree with Hilsman who states that there are two independent spermatogenetic cycles corresponding with the two "breeding" seasons.

Experimental Results

Experiment I.—The first experiments were designed to determine if light or temperature or combinations of these factors would have any effect during the period of active gametogenesis. Sixty animals were collected on July 27, 1939, and divided into six groups of ten each. For comparative purposes there was added a seventh group which was collected on September 4 and killed on that date.

(1) Ten control animals, normal daylight through glass, temperature, 20°–24° C.

(2) Ten animals with constant light from a fluorescent lamp delivering 32 foot candles at the surface, 13 f. c. at the tank bottom; temperature, 20°–24° C. Two of these animals died from a fungus infection.

(3) Ten animals, bilaterally enucleated (entire ocular globe removed), constant light and temperature as in group 2.

(4) Ten animals in constant darkness; temperature, 20°–24° C. Six of these animals died from a fungus infection.

(5) Ten animals in constant darkness; temperature, 11°–15° C. until September 10 and then lowered to 6°–7.5° C. until September 28. (A change in the refrigeration unit caused this lowering of temperature.)

(6) Ten animals in constant light from a fluorescent lamp delivering 32 f. c. at tank surface and 16 f. c. at bottom; temperature as in group 5.

(7) Five animals collected on September 4 and killed on that date.

Except for group 7 all of these animals were killed on September 28 after a period of 63 days. The testes were sectioned with planimeter measurements being made of every tenth section.

An analysis of the results (Figure 1) indicates: (1) At room temperature all animals showed the same degree of spermiogenesis. That

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Plate I

Sg = Spermatogonia; Sc = spermatocytes; St = spermatids; S = sperm; DgCy = degenerating cysts.

All figures are sections of testes from animals in Experiment I.

Figure 1. From an animal kept in constant light at room temperatures (20°–24° C.) (Group 2). Mag. about × 10.

Figure 2. Control animal; daylight through glass, room temperatures (Group 1). Mag. about × 10.

Figure 3. From an animal kept in constant light at low temperatures (12° C.) (Group 6). Mag. about × 18.

Figure 4. From Group 5 under similar conditions as in Figure 3 except in constant darkness. Mag. about × 18.

Figure 5. In constant darkness, room temperatures (Group 4). Mag. about × 10.

Figure 6. In constant light, room temperatures, with both eyes removed (Group 3). Mag. about × 10.

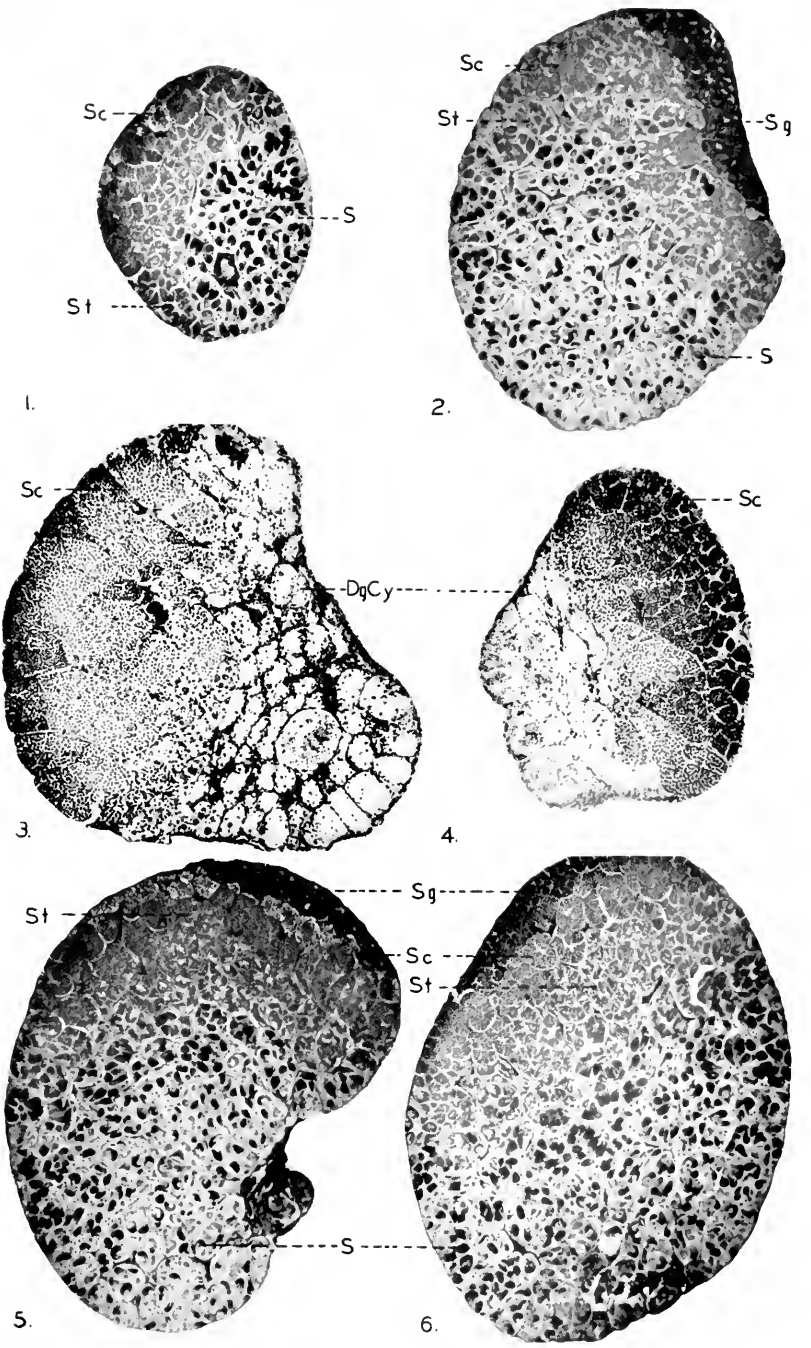

PLATE I

is, approximately equal percentages of mature sperm were found in each of the room-temperature groups irrespective of lighting conditions. The differences in these average percentages were no greater than the differ-

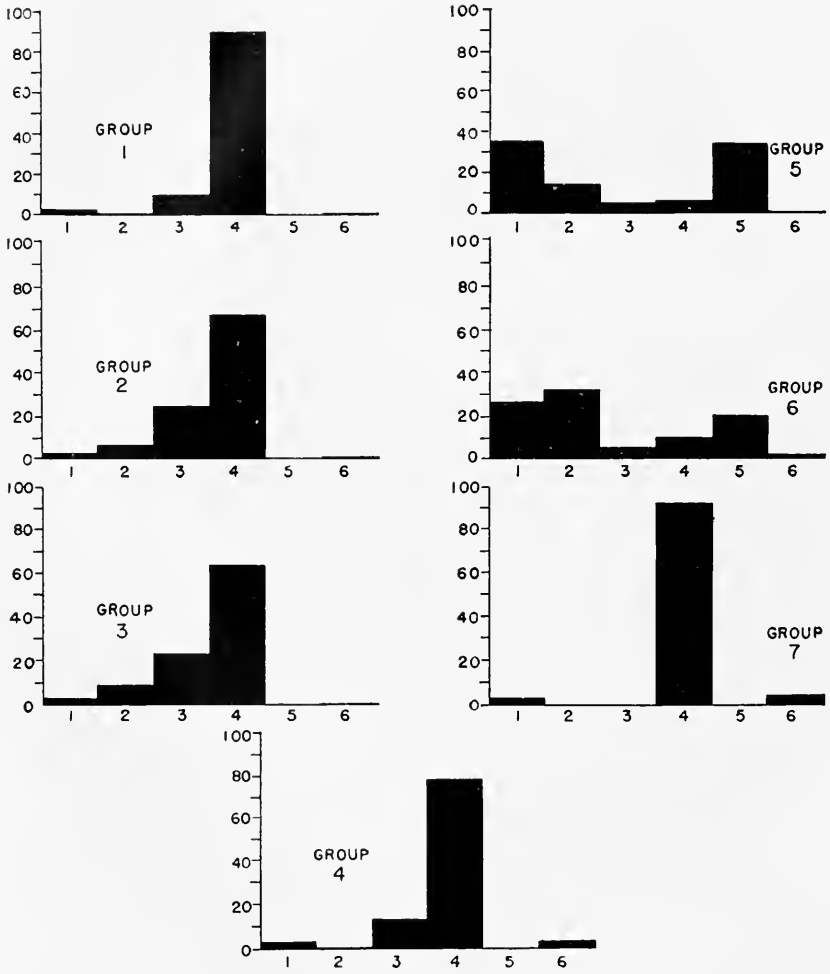

FIGURE 1. Results of Experiment 1 showing the average percentage of the testis occupied by the various germ cells. Refer to the text for the experimental conditions of the groups. Key: 1, Spermatogonia. 2, Spermatocytes. 3, Spermatids. 4, Sperm. 5, Degenerating cysts. 6, Non-germinal.

ences within the individual groups. It is evident, therefore, that light was not necessary for normal spermiogenesis, nor did constant light produce a greater degree of spermiogenesis. In the control group there was considerable variation, ranging from 69.3 per cent sperm to 96.9

per cent. Some spermatids were still present so that the spermiogenesis was not complete except in two of the animals. The analysis of the five normal animals (Figure 1, group 7) collected on September 4 showed that spermiogenesis was more rapid in nature as there were no spermatocytes or spermatids present; this was probably due to the fact that the water-temperatures in the pond (range of 25°–30.5° C. from June 28 to September 4) were higher than those in the laboratory experiments. (2) At low temperatures (groups 5 and 6) spermiogenesis had ceased since a high percentage of spermatogonia and spermatocytes was present while a low percentage of sperm areas was found. These sperm were noted, upon histological examination, to be not newly formed but were undischarged ones remaining from the spring breeding season. Undischarged sperm of this type have a characteristic appearance, the sperm being "loosely" arranged in the cysts in contrast to the compactness of newly formed sperm. The cysts are often partially discharged and fragmented sperm are sometimes found. There were no significant differences between the animals in constant light and those in the dark. A noticeable low-temperature effect was the extensive areas of cysts in which the germ cells were degenerating. Two types of cells were involved in this degeneration; certain phases of the spermatocytes were completely degenerated leaving only empty cysts, while the spermatids had typically pycnotic nuclei. The cysts did not collapse as occurs following normal evacuation. The following measurements of the long axes of various types of cysts indicate the approximate average sizes:

Normal newly formed sperm cysts	440 microns
Normal mature sperm cysts	300 microns
Normal spermatid cysts	360 microns
Normal spermatocyte cysts	240–320 microns
Normal evacuated cysts	0–160 microns
Degenerating spermatid cysts	360 microns
Degenerating spermatocyte cysts	260 microns

The cysts containing the degenerating cells maintained the normal size for cysts of that type. Spermatogonia were not affected by the low temperatures in this experiment.

The differences between the testes' weights of the various groups give additional evidence of the inhibitory effect of low temperatures. The most anterior lobe on the left side of each animal was weighed and the percentage of the body weight was determined. The average percentages of the body weights of each group are given below (Figure 2).

The differences between the sperm areas in the various experimental groups were analyzed by Fisher's (1938) method for determining the significance of differences between small samples; Table I summarizes

the results. The low-temperature groups are not comparable with the others because of the previously mentioned fact that the sperm present were undischarged from the previous cycle and, therefore, were not

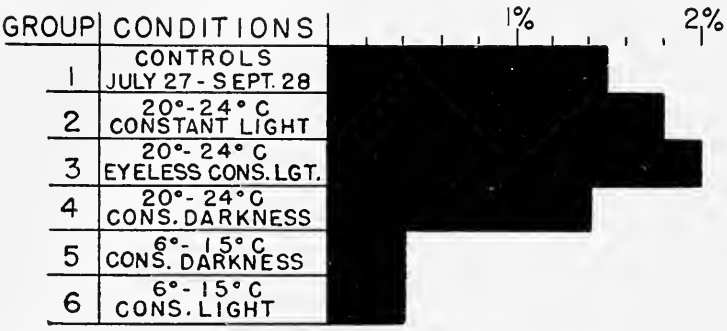

FIGURE 2. Per cent of the body weights formed by the testes in the animals in Experiment 1.

indicative of spermiogenesis occurring at the time of the experiment. Statistically, constant light at room-temperatures apparently inhibited spermiogenesis and while this may be true, obviously it is not a factor in the normal cycle.

To summarize the results of this experiment, it is clear that temperature and not light is the important external environmental factor con-

TABLE I

Statistical Analysis of Experiment 1

	Control	Constant light, 20°–24° C.	Eyeless, constant light, 20°–24° C.	Constant darkness, 20°–24° C.	Constant darkness, low temperature	Constant light, low temperature	Normals
Control		P = .05–.02 S	P = .01–	P = .3–.2	N.C.	N.C.	P = .3–.2 N.S.
Constant light, 20°–24° C.	P = .05–.02 S		P = .5–.4 N.S.	P = .05–.02 S	N.C.	N.C.	P = .01– S
Eyeless, constant light, 20°–24° C.	P = .01– S	P = .5–.4 N.S.		P = .05–.02 S	N.C.	N.C.	P = .01– S
Constant darkness, 20°–24° C.	P = .3–.2 N.S.	P = .05–.02 S	P = .05–.02 S		N.C.	N.C.	P = .01– S
Constant darkness, low temperature	N.C.	N.C.	N.C.	N.C.		P = .1–.05 N.S.	N.C.
Constant light, low temperature	N.C.	N.C.	N.C.	N.C.	P = .1–.05 N.S.		N.C.
Normals	P = .3–.2 N.S.	P = .01– S	P = .01– S	P = .01– S	N.C.	N.C.	

P = probability.
S = significant.
N.C. = not comparable (see text).
N.S. = not significant.

trolling spermiogenesis. All of the animals kept at room temperatures (20°–24° C.) showed approximately equal degrees of sperm formation irrespective of lighting conditions, while in the animals kept at low temperatures spermiogenesis was not only stopped but large percentages of spermatocytes and spermatids degenerated.

Experiment II.—A second set of experiments was designed to test temperature effects at an earlier phase in the spermatogenic period and also to find if food supply influenced the cycle.

Forty-seven animals collected on May 2, 1940, were divided into six groups, as follows:

(1) Eight animals, killed at the beginning of the experiment on May 2. Temperature of pond at collection date, 15° C.

(2) Seven animals, without food; normal light, at room temperature (20°–25° C.).

(3) Seven animals, fed regularly; other conditions as in group 2.

(4) Ten animals, fed regularly; constant light from a fluorescent lamp delivering 32 f. c. at tank surface and 16 f. c. at bottom; at temperature of 5°–8° C.

(5) Eight animals, constant dark with other conditions as in group 4.

(6) Seven animals, as in group 5, except for temperature at 1.5°–3.5° C.

All of the animals (except group 1) were killed on July 1; Figure 3 shows the results of this experiment.

An analysis of these results again indicated the importance of temperature as a controlling factor in spermatogenesis. At the beginning of the experiment, as the normal animals (group 1) show, the testes were discharging sperm and a high percentage (51 per cent) of evacuated cysts were present. The remainder of the testes consisted of spermatogonia (25.4 per cent) and undischarged mature sperm (23.4 per cent). All of the animals (groups 4, 5, and 6) at low temperatures were essentially in the same stage on July 1 as the animals at the beginning of the experiment. The sperm present were undischarged mature sperm, not newly formed ones. This indicated again that low temperatures inhibited spermatogenesis irrespective of light conditions as none of the cell type percentages were greatly different whether the animals were in light or darkness. There were no significant differences between the animals at 5°–8° C., constant darkness, and those at 1.5°–3.5° C., constant darkness. None of the low-temperature animals had degenerating cysts, and since no spermatocytes or spermatids were found at this phase of the cycle, the observation that cold affects only these types of cells was confirmed.

The animals kept at room temperature (groups 2 and 3) underwent active spermatogenesis with the production of spermatocytes, spermatids and new sperm. The evacuated cysts completely disappeared. There was little difference between the fed and non-fed animals, a fact which indicated that the food supply had no influence on the germ-cell cycle in this experiment.

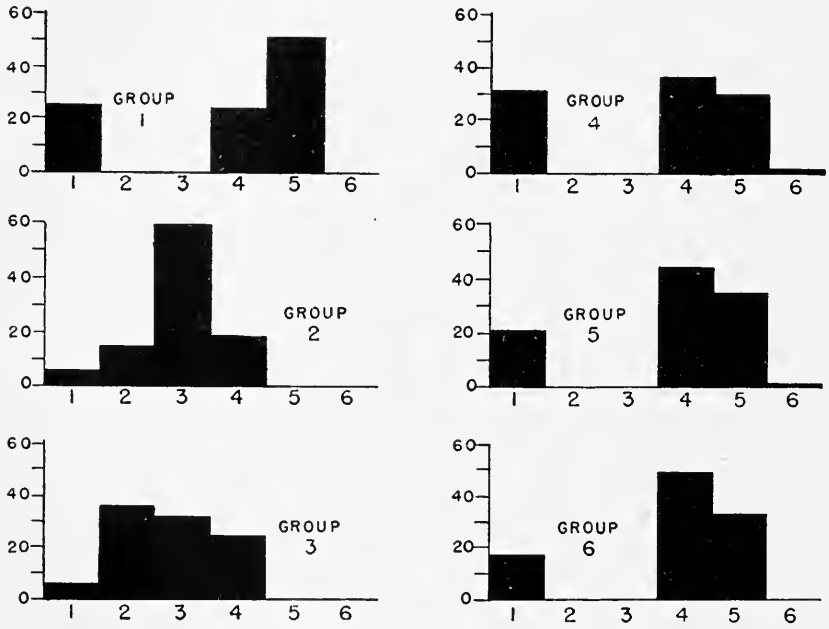

FIGURE 3. Results of Experiment 2 showing the average percentage of the testis occupied by the various germ cells. Refer to the text for the experimental conditions of the groups. Key: 1, Spermatogonia. 2, Spermatocytes. 3, Spermatids. 4, Sperm. 5, Evacuated cysts. 6, Non-germinal.

Experiment III.—The next series of experiments were made during the winter months to find if a steadily increasing light period would have any influence on either gametogenesis or on sperm discharge with temperatures at approximately the threshold value for germinal activity. The threshold temperature was considered to approximate 15° C. as temperatures just below were inhibitory, while under natural conditions breeding begins at this temperature. The 59 newts collected on November 3, 1939, were kept at temperatures averaging 15° C. until February 7 when they were placed in the experimental groups listed below. An additional 21 animals collected on February 6 were included in the experiments.

(1) Five animals collected on November 3, kept at 14°–16° C. until February 7 when they were killed.

(2) Five animals collected on February 6, killed on February 7.

(3) Nine animals collected on November 3 under same conditions as group 1 until killed on March 7. Light through glass during midday 43 f. c.

(4) Ten animals collected on November 3, temperature, 14°–16° C.; light increasing 15 minutes per day from 10 hours on February 7 to 16.5 hours on March 7 with an intensity of 21 f. c.

(5) Eight animals collected on November 3 kept under constant light, 16 f. c. from February 7 to March 7; temperature, 14°–16° C.

(6) Ten animals collected on November 3 kept in constant darkness from February 7 to March 7; temperature, 15°–17.5° C.

(7) Eight animals collected on November 3 kept in constant darkness from February 7 to March 7; temperature, 5°–8° C.

(8) Nine animals collected on November 3 kept in constant light (fluorescent lamp, 43 f. c.); temperature, 5°–8° C.

(9) Six animals collected on February 6 kept under aquarium conditions from February 7 to March 7, midday natural light throughout glass, 47 f. c.; temperature, 12°–17° C.

(10) Ten animals collected on February 6, temperature, 14.5°–17° C.; light increasing 15 minutes per day from 10 hours on February 7 to 16.5 hours on March 7 with an intensity of 18 f. c.

Figure 4 summarizes the results. Since normally during the winter months the inactive testes do not contain spermatocytes it seemed reasonable to suppose the presence of spermatocytes at this time was an indication of germinal activity. The animals collected on November 3 (group 1) and killed on February 7 did show some indications of germinal activity since 8.0 per cent of the testes were occupied by spermatocytes, while the animals collected on February 6 (group 2) had no spermatocytes and were in the typical winter condition. It is therefore clear that temperatures from 12°–17° C. were capable of initiating spermatogenesis at least to the extent of spermatocyte formation. As might be expected under these conditions the longer the animals were kept, the greater the number of spermatocytes were formed. Group 3, kept from November 3 to March 7, had this increase with 14.5 per cent spermatocytes. A striking increase to 33.8 per cent was found in the animals which had been subjected to increasing light (group 4). This might be interpreted as proof of a light effect, but the animals which had been kept in constant darkness had nearly as great an increase with 26.4 per cent spermatocytes, while animals under constant light had only 12.4 per cent

spermatocytes. As has already been pointed out there is considerable variation within groups; consequently, from these data it cannot be concluded that light has any effect. The low temperature groups (7

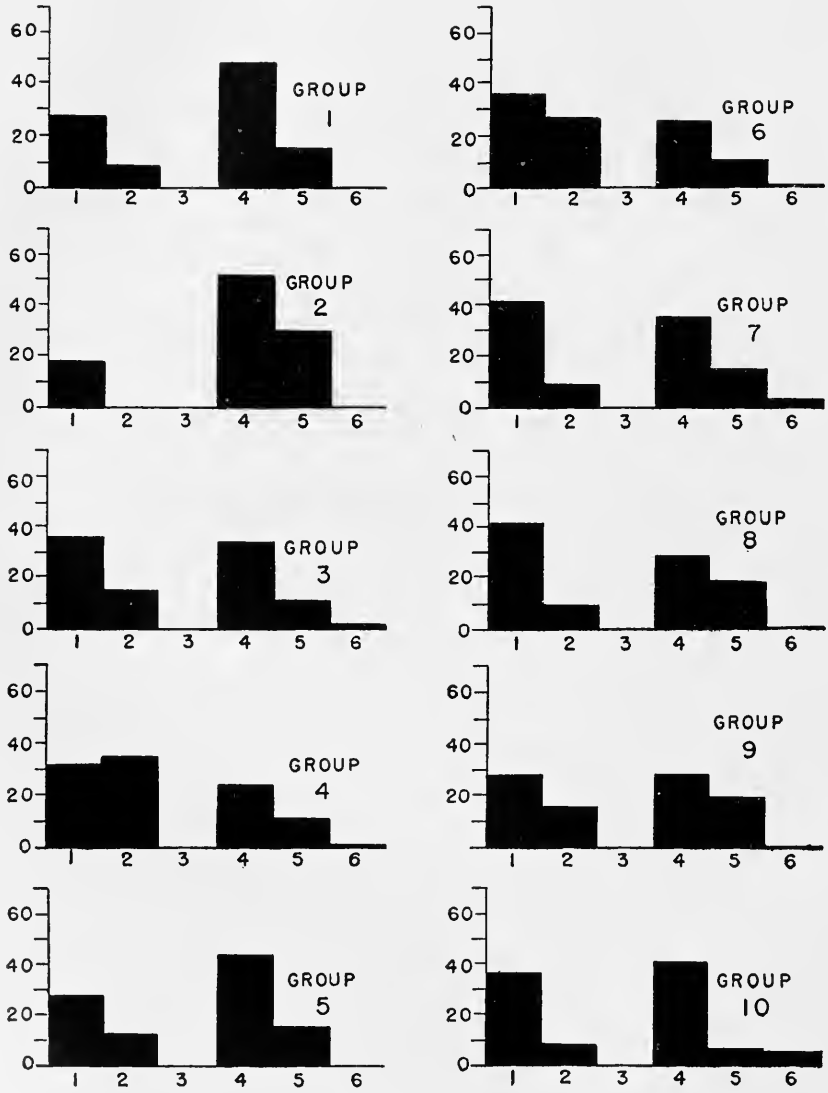

FIGURE 4. Results of Experiment 3 showing the average percentage of the testis occupied by the various germ cells. Refer to the text for the experimental conditions of the groups. Key: 1, Spermatogonia. 2, Spermatocytes. 3, Spermatids. 4, Sperm. 5, Evacuated cysts. 6, Non-germinal.

and 8) again tended to show that the germinal activity of the animals involved had been stopped as the spermatocyte percentages of 8.8 per cent and 9.7 per cent approximated the 8.0 per cent value of the animals killed at the beginning of the experiment (group 1). No degenerating cells were seen in the low temperature groups, possibly because of the shortness of the period of exposure. There were no significant light differences at the low temperatures.

The animals collected on February 6 also produced spermatocytes (compare groups 9 and 10 with group 2). The animals exposed to an artificial increase of light of 6.5 hours were not significantly different in spermatocyte production from those subjected to the natural daylight increase of 1.3 hours.

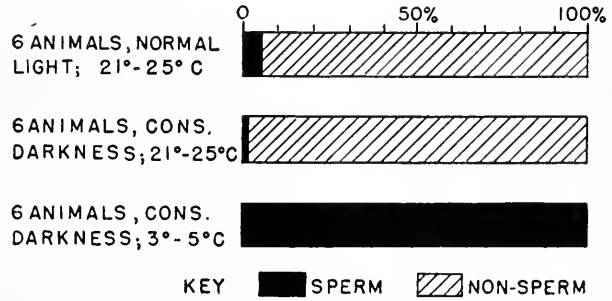

FIGURE 5. Results of Experiment 4 showing the percentage of the total weights of the testes occupied by sperm and non-sperm.

Statistical analysis (Fisher's method, 1938) showed no significant difference between the animals kept in constant darkness (group 6, 15°–17° C.) and those in the increasing light (group 4, 14°–16° C.) ($P = .4–.3$). Groups 9 and 10 were also not significantly different ($P = .6–.5$). Despite the fact that there was a significant difference between the increasing-light group (4) and the controls (group 3) ($P = .05–.02$) but not between the controls and the constant-darkness group ($P = .2–.1$) the total evidence does not point to increasing light as affecting spermatocyte production.

Experiment IV.—In order to determine the effects of light and temperature on the discharge of sperm, 18 animals were collected on October 28, 1940, and divided into three groups of six animals each. One group was placed under aquarium conditions with daylight through glass and room temperature 21°–25° C. The second was placed in constant darkness at 21°–25° C. A third was placed in constant darkness at 3°–5° C. The animals were killed on December 10 (period of 43 days), the testes weighed, separated into non-sperm and sperm regions,

and the percentage of the total testes' weight formed by each of these parts was determined. Figure 5 summarizes the results. Ordinarily at this season of the year (October 28–December 10) sperm are not discharged and the testes contain spermatogonia and a high percentage of sperm, but the animals at 21°–25° C. did discharge sperm to an approximately equal extent. The low-temperature (3°–5° C.) group showed the condition similar to that found in winter since their testes contained 99.41 per cent sperm. It is obvious from Figure 5 that light did not affect sperm discharge but temperature did.

Summary of Experimental Work

In Experiment I low temperatures halted spermatogenesis during summer, while on the other hand, room temperatures permitted the maturation processes to continue. Spermatocytes (probably secondary) and spermatids degenerated at the low temperatures. A similar low temperature effect was noted by Galgano (1936) in *Rana esculenta*. He found the germ cells increasingly sensitive to cold in the following order; primordial germ cells, spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids. *Triturus viridescens* did not show the same order of degeneration since spermatogonia and primordial germ cells were unaffected and the secondary spermatocytes were the most affected. Spermatids had pycnotic nuclei, but the secondary spermatocytes had completely degenerated thus leaving empty cysts. Since the cysts were empty it is not possible to state definitely that they were occupied by secondary spermatocytes but there is good evidence for this opinion since these cysts were located in the region between the primary spermatocytes and spermatids where secondary spermatocytes are usually found; furthermore, there were considerable amounts of normal primary spermatocytes present. It seems logical to assume that mitotic stages of the germ cells are more sensitive to cold than others since interference with mitosis often leads to abnormalities.

In Experiment II low temperatures were again seen to arrest germinal activity, this time at an earlier stage in the cycle. Degenerated cysts were not found, since spermatocytes and spermatids were absent at that time (May 2). In Experiment III winter animals were subjected to a temperature of approximately 15° C. At this temperature spermatocytes were formed but there was no measurable discharge of sperm, indicating, therefore, that different phases of the cycle require higher temperatures than others. At experimental temperatures of 21°–25° C. in winter, sperm were discharged and a new sperm cycle initiated (Experiment IV).

Light variations (constant light, increasing light, total darkness) did not alter spermatogenesis at any phase of the cycle at any of the temperatures used. A justifiable criticism of the light experiments, however, is that all of the intensities used may have been less than the threshold value or inadequate as to wave length; if this were true, differences in light rations could not reasonably have been expected to have been effective. In other forms, however, such as the starling, light intensities as low as 2.5 f. c. and of similar wave lengths were effective (Bissonnette, 1932) so that the 13 f. c. (lowest intensity used) and the wave lengths were probably entirely adequate. In all events the experiments show clearly that normal spermatogenesis can occur in the complete absence of light if the temperature is adequate. Therefore, it may be concluded with reasonable certainty that light is not an essential factor in the environmental control of the sperm cycle in Triturus.

Similarly lack of food did not inhibit spermatogenesis during the active season. While an extended study of this factor was not made, the evidence indicates that food supply plays no part, or at best a minor one, in the production of the normal rhythm.

Applying the evidence obtained from the experiments to the facts concerning the sperm cycle in nature, it is logical to conclude that temperature is the chief environmental factor influencing the sperm cycle. In fact it appears that temperature changes are responsible for the annual cycle in Triturus since the low temperatures of winter merely interrupt what would normally be a continuous spermatogenesis. Therefore, *Triturus viridescens* apparently does not possess an inherent annual sperm rhythm but has a yearly cycle imposed by environmental temperatures.

Discussion

The experimental evidence presented in this paper has led to the conclusion that temperature conditions are responsible for the annual sperm cycle in *Triturus viridescens*. This conclusion agrees with that of Galgano (1936) in his experiments with the frog, *Rana esculenta*. While he did not test light effects, he was able to induce spermtogenesis by increasing the temperature without additional illumination. In contrast to these results Bullough (1939) reports that Spaul and Gladwell (personal communication to him) found light was a requisite for the production of mature gametes and spawning in *Rana temporaria*.

The simplest explanation for the diversity between *R. temporaria* and *R. esculenta*, or any other group, may be that it is an actual one based on species differences. In fact, there may even be racial diversities as Witschi (1924) found in *R. temporaria*. There is, of course, good evi-

dence for species differences to be found in other classes. For example, Baker and Ranson (1938) list a number of species of southern hemisphere birds whose breeding cycles remain unchanged when brought to the northern hemisphere while the breeding cycles of others are modified by the new environment.

There are other possibilities to account for these variations and chief of these seems to be the lack of a uniform criterion for determining the environmental effects on the sexual cycle. The differences between *R. esculenta* and *R. temporaria* might be attributed to this cause, for the presence of sperm in the testes was Galgano's criterion of testicular activity while apparently Spaul and Gladwell used spawning as an indication of gonadal activity. Since this work is as yet unpublished, further discussion of it must be deferred. However, the possibilities that the sexes might respond differently to similar environmental changes should be considered. As an example, apparently there are sexual cyclic differences in *Triturus viridescens*, for during the "false" breeding season the males discharge sperm but the females do not ovulate (Pope, 1924). The cause of this disparity is not clear and is even more puzzling since, according to Adams (1940), the ovaries are fully mature at that time. This example indicates that sexual differences do exist and consequently in comparing results of different experiments it is not possible to use spawning as the criterion in one case and mature gametes of one of the sexes for the other.

Another source of confusion in the literature is the lack of quantitative methods for the measurement of the degree of gametogenesis. In experiments designed to indicate whether an environmntal factor, such as light, is necessary for gametogenesis a quantitative study is not necessary providing the evidence is clear cut, as when one experimental group shows gametogenesis, and the other does not. The early experiments of Rowan (1925, 1926) were of this type since the gonads of the juncos subjected to increased light were activated while the controls were not. However, quantitative methods are necessary for valid results when various degrees of spermatogenesis are present but it is sometimes difficult to find a suitable method particularly with mammals and birds. This probably accounts for the many contradictory conclusions that have been reached in the experiments on these forms. The lack of experimental agreement makes it apparent that there is insufficient evidence upon which to base any general conclusions as to the environmental control of vertebrate breeding cycles. Thus Bullough suggests that marine fishes might be controlled by temperature because of the regularity of that factor in the sea, while fresh-water forms might find temperature erratic in shallow bodies of water and hence would find the regular fluc-

tuation of light more advantageous. Whether such control would be more advantageous to fresh water forms is difficult to determine. At any event, the sperm cycle of *Triturus viridescens,* a fresh water amphibian, apparently is limited by the temperatures of the environment.

Summary

1. The observations of Adams (1940) on the normal sperm cycle of *Triturus viridescens* were confirmed. An active period of gametogenesis following the spring breeding season results in the formation of mature sperm by September. Mature sperm are discharged in the fall but during winter the testes are inactive, consisting of spermatogonia, sperm, and evacuated cysts. Sperm discharge recurs in the spring.

2. At all periods of the cycle temperatures below 12° C. prevented spermatogenesis and sperm discharge and caused the degeneration of spermatocytes and spermatids. Temperatures above 12° C. induced spermatocyte formation in winter; 21°–25° C. in winter caused sperm discharge and in summer these temperatures allowed normal spermatogenesis.

3. Variations in light rations (constant light, increasing light, constant darkness) did not affect the cycle. Lack of food did not inhibit spermatogenesis.

4. It is concluded that temperature is the principal environmental agent influencing the sperm cycle of *Triturus viridescens.*

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THE AUTOLYSIS OF MUSCLE OF HIGHLY ACTIVE AND LESS ACTIVE FISH

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Most of the studies of autolysis have been made upon such mammalian tissues as liver, spleen, kidney and muscle. Sufficient data have also accumulated to indicate that all vertebrate tissues behave essentially like mammalian tissues and may be presumed therefore to contain the same enzyme complex responsible for the autolysis and atrophy of mammalian tissues. Primary cleavage is accomplished by cathepsin—which may be a complex rather than a single proteinase—with an optimum at about pH 4. At pH 2 there is no digestion and the enzyme is destroyed rapidly; at pH 7 digestion is zero or nearly so. At pH 4 to 6 the enzyme may survive several weeks (Eder, Bradley, and Belfer, 1939), so that cessation of digestion within this pH range and period of time cannot be attributed to loss of enzyme activity, but to lack of substrate.

In some preliminary studies of fish muscle (Chen and Bradley, 1924) there was evidence of a correlation between the activity of the organism as a whole, and the speed and extent to which autolysis would proceed. These data were secured before accurate pH control could be assured, and by methods which gave total cleavage rather than primary proteolysis. The measurements therefore were an expression of the composite activity of proteinase together with the various peptidases present in the tissues.

It seems desirable therefore to reinvestigate this phenomenon with the more adequate technique now available, and to extend the observations.

The material was obtained for the most part alive. In two instances fish were frozen and shipped under refrigeration to the laboratory at Madison from the Pacific Coast. It has been shown that the autolysis of such material is essentially like that of freshly killed tissues (Callow, 1925). In addition to the highly active group and the rather sluggish group reported in detail here, we have examined a number of others of intermediate activity, such as the mackerel shark, sand shark, electric ray, flounder, scup and haddock. The muscles of these fish autolyze with the same characteristic pattern and to an intermediate extent. Inasmuch as we have no reliable data on the degree of activity, rate of

metabolism, speed, strength and endurance of any fish, the material we present here was secured from two groups differing very obviously in speed and activity. The rather sluggish or moderately active group comprises the carp (*Cyprinius carpio*), and gar (*Lepisosteus osseus*) from the Wisconsin streams and lakes, and the cod (*Gadus callarias*) and dogfish (*Squalus acanthias*) taken from the Atlantic ocean near Woods Hole, Massachusetts. A second group picked for its outstanding activity, speed and gamey qualities consisted of the Pacific mackerel (*Scomber scombrus*), albacore (*Germo alalunga*); the bonita (*Sardia sarda*), mackerel (*Scomber scombrus*) and swordfish (*Xiphias gladius*) from the Atlantic.

Experimental Procedure

The fish muscle dissected from several specimens to give a representative composite value was ground fine and weighed out to make a 20 per cent mixture in water, preserved with 5 per cent toluol. The material was homogenized in a Waring Mixer to the consistency of a smooth cream, taking care to avoid the inclusion of air bubbles. One hundred cc. digests were made from this stock, and the pH of each digest adjusted to the desired level by means of 5N HCl or N NaOH, using the glass electrode. Samples were removed at once and from time to time later; diluted with one volume of water and precipitated with two volumes of 10 per cent trichloracetic acid. At this concentration none of the native proteins appear in the filtrate, but the fragments from early cleavages do. Digestion was determined by increase in the soluble nitrogen and by the increase of the tyrosine reaction of Folin and Ciocalteu (1927) measured either by colorimeter or with the Photelometer. Nitrogen was determined by the macro-Kjeldahl method when available, and by the micro-Kjeldahl technique of Folin when not. The latter is less accurate but all results represent duplicate analyses. These two methods under the conditions used give independent evidence of the early cleavage stages of the tissue proteins and very closely parallel each other. The pH of each digest must be readjusted frequently during the first three days, since it shifts rapidly as digestion proceeds. After three days it remains substantially constant indefinitely. All digests were maintained at 38° C. Identical series were carried out to which 3 cc. of 20 per cent hemoglobin, purified as described by Anson and Mirsky (1933), were added as additional available substrate.

Each digestion series was prepared from the muscles of several specimens. The results are therefore composite and probably represent the average behavior of the species better than the muscles of a single specimen. Many of the experiments were repeated during the following year

to check previous findings. While there are differences between single specimens and groups of specimens obtained at different times, the essential behavior is reproducible. Thus carp muscle, tested many times, always autolyzes slowly and to a small extent; mackerel rapidly and more extensively.

We present a typical protocol obtained with muscle of the gar, in Table I. All of the series were carried out in the same way and under identical conditions.

TABLE I

Muscle Gar No. 1

	Muscle 20 gm./100 cc.	pH	Mg. Tyrosine/1 cc. filtrate					Mg. N_2/1 cc. filtrate				
			Initial	Increase in Tyrosine				Initial	Increase in sol. N_2			
				Days					Days			
			0	1	3	5	10	0	1	3	5	10
1	Control	6.4	.009	.003	.006	.008	.017	.134	.007	.028	.028	.039
2	"	6.0	"	.006	.014	.019	.033	.144	.005	—	.033	.039
3	"	5.0	"	.016	.030	.038	.057	.140	.043	.073	.113	.130
4	"	4.4	"	.026	.041	.049	.071	.133	.066	.133	.186	.229
5	"	3.6	"	.052	.073	.080	.102	.122	.173	.277	.357	.423
6	"	2.6	"	.032	.052	.057	.083	.128	.088	.119	.204	.298
7	"	1.7	"	.005	.007	.007	.012	.134	.025	.052	.078	.122
8	"	1.0	"	.002	.005	.008	.012	.133	.024	.081	.080	.113
9	"	7.5	"	.002	.004	.005	.007	.144	.006	.006	.009	.022
10	Control+3 cc. 20% hemoglobin	6.5	.010	.004	.009	.013	.019	.133	.020	.040	.061	.067
11	"	6.2	"	.009	.021	.027	.038	.144	.022	.029	.069	.103
12	"	5.4	"	.032	.058	.070	.101	.136	.090	.172	.223	.239
13	"	5.0	"	.052	.084	.096	.121	.146	.186	.290	.332	.365
14	"	4.0	"	.093	.148	.174	.206	.136	.321	.616	.730	.795
15	"	3.4	"	.089	.145	.164	.191	.146	.396	.584	.665	.726
16	"	2.0	"	.017	.023	.028	.031	.146	.047	.054	.079	.116
17	"	1.3	"	.003	.007	.009	.015	.144	.003	.042	.069	.122
18	"	7.5	"	.004	.007	.009	.011	.146	.012	.016	.020	.080

In presenting the rest of our experimental data we shall use the graphic method, and are omitting the three- and five-day digestion curves for the sake of simplicity. Digestion in one day represents essentially the *speed* of the reaction, since there still remains a large excess of substrate. Ten-day digestion approximates final equilibrium and represents therefore the *extent* of autolysis.

In Figure I is shown the autolytic pattern of carp muscle, which represents very well the other members of the less active group. In the

ten-day period about 30 per cent of the total muscle protein had digested. Added hemoglobin is nearly completely digested in the time allotted to the experiment. Digestion was still in progress at the end of ten days, and somewhat more of the muscle proteins would have undergone cleavage if the time had been extended.

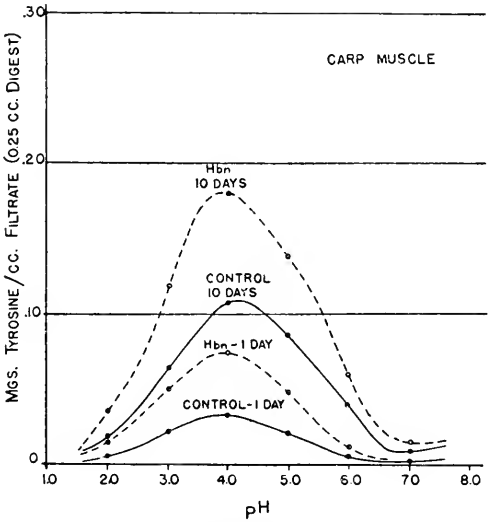


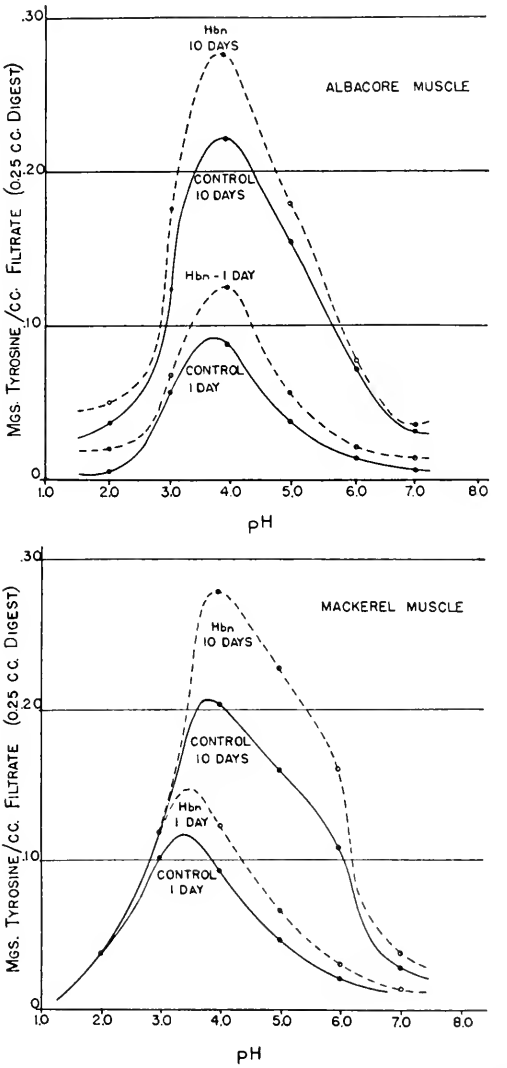
FIGURE I. The curves indicate cleavage as measured by the tyrosine color, attained by a series of digests in 24 hours where pH levels were set and maintained. Three- and five-day curves are omitted. Tissue alone is represented by solid lines; tissue and hemoglobin by dotted lines. The vertical distance between the two curves of the same duration represents the amount of tyrosine referable to the added substrate hemoglobin. In this muscle it is evident that half of the total tyrosine liberated in 24 hours came from hemoglobin cleavage.

Autolysis of carp muscle may be compared with that of albacore and Pacific mackerel under identical conditions, Figures II and III.

It will be noted that relatively more hemoglobin digests the first day in carp muscle autolysis than in the autolysis of mackerel or albacore muscle. On the other hand there is much more of the active mackerel and albacore muscle protein digested in one day than in the case of the carp. This difference is summarized in Table II.

Assuming that the tissue proteinase distributes itself between the muscle proteins and the hemoglobin in proportion to the relative masses of the two proteins, then it would appear that carp muscle is less easily fragmented than hemoglobin, while mackerel and albacore proteins are more easily fragmented by the tissue proteinase. The data suggest that the muscle proteins of the active fish are more fragile to the enzymes

present than are the proteins of the less active fish muscles. Whether there is more enzyme present in the more active muscles than in the less active is not clear from our data and will require the development of



FIGURES II and III. The rapid autolysis of these muscles is evident from the curves. Available muscle proteins are abundant so that added substrate does not greatly increase tyrosine liberated in 24 hours. By the tenth day the increased tyrosine from hemoglobin represents the nearly complete cleavage of the latter. It will be noted that digestion proceeds more slowly from five to six but in ten days is very considerable.

TABLE II

Subtracting the tyrosine produced by fish muscle alone from that produced from muscle plus hemoglobin, gives the amount referable to cleavage of hemoglobin. It will be seen that while the total tyrosine is larger in the game fish muscle digests in one day, the proportion derived from hemoglobin in the latter is considerably less, and is actually somewhat less also. This we believe indicates a preferential splitting of muscle proteins in the case of the game fish group, which indicates their greater fragility.

	Mg. Tyrosine in 1 day from			Per cent Tyrosine in 1 day from	
	Muscle	Hemoglobin	Total	Muscle	Hemoglobin
Carp	0.33	0.45	0.75	44	56
Albacore	0.95	0.32	1.27	75	25
Mackeral	1.17	0.30	1.47	80	20

adequate technique for extracting all of the enzyme present in the tissue for comparison when using a standard substrate like hemoglobin. We expect to investigate this point further.

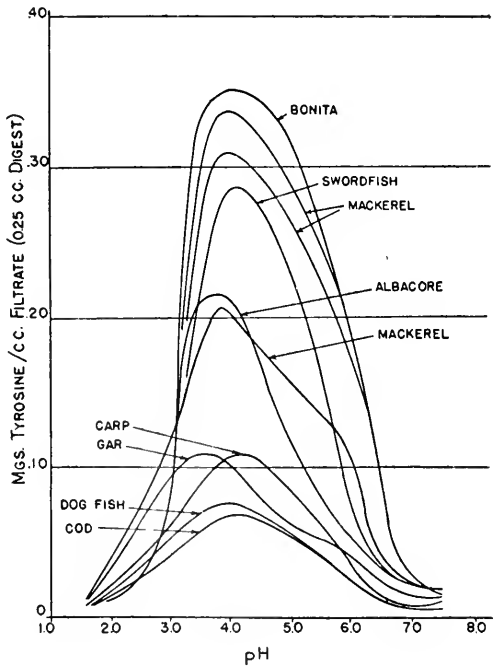


FIGURE IV. The curves above illustrate the contrast in protein cleavage between the active and less active group. Digests are of muscle only.

In Figure IV we have grouped the results of typical autolyses carried on for ten days in which the behavior of the muscles of the sluggish types is contrasted with that of the highly active. No hemoglobin was present in these digests, which indicate the extent of tissue cleavage accomplished in the ten-day period in the two groups. In the active group the cleavage level represents substantially the final equilibria attainable. In the less active forms, where digestion is slower, cleavage has not quite reached final equilibrium.

The difference in autolysis between the two groups emphasizes again the correlation between the extent and speed of digestion attained in ten days and the normal functional activity of the muscles. Pending further studies we believe the rapid autolysis of the game fish muscle proteins represents an ability to mobilize tissue proteins rapidly for the maintenance of high level activity, when the usual stores of glycogen and fat have been depleted. Greene (1919) has shown that in its fasting migration to the spawning beds the king salmon mobilizes 30 per cent of its muscle proteins without sacrifice of essential contractile structures. We assume that in the rapid autolysis of the muscles of game fish here described we have the machinery for similar mobilization of stored protein for fuel requirements when emergencies develop for these rapidly metabolizing species.

Summary

1. All fish muscles examined show the typical pattern of catheptic activity characteristic of such mammalian tissues as liver, kidney, spleen and muscle.

2. Muscles of the relatively sluggish fish such as carp, gar, dogfish and cod autolyze slowly and to a relatively small extent in the ten-day period of the experiments.

3. Muscle from the game fish mackerel, albacore, bonita and swordfish autolyze much more rapidly and completely in the same time, and under the same conditions.

4. A correlation appears to exist between the normal functional activity of the muscles and the speed and extent to which they digest. This represents we believe a mechanism for maintenance of activity through mobilization of tissue proteins during periods of food scarcity.

5. The data suggest that muscle proteins of the active species are more readily fragmented by the enzymes present than are the proteins of less active species.

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TRITURUS TOXIN: CHEMICAL NATURE AND EFFECTS ON TISSUE RESPIRATION AND GLYCOLYSIS

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Introduction

The eggs and embryos of certain Triturus salamanders contain a toxic principle which selectively paralyzes the motor portion of the nervous system of Amblystoma salamander larvae (Twitty, 1937). This toxin, which seems to be neither an alkaloid nor a substance related to the cardiac toad poisons, caused death in mammals (usually preceded by convulsions) by direct depression of the central respiratory mechanism (Horsburgh, Tatum and Hall, 1940). Further investigations into the chemical nature and the mode of physiological action of the toxin are reported in this paper.

Chemical Section

While the toxin has not yet been identified in a chemical sense, it has been prepared in more concentrated solutions than were hitherto available. It is now possible to rule out the possibility of identity with a number of biologically active agents. The evidence on this point follows.

Materials and Methods

Eggs of *Triturus torosus* were crushed, pressed, and the juice filtered through cloth. Ethyl alcohol was added to the filtrate to a concentration of 50 per cent, so that the solution could be stored until needed. In all, 24 liters of this solution were obtained from seven or eight gallons of egg clusters, with a total activity of 600,000 *mouse units*.

The toxin content of all preparations was assayed biologically as previously described (Horsburgh, Tatum and Hall, 1940) and calculated in terms of the "*mouse unit*" (m.u.). One *m.u.* is that amount which on subcutaneous injection completely stops the respiration of a white mouse in 10 minutes.

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Preliminary investigations. The stability of toxin solutions to heating and to acid and alkali was found to be essentially as previously given (Horsburgh, Tatum and Hall, 1940). It was stable at 100° C. at pH 6 or 7, was 80 per cent inactivated in 30 minutes at pH 4, and 60 per cent inactivated at pH 8 and pH 10. At pH 9 and 120° C. the toxin was over 99 per cent inactivated in 10 minutes. Inactivated toxin solutions were prepared in this way for use in the respiratory studies. It was completely destroyed at pH 12 even at room temperature in a few minutes.

Although the solubilities of the toxin were essentially as previously reported (Horsburgh, Tatum and Hall, 1940), the present material appeared to be much more unstable in higher concentrations of alcohol. It was partially or largely inactivated in concentrations of ethyl alcohol and acetone over 75 per cent, and in methyl alcohol over 90 per cent. However, in the presence of NaCl, it is quite stable, even in absolute methyl alcohol, in which it is completely soluble. This stabilizing effect of salt is evident even in water solutions of the toxin, which gradually decrease in activity on standing unless around 1 per cent NaCl is present. No explanation of this salt effect is available at present.

The chemical evidence substantiates the earlier conclusion that the toxin is not basic in nature. It was not precipitated from concentrated solutions by any basic precipitants tried, including chlorplatinic, phosphotungstic, picric, picrolonic, and flavianic acids.

Attempts were made to purify the material by selective adsorption of the toxin on activated charcoal and on kaolin. Both of these either adsorbed or inactivated the toxin, but no active material could be recovered from the adsorbent, although a number of solvents (pyridine, alcohol and acetone), at several pH values, were tried.

High vacuum molecular distillation of the toxic principle was also unsuccessful. No active material distilled at 10^{-5} mm. Hg at temperatures of 80° C. or higher. After heating to 140° C. the residue was also inactive.

An estimation of the molecular weight by measuring the rate of diffusion through agar blocks (Tatum and Beadle, 1938) and testing the concentration of toxin in each block by the mouse test, gave results indicating a molecular weight between 200 and 400. This value and the ease with which the toxin passes through a dialyzing membrane rule out high-molecular compounds such as proteins or peptones. (See also evidence of rapid diffusion in embryos, Twitty, 1937.)

Final purification procedure. In spite of the rather great instability of the toxin, it has been possible, by making use of the most successful and

least destructive treatments, to purify the active principle to a considerable extent. It was found necessary to work up only small batches at a time, since otherwise too much activity was lost. The most satisfactory procedure was essentially as follows:

Preparation I: 3 liters toxin solution in 50 per cent ethyl alcohol (14.4 gm. dry weight; 72,000 *m.u.*). Activity: 5,000 *m.u.* per gm.; treated with super cel; filtered.

Preparation II: Filtrate (60,000 *m.u.*). Concentrated in vacuo to 75 cc.; dialyzed against 1 per cent NaCl solution.

Preparation III: Dialyzate (60,000 *m.u.*). Concentrated to dryness in vacuo; extracted with hot absolute methyl alcohol.

Preparation IV: Extract (54,000 *m.u.*). Dried, extracted with hot chloroform.

Preparation V: Residue (1.61 gm. dry weight; 50,000 *m.u.*). Activity: 31,000 *m.u.* per gm.; dissolved in 5 cc. 1 per cent NaCl solution; phosphotungstic acid in 1 per cent NaCl solution added until precipitation was almost complete; centrifuged.

Preparation VI: Centrifugate (0.66 gm. dry weight;[3] 42,500 *m.u.*). Activity: 64,400 *m.u.* per gm.

It was found that the recovery of the toxin in the last step was most satisfactory if an excess of phosphotungstic acid was avoided. Although the toxin was not precipitated by an excess of phosphotungstic acid, even in the presence of sulfuric acid, the resulting preparation lost its activity very much more rapidly. This was true even in a 1 per cent NaCl solution, in which the toxin as ordinarily prepared was quite stable. It is possible that a precipitation with slight excess of phosphotungstic acid removed some protecting substances from solution.

The effect of various treatments on this purified preparation was investigated. It was easily decomposed by alkali, but without any detectable evolution of amines. The activity was unaltered by treatment with dilute bromine water, with Chloramine T, with formaldehyde, and by acetylation with acetic anhydride in methyl alcohol. The stability of the toxic principle to these reagents indicates that it probably does not contain an aliphatic amino group, and suggests that the amino acids present in this fraction are inactive contaminants.

Analyses of the most purified preparation. The best toxin preparation (VI) was analyzed for total-N (Pregl, 1935), and amino-N (Van Slyke, 1912) before and after hydrolysis at 120° C. with 10 per cent sulfuric acid, for ammonia-N and for reducing sugars (Stiles, Peterson and Fred, 1926). The results are given in Table I and are calculated on the basis of total organic material present in the preparation.

The results of the analyses show that 10 per cent of the organic dry material in this preparation was sugar, which presumably was inactive.

[3] Organic material.

No glycosidic sugars could be detected. No peptide-N was detected, and practically all of the total-N was accounted for by the amino- and ammonia-N. Although acetylation of the amino nitrogen was almost complete, there was no accompanying change in activity. This fact, together with the stability of the toxin to treatment with bromine water, chloramine-T, and to formaldehyde, suggests that the toxin itself contains no ordinary aliphatic amino-N. It may be significant that the ammonia-N, the non-acetylated amino-N and the amino-N lost on acid hydrolysis are of nearly the same magnitude.

TABLE I

Analysis of Toxin Preparation VI

Constituent	Per cent
Reducing sugars as glucose	10.9
Total-N	6.4
Ammonia-N	0.3
Amino-N	5.8
Amino-N after hydrolysis [1]	5.5
Amino-N after acetylation [2]	0.4

[1] Hydrolyzed with 10 per cent H_2SO_4, 3 hours at 120° C.

[2] Treated with acetic anhydride in absolute methyl alcohol solution; reagents removed in vacuo.

The activity of the preparation used for the analyses was 64 *m.u.* per milligram, which compares quite well with the previously reported activity of 75 *m.u.* per milligram (Horsburgh, Tatum and Hall, 1940). In order to estimate the potency of the pure toxin itself, the activities of various fractions of the preparation have been calculated from the analytical data given in Table I. For purposes of the calculations the amino acid content was taken as the amino-nitrogen value $\times$ 6.25. The activity of the material with the amino-acids and sugar removed, would then be around 120 *m.u.* per mg. However, it seems probable by analogy with other physiologically active materials that the toxin does contain nitrogen. The concentration of active material was therefore calculated on the basis of the non-acetylated amino-N, assuming a molecular weight of 400 for the toxin (the maximum value from the diffusion experiments) and 2 N atoms per molecule. The value obtained approximates 2,000 *m.u.* per mg. Similar values are obtained on the basis of ammonia-N or amino-N lost on acid hydrolysis. Even with half of this activity (1,000 *m.u.* per mg.), the material would still be extremely active as compared with other known toxic substances.

Physiological Section

The influence of Triturus toxin, prepared as described above, on respiration (preparation VI) and anaerobic glycolysis (preparation V) in isolated rat cerebral cortex and on respiration in isolated rat kidney cortex and liver is reported here.[4]

Materials and Methods

Fourteen adult albino rats were used. These were decapitated, the brains removed rapidly and placed in a moist box to minimize changes in water content during slicing (Sperry and Brand, 1941). Methods of slicing (Crismon and Field, 1940) and of preparation of toxin solutions for use in respirometer vessels (Fuhrman and Field, 1941) have been described elsewhere. Oxygen consumption and anaerobic glycolysis were measured by the usual manometric methods (Dixon, 1934) and the gas consumption or production was calculated in μ l., N.P.T., per mgm. wet weight per hour (Q_{O_2} and $Q_{N_2}^{CO_2}$ respectively). The thermostat temperature was $37.5° \pm 0.01°$ C. Toxin solutions to be added were adjusted to the pH of the suspension medium, and frequent checks with a glass electrode showed that little change in pH of the suspension medium occurred during a run.

Toxin was first added to the experimental vessels from the sidearm after a preliminary run of 30 minutes. At least two vessels were run at each concentration (active or inactivated) and, as controls, two vessels receiving no toxin. Frequent checks (Horsburgh, Tatum and Hall, 1940) showed that the toxin did not decrease in potency during the runs.

Results

1. *Respiration of Brain, Kidney and Liver.*—It is shown in Table II, part 1, that differences in Q_{O_2} between the control and experimental runs were rather small and transient, and that the Q_{O_2} of cerebral cortex and kidney slices was affected only by the most concentrated toxin preparation available (850 *m.u.* per ml.), while the respiration of liver slices was stimulated slightly at a toxin concentration of 85 *m.u.* per ml. However, it is not clear that these differences in respiration could be attributed to the toxin, since concentrations of 85 and 850 *m.u.* per ml. involved the presence of 1.33 and 13.3 mgm. of organic matter respectively. These substances may have served as additional substrate.

Since addition of partially inactivated toxin gave results unlike those obtained with untreated dilute toxin of the same potency and since these

[4] A preliminary note dealing with the effects of toxin preparations on tissue oxygen consumption has been published (Fuhrman and Field, 1941).

results were unlike those observed in controls, it appears probable that inactivation involved more complex changes than simple destruction of toxin. Accordingly the partially inactivated toxin cannot be used to test the possibility that substrate rather than toxin was responsible for the questionable initial rise in Q_{O_2} at high toxin concentrations.

TABLE II

Part 1

Comparison of respiratory rates of rat cerebral cortex, kidney cortex and liver slices in the presence of high concentrations of Triturus toxin with that of controls (arbitrarily taken as 100). Time indicated is after start of run.

	Toxin				"Inactivated toxin"	
	850 *m.u.* per ml.		85 *m.u.* per ml.			
	30 min.	120 min.	30 min.	120 min.	30 min.	120 min.
Cerebral cortex	111	104	99	96	118	115
Kidney cortex	134	107	100	97	108	84
Liver	174	113	119	128	108	60

[1] Residual potency 85 *m.u.* per ml.

TABLE II

Part 2

Comparison of rates of anaerobic glycolysis in rat cerebral cortex in the presence of high concentration of toxin with that of controls (arbitrarily taken as 100)

	Toxin (*m.u.* per ml.)		Inactivated toxin [1] (initial *m.u.* per ml.)	
	37	112	37	112
Average rate during first 10 minutes after addition of toxin	253	344	290	250
Average rate for period 10 to 70 minutes after addition of toxin	135	36	167	70

[1] Residual potency less than 0.5 *m.u.* per ml.

2. *Anaerobic Glycolysis in Cerebral Cortex.*—The results of addition of toxin, in final concentrations of 37 and 112 *m.u.* per ml., are shown in Table II, part 2. There is a transient initial increase in the rate of carbon dioxide displacement which is probably due to some factor other than the toxin, since it was obtained with the inactivated as well as the active preparation. During the subsequent 60 minutes the higher concentration of toxin clearly inhibited anaerobic glycolysis. The

effect of the lower concentration was inhibitory also, since in its presence the rate of carbon dioxide production was somewhat lower than in the case of the "inactivated" control.

Discussion

Administration of Triturus toxin to the anesthetized cat in doses of 50 *m.u.* or more per kgm. body weight, caused death in about one minute, due to direct depression of the central respiratory mechanism (Horsburgh, Tatum and Hall, 1940). The concomitant fall in arterial pressure was insufficient to account for this effect on the basis of an interruption in the oxygen supply to the brain (Hall). The time involved appears too short for the development of fatal hypoglycemia, since we have found that the respiration of isolated cerebral cortex, in glucose-free media, does not diminish markedly in the first few minutes of measurement (cf. also MacLeod, 1934). However, the general picture, that of convulsions followed by rapid respiratory death, did suggest interference with the metabolism of the brain (cf. Gerard, 1938), possibly by inhibition of oxygen consumption, glycolysis or both, through action upon enzymes concerned in these processes. This seemed the more probable because certain snake venoms are known to inhibit one or both of these processes (Chain and Goldsworthy, 1938; Chain, 1937, 1939; Mellanby, 1935). However, under the conditions of our experiments, concentrations causing rapid respiratory death in intact animals have no effect on cerebral cortex respiration or anaerobic glycolysis (assuming uniform distribution). Very high concentrations of the toxin caused slight transient stimulation of respiration and inhibition of glycolysis. These were respectively, 17,000 and 7,000 times the concentration killing a cat in one minute. It thus appears that another explanation of the action of the toxin must be sought. It is possible that the toxin inhibits those reactions which underlie neuron excitation (conduction and transmission) in contrast to those important in resting maintenance or recovery.

Summary

The most active toxin preparation obtained had a potency of 64,000 *mouse units* (*m.u.*) per gram of organic material. Most of the nitrogen in this preparation was probably contained in inactive amino-acids, and the activity as calculated on the basis of the remaining nitrogenous material was between 1 and 2 million *m.u.* per gram.

The effect of the toxin on respiration and glycolysis in certain organs of the rat was investigated. Oxygen consumption of liver slices ap-

peared to be slightly and temporarily increased in the presence of 85 *m.u.* of toxin per ml. Similar effects were observed on the respiration of kidney and brain slices with a toxin concentration of 850 *m.u.* per ml. However, control studies with inactivated toxin makes it questionable whether these changes were attributable to the toxin *per se*.

Anaerobic glycolysis in rat brain slices was progressively inhibited with higher concentrations of the toxin preparation. However, this effect was obtained only with concentrations far higher than those required to kill a two-kilogram cat in one minute.

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AN ANALYSIS OF THE ACTION OF ACETYLCHOLINE ON HEARTS, PARTICULARLY IN ARTHROPODS

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Two organic compounds have been shown to be liberated as mediators at nerve endings, adrenin-like sympathin and acetylcholine. The distribution of these substances throughout the animal kingdom is nearly universal. Adrenalin seems to be excitatory on all adult hearts although many molluscan hearts are relatively insensitive to it. Acetylcholine has an inhibitory action on the systemic hearts of adult vertebrates and of molluscs; acetylcholine has no action on the hearts of early Fundulus embryos; it has an accelerating action on the hearts of decapod Crustacea, of Limulus, the grasshopper Melanoplus, and of the annelids Arenicola and Lumbricus; (see Table II for references). It might be possible to explain these three types of action of acetylcholine if a wide variety of hearts was compared with respect to drug effects and histological characteristics. Baylor (1942) has recently found acetylcholine to inhibit the Daphnia heart. This indicates that acceleration is not characteristic of all arthropods and it becomes important to know which groups show acceleration, which inhibition, and which no effect of acetylcholine.

In establishing the physiological action of a drug the effects must be reversible and a threshold concentration must be demonstrable. These two criteria have been adhered to in the following experiments. The mere fact that acetylcholine acts in a given manner on an effector does not imply that the nerves supplying that effector liberate acetylcholine. Mediation by a substance can be proved only when that substance is obtained in a perfusate and when potentiating and inhibiting drugs are combined with nerve stimulation. The following experiments are concerned only with drug effects, not with the liberation of substances at nerve endings.

Penetration of Acetylcholine into Intact Animals

The effects of acetylcholine upon higher arthropods have been examined by direct perfusion of isolated hearts. In working with the smaller transparent crustacea the only feasible method of applying the drug is to immerse the animals in a solution of it. To test the validity of this method a number of experiments have been performed on crayfish. Davenport, Loomis and Opler (1940) showed by perfusion that acetylcholine (10^{-10}) accelerates, raises the tone, and may stop in systole the heart of *Astacus trowbridgei*. We find the hearts of *Cambarus virilis* and *C. propinquus* to be accelerated likewise by perfusion in low concentrations. When acetylcholine is dripped over the outside of a heart which is left in situ the threshold is much higher (10^{-6} to 10^{-5}) than when perfused.

To learn whether acetylcholine would penetrate in appreciable amounts into intact animals the hearts of crayfish were exposed by removing a piece of the dorsal carapace and the animals allowed to stand in water just over the lateral margin of the carapace so that water never came in contact with the heart. The heart rates were recorded kymographically or were counted. The effect of acetylcholine applied in this way is shown in Figure 1*A*. A concentration of 10^{-4} in water accelerated in this way about as much as did 10^{-5} in saline when dripped on a heart similarly exposed. Acceleration by potassium and inhibition by calcium in the water were readily demonstrated. Eserine (physostigmine) definitely potentiated the acetylcholine acceleration. Small specimens were more sensitive than large ones, due probably to differences in dilution in the body and in the absolute amount of choline-esterase present. Maluf (1940) showed with dyes that crayfish take in very little water by mouth but that dissolved material enters by way of the gills. Since acetylcholine does not stimulate the central nervous system of the crayfish (Prosser 1940a) its action in the immersion experiments is probably directly on the heart.

It is concluded that sufficient acetylcholine enters the body of a crayfish by the gills from a solution of 1 : 10,000 to accelerate the heart by at least 20 to 25 per cent. Therefore direct application of a drug to an exposed heart is not necessary to establish its qualitative action, although thresholds by perfusion are very much lower. Penetration through the body surface (gills, etc.) has frequently been used for studying the effects of other inhibitors and accelerants on hearts, and toxic substances of polluted water must frequently penetrate animals in this way.

Effect of Acetylcholine on Non-decapod Crustacea

Amphipoda: Two species were used, *Talorchestia longicornis* and *Bactrurus mucronatus.*

Talorchestia: When a small spot of light is focused on the dorsal side of the marine amphipod Talorchestia the heartbeat can be counted very readily with the aid of a low-power dissecting microscope. The heart is very rapid and to count the beats it was slowed by cooling. Single specimens were restrained by insertion into a glass tube of slightly larger bore than their body diameter. Appropriate solutions passed through this tube. The tube was placed in a water bath which was maintained at 15 ± 0.5° C. Even at this temperature the rate is fast (about 175–200/min.) but can be counted accurately after a little practice. The counts were checked by other persons on several occasions.

Six different experiments were performed and in each of these several applications of acetylcholine were tried. The time for ten beats was counted from five to 12 times approximately every five minutes. Probable errors for individual sets of counts were 0.05 to 0.1 second, while significant differences were 0.3 to 0.5 second. The dilutions of acetylcholine were prepared by adding a stock solution (10^{-3}) to sea water. Similar dilution of sea water with distilled water (90 per cent sea water) had no effect upon the heart rate over the periods used.

Results of one typical experiment are shown in Figure 1*B*. Acetylcholine accelerates the heart of Talorchestia. Threshold by external bathing lies between 10^{-5} and 10^{-4}. There may be some eserine potentiation. Similar results were obtained on five animals.

Bactrurus: Specimens of this freshwater Amphipod were collected at the mouth of a drain from a field and were studied within a day after collection. Individual animals were placed in separate watch glasses containing test solutions. For counting, they were held under a coverslip supported at two sides by a number of coverslips held by vaseline. The number of supporting coverslips was varied according to the size of the specimen so that the abdomen was freely movable and the thorax held lightly in place. The hearts of freshly collected specimens were very steady and easily counted. Twelve experiments were performed. Acetylcholine (10^{-4}) accelerated the heart markedly (15–20 per cent) in each of eight specimens (Figure *C, D*). There was slight acceleration in two at 10^{-5} and no acceleration in two at 10^{-6} (Figure 1*D*).

It is concluded that the hearts of amphipods are accelerated as are the hearts of decapod Crustacea.

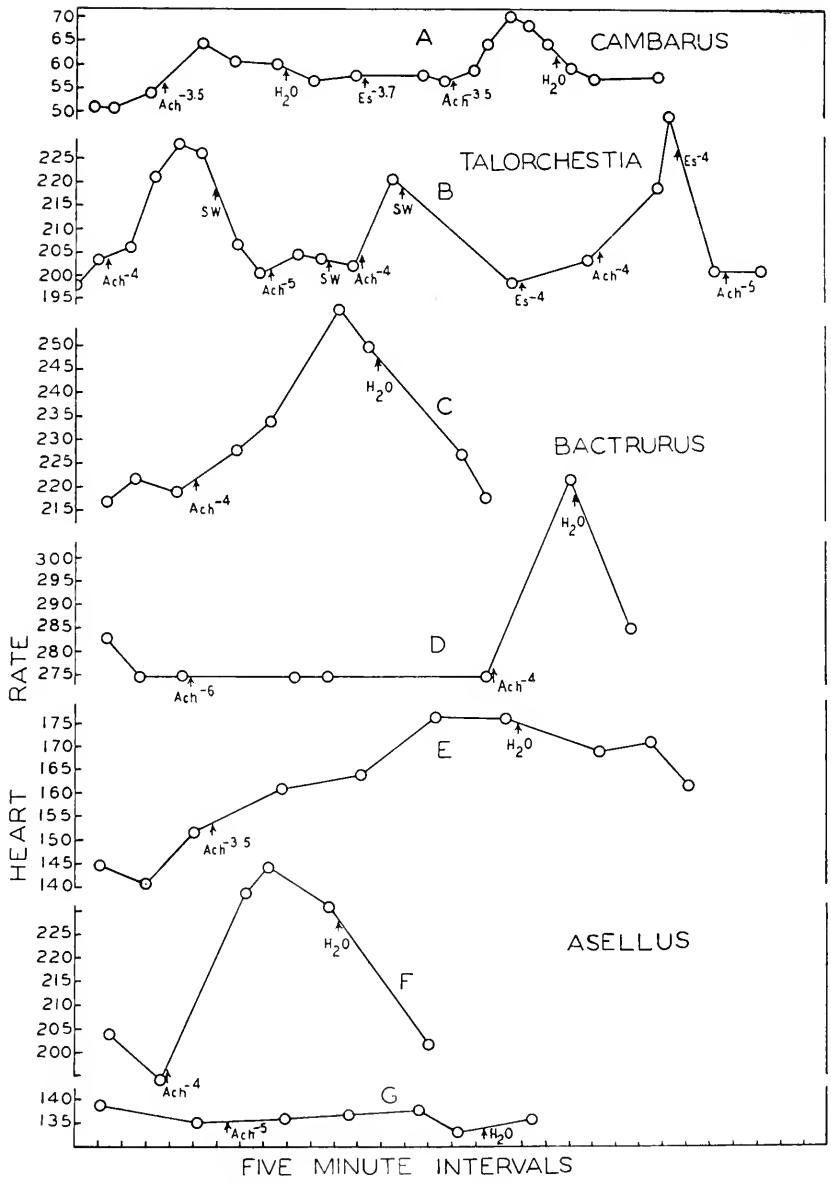

FIG. 1. Effect of acetylcholine upon the heart rate in beats per minute of (*A*) *Cambarus virilis*, (*B*) *Talorches tialongicornis*, (*C*, *D*) *Bactrurus mucronatus* and (*E*, *F*, *G*) *Asellus tridentatus*. Drugs applied by immersion. Concentrations given in negative exponents of 1×10.

Isopoda:

Specimens of the transparent eyeless freshwater isopod *Asellus tridentatus* were collected along with the Bactrurus.[1] Their heart rate varies more with body activity than that in any of the Crustacea which we have observed. When leg movement occurs the heart speeds up and then slows when the animal becomes quiet. In general the resting rate was taken. The animals were restrained in the same chambers as the Bactrurus and were not left on the observation slide more than five to ten minutes at a time. Freshly collected specimens had more regular hearts and were more sensitive to acetylcholine than specimens which had been in the laboratory for several days. Twenty-four experiments were performed. Acetylcholine $10^{-3.5}$ accelerated the heart in each of four specimens (Figure 1*E*). Acetylcholine 10^{-4} accelerated in eight (by approximately 20 per cent) (Figure 1*F*), and was without effect in two. In three specimens there was some acceleration with acetylcholine 10^{-5} and in four no effect (Figure 1*G*), while in three specimens there was no effect at 10^{-6}. Significant differences were three to five times probable errors. The variability of heart rate, the differences in sensitivity, and different patterns of acceleration (Figures 1*E* and 1*F*) make the preparation less satisfactory than Bactrurus but there is no doubt qualitatively that acetylcholine accelerates the heart.

Copepoda:

Diaptomus. Two species of freshwater copepod were studied, *Diaptomus oregonensis* from winter plankton and *Diaptomus sanguinensis* collected in temporary spring ponds. Nineteen experiments were performed, fifteen of these with *Diaptomus sanguinensis.* The hearts are very rapid (ten beats in less than two seconds) and the animals are too opaque to use the stroboscopic method employed by Baylor with Daphnia. Hence they were entangled in cotton placed in depression slides and these floated on a water bath held at $11 \pm 0.1°$ C. It was possible to get reproducible counts if the rate did not exceed ten beats in 1.6 seconds. The accuracy is not high but qualitatively there is little difficulty in distinguishing between a heart beating ten times in 2.0 seconds and one beating ten times in 1.6 seconds. Probable errors are of the order of 0.05 sec. and significant differences 0.2 sec. Acetylcholine acceleration was observed with recovery in pond water in each of three specimens in a concentration of $10^{-3.5}$, in each of ten specimens in 10^{-4} and slight acceleration in each of three at 10^{-5}. No acceleration was noted in two at 10^{-6}. Typical curves are shown in

[1] I am indebted to Dr. Leslie Hubricht of the Missouri Botanical Garden for identification of the specimens of Bactrurus and Asellus.

Figure 2 *II*, *I*. It is evident that acceleration by acetylcholine is not restricted to the malacostracan Crustacea.

Phyllopoda:

Artemia salina. Eggs of Artemia were hatched in sea water to which sea salt had been added to give a solution with a freezing point of −2.8° C. The animals were fed yeast. Most of the specimens tested were adults although some were in the last or next to last instar. Attempts to mount specimens in small tubes through which test solutions passed were unsuccessful because the animals were very easily

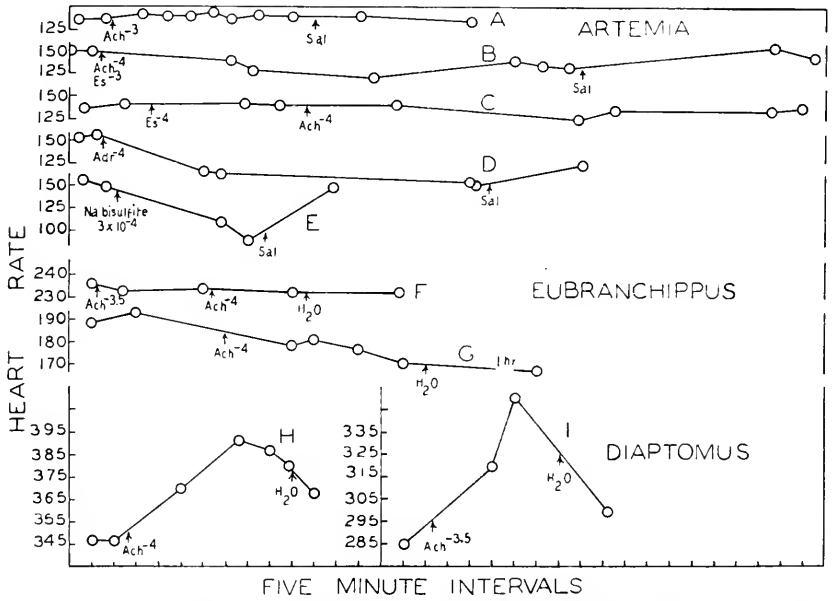

FIG. 2. Effects of drugs on the hearts of *Artemia salina* (curves *A*, *B*, *C*, *D*, *E*), *Eubranchipus serratus* (curves *F*, *G*) and *Diaptomus sanguinensis* (curves *H*, *I*). Ach, acetylcholine, Es, eserine, Adr, adrenalin. Heart rate in beats per minute.

affected by slight currents. Restraint in cotton or lens tissue was also ineffective. The best method found was to hold the specimens in a chamber supported by a variable number of coverslips as described above for Bactrurus. The specimens were placed in watch glasses containing approximately 10 ml. solution and were transferred from time to time to the restraining slides for examination. Prolonged restraint sometimes slowed the heart. Drug solutions were made up in culture fluid.

The heart was easily seen from either dorsal or lateral aspect. Sometimes a margin of the heart was observed, sometimes the move-

ment of the valvular ostia and often the rhythmic propulsion of corpuscles. The contractions of the heart occasionally seem synchronous with the movements of the gill appendages but careful count usually shows a difference in the two rhythms, the heart being slightly faster.

Acetylcholine is without effect on the heart of Artemia (Figure 2 *A*, *B*, *C*). Specimens were immersed in concentrations as high as 10^{-4} and 10^{-3} in culture fluid for periods of one to two hours. Of six animals in acetylcholine 10^{-4}, five showed no effect and one a slight slowing of heart rate. Five specimens in acetylcholine 10^{-3} showed no effect (Figure 2*A*). Eserine 10^{-4} was applied to other specimens both before and concurrently with acetylcholine. In four specimens treated with eserine for about a half hour before treatment with acetylcholine there was no alteration of heart rate by the acetylcholine. Six specimens were placed in culture fluid containing both eserine (10^{-4}) and acetylcholine (10^{-3} or 10^{-4}). In three of these there was no effect (Figure 2*C*). Three, however, showed a slight slowing of the heart (Figure 2*B*). Eserine alone applied for periods of one half hour or longer slowed in three animals and had no effect in three others. Thus the slowing in the mixture of eserine and acetylcholine may have been due to the eserine rather than to the acetylcholine. All specimens treated with eserine showed locomotor paralysis; the gill appendages often stopped beating altogether. Eserine had a much greater paralyzing effect on gill movement than on the heart. Recovery was rapid in non-eserinized culture fluid.

Adrenalin (Parke, Davis solution) was applied in concentrations ranging from 10^{-7} to 10^{-4}. Adrenalin 10^{-7} and 10^{-6} were without effect. Adrenalin 10^{-5} slowed the heart slightly and 10^{-4} slowed it by over 33 per cent (Figure 2*D*). This effect was surprising and the preservatives in the adrenalin were suspected. Chloretone in twice the concentration present in 10^{-4} adrenalin (chloretone 2×10^{-4}) was without effect. Powdered adrenal medulla was tried and there was no effect on the Artemia hearts within one half hour. However this adrenalin became partly oxidized in this time as indicated by the development of a pink color. Sodium bisulfite was added to the crystalline adrenalin solution and there was a marked slowing. When placed in sodium bisulfite 10^{-4} alone a marked slowing was observed in several specimens (Figure 2*E*). Threshold was about 10^{-5}. The apparent slowing by adrenalin is due, therefore, not to adrenalin but to the sodium bisulfite present as a reducing agent.

The lack of effect of acetylcholine and adrenalin raises the question whether these substances get to the heart. Artemia is famous for its resistance to acid fixing solutions. Krogh (1939) found that its permeability to heavy water is low but appreciable. An attempt was made to inject drug solutions. A micropipette attached by micromanipulator tubing to a syringe was used. The micropipette was inserted by hand in the thoracic region and 0.008 ml. of solution injected. Solutions of culture fluid and of acetylcholine were injected, and the pipette inserted without any injection in different individuals. The heart always stopped beating for some distance ahead of the point of insertion. Sometimes local beats would arise, particularly in the abdomen. The animals were upset and often died as the result of the mere insertion of the micropipette. It was impossible to observe any effect attributable to injected acetylcholine.

A study of penetration of dyes was then made. Animals were placed in dilute solutions of methylene blue and of neutral red. Within fifteen minutes the dyes could be seen in the intestine and in about half an hour they were noted in various tissues, even in the blood sinuses in the appendages. In contrast to the crayfish, the dyes were not taken up by the gills but were swallowed. Considerable variation was noted in the amount swallowed by different specimens. Dyes were used in the acetylcholine solution to show that the solutes definitely reached the interior of the animals. The imperviousness to fixatives may be due to the very low gill permeability and lack of swallowing in the fixative. The fact that eserine and sodium bisulfite have definite effects upon the heart and upon locomotion shows that these substances, like the dyes, enter the animal. The acetylcholine could hardly be destroyed since the medium was slightly acid and eserine had no effect. It is concluded that acetylcholine and adrenalin are without effect upon the heart of Artemia and that this lack of effect is not due to lack of penetration. It was noted that during periods of activity of thoracic appendages the heart was not accelerated over the rate at rest.

Eubranchipus:

Specimens of *Eubranchipus serratus* were collected in temporary ponds in March and April of two successive years. For observation most of the specimens were placed in small tubes through which test solutions could pass, as with the Talorchestia; a few were kept in watch glasses and mounted for observation, as were the Artemia. Essentially the same results were obtained with fourteen specimens. Acetylcholine (10^{-4} or 10^{-3}) in pond water was without effect (Figure 2*F*, *G*). Thus Eubranchipus behaves like Artemia.

Limulus embryos

It was shown by Carlson and Meek (1908) that the heart of Limulus begins beating on the twenty-first day of development, that this is before the heart ganglion is developed, that the beat continues to be myogenic until about the thirtieth day when the ganglion is first demonstrable histologically. Crozier and Stier (1927) found the temperature characteristic of the heart rate during the myogenic period to be usually $\mu = 11{,}500$ or 16,400 by contrast to $\mu = 12{,}200$ for the adult neurogenic heart. If there is a difference in the pharmacology of neurogenic and myogenic hearts one would expect the hearts of Limulus embryos to behave differently during the two periods of development. The heart of Limulus is accelerated by acetylcholine (Garrey, 1941).

Two batches of eggs were obtained at the time of laying. The several hundred eggs were kept in evaporating dishes through which sea water ran. The embryos developed during about a month and a half.

Heartbeat was first clearly visible on the twenty-first day of development. At that time about 15 embryos were mounted in numbered watch glasses so that they could be followed during the course of development. The egg capsule was removed, the chorion slit longitudinally in the mid-dorsal line so that it could be folded back at the sides of the animal. The edges of the chorion were held down by vaseline and the embryos remained permanently in this position. The watch glasses were kept in a sea water tray with water in the dishes changed at least daily. Some animals died and were replaced by others from the large evaporating dishes. Development proceeded at about the same rate in the eggs both in the watch glasses and in the large dishes. A small spot of light was focused on an embryo and observations made with a dissecting binocular. The heart rate was occasionally faster during the first one to three minutes of observation than after that time. The embryos were covered with sea water approximately 5 mm. deep and sometimes an additional water filter was used to remove any heat, but the brief initial acceleration continued. It is possible that light has a slight stimulating action on the heart. Counts were made after equilibrium was reached.

Solutions were changed in the watch glasses by pipettes and the heart rates counted (time for ten beats) from five to ten times at intervals of a few minutes for several hours. Between periods of counting, the watch glasses stood in sea water the temperature of which remained constant to within 1° C. This procedure was repeated on the same animals on alternate days for approximately two and a half weeks.

It was much easier to observe the hearts during the early period when the carapace was soft and fairly transparent. At the age of about four weeks pigment developed, the carapace hardened, and the heart could not be seen easily. It was found that in the older embryos a small area behind the median eye remained fairly translucent and light reflected from the anterior end of the heart could be observed there. After about 30 days of development the heart rates became somewhat more irregular.

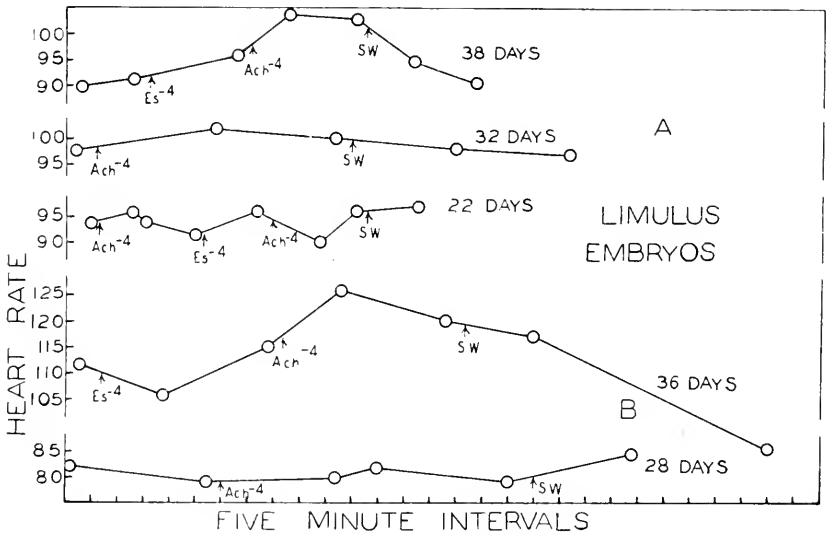

FIG. 3. Effect of acetylcholine and eserine on hearts of two Limulus embryos. Embryo *A* at 22, 32, and 38 days of development and embryo *B* at 28 and 36 days.

Data were obtained on 11 embryos. In two of these the period of observation extended over only four days but in the others data were obtained for periods between seven and 19 days. Typical curves are shown in Figure 3 and the entire experiment summarized in Table I. During the myogenic period acetylcholine had no effect upon the hearts of the Limulus embryos. Specimens were kept in concentrations of 10^{-4} for periods up to one hour. Eserine 10^{-4} was applied either with the acetylcholine or prior to treatment with it and still there was no effect of the acetylcholine. On the twenty-eighth to thirtieth days when, according to Carlson, the heart ganglion is present there was still little or no action. On the thirty-first day, however, two embryos showed acceleration and by the thirty-fifth day most of the specimens were accelerated by acetylcholine as is the adult heart. Only when the

effect was completely reversible was a positive effect recorded (Table I). It appears, therefore, that as in the Fundulus embryo (Armstrong, 1935) in Limulus a non-innervated heart is unaffected by acetylcholine.

Histological Interpretation

The preceding evidence shows (1) that acceleration by acetylcholine is not a general arthropod character, and (2) that some hearts may be accelerated, some inhibited, and others unaffected by acetylcholine. We shall seek an interpretation of the differences in acetylcholine action in terms of (1) whether the pacemaker tissue is nervous or muscular, and (2) whether there are also present regulating nerves coming to the heart from outside.[2]

Table I

Days															Embryo
22	23	24	25	26	27	28	29	30	31	32	35	36	38	40	1
0	0		0			0		0		+			+	+	1
	0		0			−		0		0	+				2
	0		0												3
				+ sl	0				+ sl						4
		0		0	0				+		+				5
						0				0	0	+			6
		0		0	0						+				7
				0	0		−		0						8
							0		0						9
								0				+ sl		+	10
										0	0	0	+	+	11

Effect of acetylcholine on hearts of embryos of Limulus from the twenty-second to fortieth days of development. 0 indicates no effect, − inhibition, + acceleration, and sl slight.

Nerve cells were first described in the heart of the crayfish by Berger in 1877 and have been seen in various decapod Crustacea by all who have looked for them since that time. Some of the more important papers are listed in Table II. Alexandrowicz (1932) has shown these cells to be large, limited in number, and to be mostly multipolar. Ganglion cells of the same sort occur in the isopod, Ligia, (Alexandrowicz, 1931). In Limulus the median cardiac ganglion contains multipolar and bipolar cells and is the pacemaker of the adult heart (Carlson, 1904). Two chains of nerve cells were described on the heart of the

[2] The hypothesis to be presented was suggested by Prosser and Zimmerman, 1941. *Biological Bulletin*, **81**: 292.

cockroach by Alexandrowicz (1926) while comparable cells seem to have migrated out to part of the stomatogastric system in Aeschna (Zawarzin, 1911). Walling (1908) described cells which may be nervous in the heart of the grasshopper, Melanoplus. Few insects have been investigated and it may well be that some have myogenic hearts (Maloeuf, 1935). In decapod Crustacea, insects, and Limulus extrinsic regulating nerves have been described. In each of these groups there is little doubt from the location, the shape and size of the ganglion cells, and from operative experiments (particularly on Ligia and Limulus) that these nerve cells constitute the normal pacemakers. In each of these groups acetylcholine stimulates the pacemaker to faster discharge. These pacemaker cells are essentially autonomic ganglia; in the vertebrates postganglionic sympathetic neurones are excited by acetylcholine. In both arthropods and vertebrates, therefore, autonomic ganglia are excited by acetylcholine and in the higher arthropods where these ganglia are the cardiac pacemakers the heart is accelerated.

Recent evidence (Prosser and Zimmerman, 1942) places the hearts of some annelids in the group of acetylcholine-accelerated, neurogenic hearts.

Whether or not there are nerve cells in the hearts of molluscs has long been disputed. Dogiel (1877) described "apolar" nerve cells in several species; these could not function as normal neurones. Darwin (1876), Foster and Dew-Smith (1877) denied that these are nerve cells and Ransom (1883) showed these cells of Dogiel to be either plasma cells or connective tissue cells. These authors, as well as Motley (1933) and Esser (1934) were unable to find ganglion cells in the hearts of the molluscs they investigated. Suzuki (1934) described in the oyster heart cells which had processes like nerve cells, but he states that they were best seen in haematoxylin preparations which were hardly specific for nerve tissue. Alexandrowicz (1913) briefly stated that there are nerve cells in the heart of Octopus but did not describe them. Carlson (1905) summarized a great deal of evidence indicating that in all groups of molluscs there are cardioregulator nerves. In some forms these nerves are interrupted by ganglia which may be located directly on the heart (particularly in cephalopods). These ganglia contain secondary regulating neurones and can be removed without stopping the heart (Ransom, 1883). In most molluscs the beat can be seen to originate periodically at different points over the heart. It seems likely, therefore, that many cells which have been described as nerve cells in molluscan hearts are either blood cells or connective tissue cells, or are secondary neurones, and that all molluscan hearts are myogenic.

Table II shows that the hearts of all molluscan classes investigated are inhibited by acetylcholine.

The beat of the vertebrate heart arises in the sinus or in the sinauricular node which is muscular in nature (His, 1894, et al.). The myogenicity of the beat was proven by Gaskell (1900) who has been amply supported by later investigations. Nerve cells have been recognized in the hearts of all classes of vertebrates; they are distributed in the sinauricular region, over the auricle and near the auriculo-ventricular fissure. (See Woollard, 1926, for recent data and references.) Gaskell and Langley proved these to be secondary vagal neurones.

In both the molluscs and vertebrates, therefore, the heart is myogenic but innervated, and is inhibited by acetylcholine. Baylor (1942) has found the heart of Daphnia to be inhibited by acetylcholine. Ingle (personal communication), after using a variety of nerve-staining methods, has failed to find nerve cells in the heart of Daphnia. This arthropod may, therefore, have mutated to myogenicity.

Waterman (1942) studied the effect of acetylcholine on the heart of intact specimens of the ascidian, Perophora. He found the duration and number of beats of the abvisceral phase to be increased, and relative dominance of this phase over the advisceral phase to be strengthened by acetylcholine. Waterman has given me the opportunity to calculate heart rates from his original data. In each of six experiments with acetylcholine and in two with mecholyl a smooth marked acceleration in rate occurs. The frequency in beats per minute for successive abvisceral phases each lasting $1\frac{1}{2}$ to 2 minutes in one typical experiment follows: in sea water 56.7, 55.2, 55.6; then in acetylcholine (10^{-5}) 50.8, 64.3, 64.1, 62.1, 66.6, 65.3, 66.8. Acceleration occurs in both pacemakers but the threshold for the abvisceral one is lower than that for the advisceral beat. Bacq (1934a, 1935) stated that in physiological concentrations of acetylcholine there is no effect on the heart of Ciona but that strong concentrations stop the heart in systole and on recovery the beat is faster. Schultze (1901) and Ransom (1883) failed to find ganglion cells in hearts of Salpa; Alexandrowicz (1913) claimed to find them in Ciona but gave no figures. Hunter (1902) figured bipolar ganglion cells at the ends of the Molgula heart. According to the relation between acetylcholine acceleration and neurogenicity presented in this paper, Waterman's evidence supports Hunter that at least some ascidian hearts are neurogenic. One must conclude either that the ascidians show aberrant mutation from the chordate line or else arose long before the main chordate characters were established. In support of the latter view is the fact that ascidian

muscles contain the invertebrate arginine phosphate rather than the vertebrate phosphocreatine (Baldwin, 1937).

The embryonic Fundulus heart is insensitive to acetylcholine until the vagal secondary neurones have migrated to the heart (Armstrong, 1935). The chick heart is relatively insensitive to muscarine until innervation at about 100 hours (Pickering, 1893). We have shown above that the embryonic Limulus heart is unaffected by acetylcholine. It seems likely, therefore, that a non-innervated heart, whether it is to be myogenic or neurogenic, is insensitive to acetylcholine. Apparently either innervation sensitizes the heart or else acetylcholine acts at the nerve terminations. If this reasoning is correct, the hearts of Artemia and Eubranchipus are non-innervated. This view is supported by the fact that locomotor rhythms in Artemia could be altered by pressure and by eserine without appreciably affecting the heart rate.

Wherever it has been tested, potassium acts in the same direction as acetylcholine, inhibiting myogenic and accelerating neurogenic hearts. Calcium slows neurogenic hearts. Its action on myogenic hearts is complicated by its effects on the contractile system. Atropine antagonizes acetylcholine, presumably by competing for the receptor substances, in vertebrates and decapod Crustacea, but in most molluscs (oyster, Jullien, 1936; Loligo, Bacq, 1934b; Aplysia, Heymans, 1924; Venus, Prosser 1940b, et al.) there are toxic effects and no antagonism is found. This may mean that the receptor substances in the heart cells are different in vertebrates and molluscs. Muscarine also yields conflicting results; it accelerates in Cancer (Davenport, 1942) and inhibits in Limulus (Nukada, 1917); it initially accelerates in Mya (Yung, 1881) and inhibits in cephalopods (Ransom, 1883). Nicotine usually stimulates neurogenic hearts; its effect on myogenic hearts varies greatly with concentration. It thus becomes difficult, if not impossible, to classify acetylcholine effects on hearts as muscarine-like or nicotine-like. Adrenalin accelerates all those hearts which it affects. The hearts of Artemia (this paper), Ciona (Bacq, 1934a) and Nereis (Federighi, 1928) are unaffected by it. Many molluscan hearts are relatively insensitive to adrenalin (Boyer, 1926; Motley, 1934, et al.). Its effect upon non-innervated embryonic hearts seems not to have been tested.

The evidence summarized in Table II shows that hearts fall into three classes with respect to the action of acetylcholine, and suggests that those hearts which are accelerated are neurogenic, those which are inhibited are innervated myogenic, and those which are unaffected are non-innervated.

TABLE II

Animal	*Acetylcholine action*	*Presence of ganglionic pacemaker*
Annelida		
Lumbricus	+ (Prosser and Zimmerman, 1942)	Yes (Stubel, 1909)
Arenicola	+ (Prosser and Zimmerman, 1942)	Yes (Carlson, 1908)
Arthropoda		
Crustacea		
Decapoda		
Carcinus	+ (Welsh, 1939*a*)	Yes (Alexandrowicz, 1932)
Maia	+ (Welsh, 1939*a*)	Yes (Alexandrowicz, 1932)
Panulirus	+ (Welsh, 1939*b*)	Yes (Alexandrowicz, 1932) Yes (Welsh, 1939*b*)
Cancer	+ (Davenport, 1941)	Yes (Alexandrowicz, 1932)
Astacus and Cambarus	+ (Davenport et al., 1940)	Yes (Berger, 1877)
	+ (MacLean and Beznak, 1933)	Yes (Dogiel, 1894)
	+ (Prosser, this paper)	Yes (Nevomywaka, 1928)
Potamobius		Yes (Alexandrowicz, 1932)
Homarus	+ (Welsh, 1940, 1942)	Yes (Alexandrowicz, 1932)
Libinia	+ (Prosser [3])	Yes (Smith by Prosser, 1940)
Squilla		Yes (Alexandrowicz, 1934)
Palaemon		Yes (Nusbaum, 1899)
Amphipoda		
Bactrurus	+ (Prosser, this paper)	
Talorchestia	+ (Prosser, this paper)	
Isopoda		
Ligia		Yes (Alexandrowicz, 1931)
Asellus	+ (Prosser, this paper)	
Copepoda		
Diaptomus	+ (Prosser, this paper)	
Branchiopoda		
Daphnia	− (Baylor, 1942)	No (Ingle, personal communication)
Artemia	0 (Prosser, this paper)	
Eubranchipus	0 (Prosser, this paper)	
Xiphosura		
Limulus	+ (Garrey, 1941, 1942)	Yes (Patten and Redenbaugh, 1899) Yes (Carlson, 1904)
Limulus embryo	0 (Prosser, this paper)	No (Carlson and Meek, 1908)
Insecta		
Periplaneta [4]		Yes (Alexandrowicz, 1926)
Melanoplus	+ (Hamilton, 1939)	Yes? (Walling, 1908)
Aeschna		Probably migrated outside heart (Zawarzin, 1911)

[3] Experiments unreported but used regularly for class demonstration.

[4] Mr. Herbert B. Saslow has recently found in my laboratory that the heart of the cockroach, *Blatta orientalis*, is reversibly accelerated by low concentrations of acetylcholine. He also finds that the heart of the honeybee is similarly affected.

TABLE II—*Continued*

Animal	*Acetylcholine action*	*Presence of ganglionic pacemaker*
Mollusca		
Gastropoda		
Helix	− (Jullien, 1936a)	No (Darwin, 1876)
	− (Jullien et al., 1939)	No (Foster and Dew-Smith, 1877)
		No (Ranson, 1883)
		Apolar cells (Pompilian, 1900)
		No (Alexandrowicz, 1913)
Murex	− (Jullien, 1936a)	
Ariolimax	− (Davenport et al., 1940)	
Aplysia	− (Heymans, 1924)	Apolar cells (Dogiel, 1877)
	− (Jullien, 1937)	
Pterotrachea		No (Ransom, 1883)
Pelecypoda		
Pecten		Apolar cells (Dogiel, 1877)
Mytilus	− (Jullien et al., 1938)	
Anadonta and other mussels	− (Prosser [5])	Apolar cells (Dogiel, 1877)
		No (Motley, 1933)
		No (Esser, 1934)
Ostrea	− (Jullien, 1936b)	Yes? (Suzuki, 1934)
Venus	− (Prosser, 1940)	No (Smith by Prosser, 1940)
Cephalopoda		
Octopus	muscarine − (Ransom, 1883)	Yes? (Alexandrowicz, 1913)
		No (Ransom, 1883, ganglion of secondary neurones)
Sepia	− (Kruta, 1936)	
Loligo	− (Bacq, 1934*b*)	Ganglion of secondary neurones (Carlson, 1905)
Chordata		
Urochorda		
Salpa		No (Ransom, 1883)
		No (Schultze, 1901)
Ciona	0+ (Bacq, 1934*a*, 1935)	No (Ransom, 1883)
		Yes? (Alexandrowicz, 1913)
Molgula		Yes (Hunter, 1902)
Perophora	+ (Waterman, 1942)	
Vertebrata		
Probably all classes	− (Dale, 1914)	Secondary vagal neurones (Woollard, 1926, Gaskell, 1900 for references)
	− (Hunt, 1915)	
Fundulus embryo	0 (Armstrong, 1935)	pre-innervation stage
Chick embryo	muscarine 0 (Pickering, 1893)	pre-innervation stage

The effect of acetylcholine on the hearts of animals named and the presence or absence of ganglionic pacemaker. 0 indicates no effect, − inhibition, and + acceleration.

[5] Experiments unreported but used regularly for class demonstration.

Summary

The effects of drugs upon the hearts of a number of arthropods have been investigated by immersion of the specimens in test solutions. Acetylcholine, potassium and other substances affect the heart of a crayfish when they enter through the body surface (gills) although the threshold is much higher than when the drugs are applied directly to the heart.

Two amphipods, the marine *Talorchestia longicornis* and the blind freshwater *Bactrurus mucronatus* show cardiac acceleration by acetylcholine. The heart of the blind freshwater isopod *Asellus tridentatus* is accelerated by acetylcholine as are the hearts of the copepods *Diaptomus oregonensis* and *Diaptomus sanguinensis*.

The heart of *Artemia salina* is unaffected by acetylcholine even in very high concentrations, with or without eserine. Eserine alone has a toxic depressing action on Artemia. The Eubranchipus heart is also unaffected by acetylcholine. Dye penetration and injection experiments show that the lack of acetylcholine action on Artemia is not due to lack of penetration.

Pure adrenalin is without effect on the Artemia hearts, but adrenalin solution (Parke, Davis) markedly inhibits due to the reducing agent sodium bisulfite.

Limulus embryos during the myogenic period of heart contraction (21 to 33 days of development) show no effect of acetylcholine. Acceleration appears along with innervation.

A comparison of the effects of acetylcholine with the presence of extrinsic innervation and of ganglionic pacemakers shows the following: The hearts of higher arthropods and of some annelids and tunicates which are accelerated are neurogenic. The hearts of adult vertebrates, of molluscs and probably Daphnia which are inhibited by acetylcholine are myogenic but innervated. Embryonic hearts of vertebrates, Limulus, and the hearts of Artemia and Eubranchipus are unaffected by acetylcholine and are probably non-innervated.

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CARDIAC PHARMACOLOGY OF THE CLADOCERAN, DAPHNIA

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Acetylcholine has been demonstrated to speed the heart of several of the decapod crustacea (Welsh, 1939 a and b; Davenport, Loomis and Opler, 1940 and Davenport, 1941), the grasshopper (Hamilton, 1939), and Limulus (Garrey, 1941). Is this reaction, which may be indicative of a neurogenic heart, a general characteristic of the arthropods? The pharmacology of the heart of the cladoceran, Daphnia, was examined to extend the observations to one of the lower groups within the phylum.

Obreshkove, 1941, has demonstrated the action of acetylcholine, eserine, and atropine on intestinal movement in Daphnia. Acetylcholine stimulates intestinal movement, its action is inhibited by atropine and potentiated by eserine.

Materials and Methods

Young animals, largely of the second instar of the species *Daphnia magna*, were used in this work to insure uniform age. At this stage, the animals are quite transparent and generally amenable to the requirements of the experiments. Older animals reacted in the same manner as younger ones but they were not used so extensively because they were more opaque.

At room temperatures the heart beat of Daphnia is too rapid to count with the naked eye. Therefore a stroboscope was devised to measure the cardiac rate at these higher temperatures. In other experiments the specimen was cooled to 10° C. and the heart was counted by eye.

Essentially the stroboscope consisted of a shaft with a light interrupter disc and a tachometer, all driven by a variable speed motor. The interrupter disc cut the light beam from a Spencer microscope lamp as it impinged on the mirror of a microscope used for viewing the preparation. The tachometer was a motor generator which was calibrated so that revolutions per minute could be obtained directly from reading the current generated.

Visual counting was made possible by lowering the temperature to $10° \pm 0.2$. At this temperature it was possible to obtain the rate directly and accurately with a stop watch.

The standard procedure was to place the animal upon a network of cotton fibers in a depression slide of about 0.5 cc. volume. The experimental fluids could then be applied and removed by means of two pipettes, one of which held the test solution and the other of which was connected to a vacuum source for efficient removal of the solution. Counts of the heart rate were made after equilibration in dechlorinated tap water. The experimental solutions were then added and the heart rate was followed. Finally, the dechlorinated tap water was replaced on the animal to allow recovery. In order to assure the concentration of any solution employed, the preparation was washed several times with the solution and the entire volume was changed at least four times.

The adrenalin used in these experiments was prepared by Parke, Davis and Company; atropine sulfate and eserine sulfate (physostigmine) were prepared by Hoffman-La Roche; acetylcholine bromide by Eastman Kodak Company.

The solutions were made up to the indicated strengths in aerated non-chlorinated tap water.

Results

The normal heart rate varies from four beats per second to ten beats per second at room temperature. Feeding causes great variations. Animals of a culture might show an average beat of four beats per second before being fed, and a day later after receiving yeast show a beat of ten per second. The large culture jars were aerated continuously. It was found that while in the depression slide additional aeration was not necessary and that the beat would maintain itself for four to five hours without it.

Acetylcholine

The results obtained from forty experiments with acetylcholine agree in showing that acetylcholine inhibits the heart rate in Daphnia. Figure 1 *A*, *B*, *C*, *D*, shows the results on single animals using a series of different concentrations. There is a direct relation between the heart rate and the concentration of the drug. The threshold appears to be 10^{-9} at which concentration one of three experiments gave a 3 per cent inhibition, the others none. A concentration of 10^{-8} in three experiments gave an average inhibition of 8 per cent; 10^{-7} in five experiments gave an average inhibition of about 18 per cent; 10^{-6} in

six experiments gave an inhibition averaging 21 per cent; and 10^{-5} in four experiments gave an average inhibition of 27 per cent.

As can be seen by the graphs, the effect of acetylcholine is not completely reversible in every case. In some cases after dechlorinated

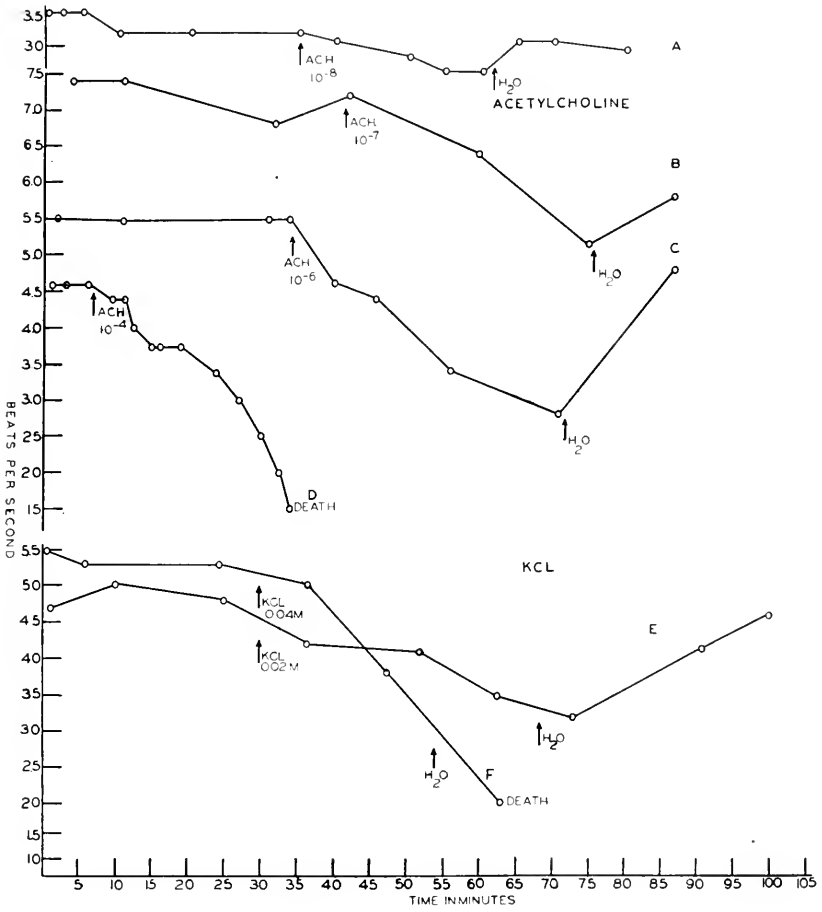

FIG. 1. Effects of acetylcholine (*A B, C, D*) and potassium (*E, F*) on the heart rate in *Daphnia magna*. Specimens placed in solutions as indicated by arrows. Ach, acetylcholine, H_2O dechlorinated tapwater.

water was added following retardation, the rate rose to only one-half the original rate. This indicates a toxic effect of acetylcholine on the heart of Daphnia, particularly at higher concentrations.

Acetylcholine in higher concentrations (10^{-6} to 10^{-4}) causes a series of events starting with a slowing of the heart rate followed by a

weakening of the strength of the beat. There is always observed a great reduction of amplitude in these concentrations. Next a sort of doubling occurs which is probably the result of the wave of contraction not being conducted completely over the heart. In a few cases, such as that seen in Figure 1 *D*, the heart stopped in diastole.

Atropine

Atropine was applied in fifteen experiments in concentrations from 10^{-8} to 10^{-2}. In these concentrations it causes a negative chronotropic effect which is probably a toxic one since the addition of water failed to bring about recovery from solutions higher than 10^{-8}. Concentrations of 10^{-7} to 10^{-5} inhibited by from 6 to 8 per cent and 10^{-2} inhibited by 15 per cent. After replacement of the atropine by water the heart beat did not return to its normal rate but remained low for many hours, after which the animals died. Experiments on the antagonism of acetylcholine by atropine, such as was observed by Obreshkove, were thus impossible to perform. These results do not agree with those obtained by Pickering, 1894, on Daphnia. He found that atropine accelerated the heart rate.

Eserine

Eserine on the heart of Daphnia causes an inhibition which is not reversible upon addition of water. Two experiments were performed at each of the four concentrations. There was no effect at 10^{-9}. In any concentration higher than and including 10^{-8}, the heart beat was inhibited in varying amounts depending on the concentration of eserine. Addition of water did not bring about recovery and the heart rate remained low and eventually stopped. Because of this toxicity, experiments on eserine potentiation rendered results which could not be interpreted except in the light of the toxicity. Two experiments using subthreshold eserine followed by acetylcholine gave no indication of potentiation. Obreshkove (1941) working on intestinal movements found potentiation of acetylcholine by previous eserine treatment. It would therefore be expected that the heart would show similar reactions. Further experiments in which the time of exposure to eserine is much reduced before treatment with acetylcholine and the time factor of the appearance of inhibition is carefully noted, should be performed before any definite statement can be made.

Adrenalin

Application of commercial adrenalin (Parke, Davis solution) to the heart of Daphnia gave erratic results (Figures 2 *B* and *C*). This was shown by Prosser (1942) in the course of experiments on Artemia to be

due to the sodium bisulfite added to the commercial adrenalin solution to prevent auto-oxidation of the ardenalin. This was true for Daphnia as well. Comparable concentrations of $NaHSO_3$ (1% diluted to 10^{-7}) without adrenalin cause cardiac inhibition as high as 20 per cent. (Figures 2 *D* and *E*.) Solutions of pure crystalline adrenalin give

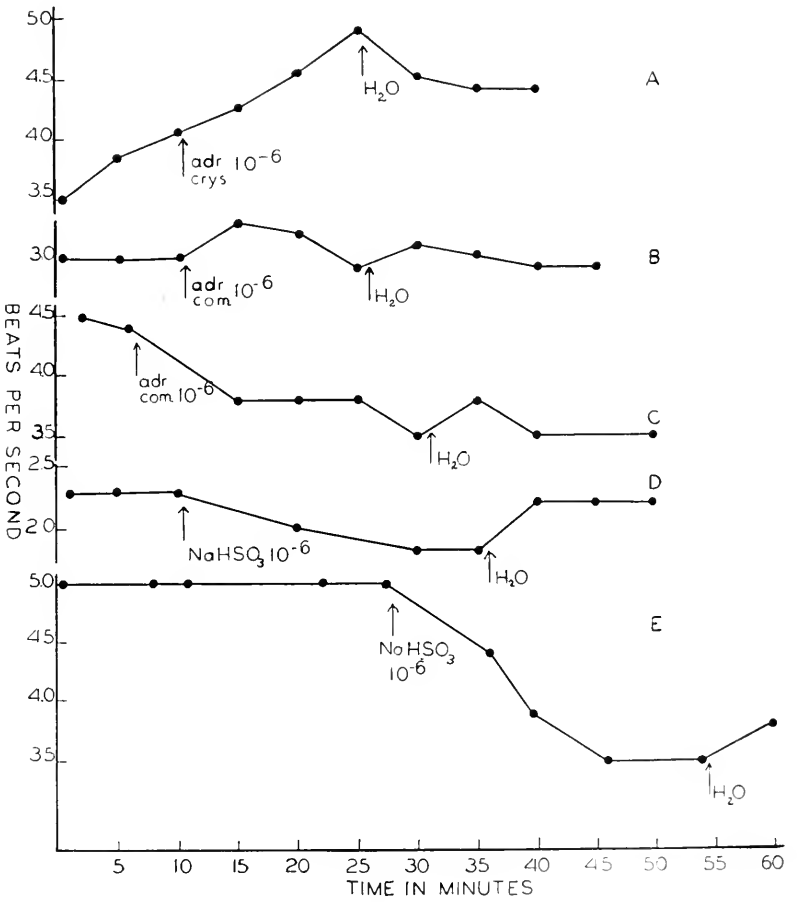

FIG. 2. Effects of: *A*, crystalline adrenalin (adr. crys.); *B*, *C*, commercial adrenalin solution (adr. com.) and *D*, *E*, sodium bisulfite on the heart rate of *Daphnia magna*.

notable accelerations which increase as the concentration increases (Figure 2 *A*).

Potassium

Potassium causes an inhibition in the rate of the beat. Eight experiments were performed. Concentration of 0.005 M was sub-

threshold; 0.01 M, in two experiments, caused an inhibition of 1.5 per cent; .02 M approximately 12 per cent (see Figures 1 *E* and *F*). The highest concentration used, 0.04 M, was toxic and did not reverse upon return to water (see Figure 1 *F*). This curve appears very similar to that of acetylcholine 10^{-4}.

Nicotine

Nicotine applied to the heart of Daphnia shows a stimulation in the weaker concentrations and an inhibition in the stronger concentrations which is not reversible. A concentration of 10^{-6} showed an acceleration of 6 per cent; 10^{-4} a 17 per cent fall; and 10^{-3} a 35 per cent fall.

Discussion

The heart of Daphnia, like the heart of the vertebrate, is inhibited by acetylcholine and potassium. In all arthropods previously considered except Daphnia there is an acceleration of the beat in response to acetylcholine and potassium. The hearts of all vertebrates appear, from histological evidence, to be myogenic and those of all decapod crustacea and Limulus appear from histological evidence to be neurogenic (Alexandrowicz, 1932; Carlson, 1904).

Obreshkove, 1941, has shown that stimulation of the gut of Daphnia by pricking causes increased peristalsis of the intestine and an inhibition of the heart. He also found similar increased peristalsis upon application of acetylcholine which, as shown by our experiments, causes a decreased heart rate. Acetylcholine in the vertebrate causes peristalsis and decreased heart rate. This, then, is a further indication of the similarity of the cholinergic systems of Daphnia and the vertebrates.

On the basis of the evidence presented in the literature, Prosser (1942) has postulated that all myogenic hearts are inhibited by acetylcholine and potassium and that all neurogenic hearts are accelerated by acetylcholine and potassium. Since the pharmacology of the Daphnia heart resembles that of the vertebrate in all respects thus far tested, and since there is no work to indicate the presence of nerve cells in this heart, we may conclude that the Daphnia heart is myogenic and predict that it will be found to have no pacemaker nerve cells. Extrinsic or regulating innervation is, however, probable.

It might be argued that in the experiments presented in this work acetylcholine affects the heart by way of the central nervous system of the Daphnia. However, the following facts oppose this argument. In the vertebrates injection of acetylcholine into the intact animal gives cardiac inhibition much like that found in isolated hearts. Also,

crayfish half immersed in solutions of acetylcholine so that any acetylcholine reaching the heart must enter through the gills and pass by way of the blood to the heart, show acceleration (Prosser, unpublished data). Furthermore, the concentrations employed in this work on Daphnia closely approximate those used by other workers on other animals where the drugs were applied directly to the hearts.

Summary

1. Acetylcholine was found to have a depressing effect on the heart rate and strength of beat of the heart of *Daphnia magna* with the threshold at 10^{-9} and a depression of 25 per cent at 10^{-5}. The effect is reversible.

2. Atropine has a depressing effect on the heart rate with the threshold at 10^{-7} and an inhibition of 15 per cent at 10^{-4}. The effect is not reversible.

3. Eserine with a threshold of 10^{-9} has a toxic effect on the heart of Daphnia causing irreversible inhibition at any higher concentration.

4. Adrenalin has an accelerating effect at 10^{-7} when prepared from the crystalline form. Commercial adrenalin solution may actually inhibit due to the reducing agent, $NaHSO_3$, which itself inhibits heart rate in concentrations as low as 10^{-6}.

5. Potassium chloride inhibits the heart rate with a threshold at 0.005 M, a 12 per cent inhibition at 0.02 M and death at 0.04 M.

6. The above facts would indicate that the heart of Daphnia more closely resembles the vertebrate heart than that of the higher crustacea in its pharmacology.

At this time I wish to express my gratitude in acknowledgment of the help and advice given me by Dr. C. L. Prosser.

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THE HYDROGEN ION CONCENTRATION OF THE CONTENT OF THE FOOD VACUOLES AND THE CYTOPLASM IN *AMOEBA* AND OTHER PHENOMENA CONCERNING THE FOOD VACUOLES [1]

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Introduction

The content of the food vacuole has been studied by various investigators in a considerable number of different protozoa. All maintain that it becomes acid shortly after the food vacuole is formed and that it later becomes neutral or alkaline, and all appear to assume that acids and bases are secreted by the cytoplasm and poured into the vacuoles. For example, Greenwood and Saunders (1894) say: "The ingestion of solid matter, whatever its nature, stimulates the surrounding cell substance to secrete acid fluid"; Nirenstein (1905) says: "Wie man sieht bedarf die Frage nach dem Sinne der Säuresekretion noch durchaus der Aufklärung"; Howland (1928, p. 131) says: "This would indicate that the acid [in the food vacuole] is secreted by the surrounding protoplasm"; and Claff et al. (1941) say: "The initial acid production around the vacuole was stimulated by the closure of the vacuole." Similar statements have been made concerning the bases.

It is generally held that the base in the vacuoles functions in the digestion of food, but concerning the function of the acid there is marked diversity of opinion. Hemmeter (1896) contends that it serves to kill the ingested organisms. Claff et al. (1941) support this contention. They say: "The prey is killed simultaneously with a sudden release of an acid into the newly-formed food vacuoles." Howland (1928) also favors it, and she seems to hold that the acid aids digestion. She says (p. 133): "It is possible that the acid functions as a killing fluid within the vacuole. . . . All vacuoles in which disintegrating food lies in the center surrounded by vacuolar fluid, appear to be in the process of active digestion. . . . The pH value of such vacuoles, as uniformly indicated by brom cresol green, is 4.3 ± 0.1." Nirenstein (1905) does not agree with these contentions. He maintains that the

[1] I am indebted to Dr. R. A. Fennell and to Dr. W. J. Bowen for very efficient assistance in this work.

acid in the food vacuole is not strong enough to kill bacteria, but he asserts that it may serve to make other substances present toxic. He holds that digestion does not begin until after the substance in the vacuole has become alkaline, but he thinks the acid may function in preparation for digestion. He says: "So wäre daran zu denken, dass die Säure bei der Aktivierung des Profermentes eine Rolle spielt; ferner könnte der Säure die Bedeutung zukommen, native Eiweisstoffe zu koagulieren, woraus sich indirekt eine gewisse Wichtigkeit der Säure für Verdauung ergeben würde." Greenwood and Saunders (1894) also maintain that digestion does not begin until after the vacuole is alkaline. They say: "The outpouring of acid is unaccompanied by any digestive change in nutritive matter."

According to these views, then, the cytoplasm around the younger food vacuoles in protozoa secretes acids which are poured into them and possibly serve to kill and digest the ingested organisms, and the cytoplasm around the older vacuoles secretes bases which are poured into them and function in digestion. These views are very largely based on results obtained in observations on ciliates. If the contention that the cytoplasm adjoining the surface of the food vacuoles pours acids into some and bases into others holds for *Amoeba*, it is a most remarkable phenomenon, for in these organisms food vacuoles with content respectively acid and alkaline are often in close contact with each other, and apparently surrounded by the same kind of cytoplasm.

Materials and Methods

Amoeba proteus, *Amoeba dubia*, *Chilomonas paramecium* and *Colpidium striatum* were used almost exclusively in the following experiments. They were raised in Hahnert solution [2] containing rice or wheat. The observations were made as follows: Amoebae were taken from the cultures and passed through fresh portions of Hahnert solution until all the organisms on which they ordinarily feed were removed. They were left in this solution until nearly all the food vacuoles had disappeared, i.e., for 24 hours or longer. Some of them were then mounted between ridges of vaseline on slides and either living or dead food stained with various indicator dyes added. Others were stained with various dyes, then mounted, and unstained living or dead food added. Cover-glasses were put on the preparations, the amoebae studied under low and high magnifications, and the length of life of the ingested organisms and the changes in the color and the structures in them observed.

[2] Hahnert (1932) "KCl, 0.004 gm.; $CaCl_2$, 0.004 gm.; $CaH_4(PO_4)_2$, 0.002 gm.; $Ca_3(PO_4)_2$, 0.002 gm.; $MgHPO_4$, 0.002 gm.; H_2O, 1000 cc. (pH 6.5)."

In ascertaining the hydrogen ion concentration of the content of the food vacuoles, Clark buffers covering the range of the dye under consideration, and differing by 0.2 pH were put respectively into small test tubes and the same quantity of the dye added to each. The content of the food vacuole in the amoebae was then compared in color with the buffers in the test tubes. In some experiments other methods were also used; these are described below.

Neutral Red

Living organisms ingested

Chilomonads and colpidia were added to culture solutions containing neutral red, and amoebae prepared as described above. The amoebae ingested the chilomonads and colpidia freely, frequently two or more at a time. Some of the food vacuoles thus formed were observed continuously for an hour or longer and briefly from time to time for 24 hours.

The newly formed food vacuoles contained large quantities of fluid in which the ingested organisms swam violently about. The amount of this fluid gradually decreased until there was little left (Fig. 1); then the organisms in it usually became quiet suddenly, after which they soon became purplish pink like the color of buffer, pH 3.6 (containing neutral red), and then very gradually pinkish (pH 6); but the fluid in the vacuoles remained colorless. Later the fluid increased in amount (Fig. 1) and became distinctly yellow, but the organisms in it were still pinkish, indicating that the fluid was now alkaline (about pH 8) and the organisms in it acid or neutral (pH 6 to 7). The fluid and the organisms then gradually became darker until the former was brownish yellow and the latter reddish brown (pH 8.8 to 9).[3] The time required for these changes varied greatly. Some details are presented in the following records taken from my notes.

(1) Specimens of *Amoeba dubia* were put into Hahnert solution containing neutral red on a slide and left about an hour, then colpidia were added. A few minutes later (1:40 P.M.) one of the amoebae ingested a colpidium. This colpidium was continuously observed for several hours and its color compared with those of a series of buffer solutions containing neutral red.

The colpidium swam vigorously in the food vacuole for nine minutes, then suddenly stopped. No change had taken place in the color of the colpidium and the fluid around it was colorless.

[3] Results presented below demonstrate that the content of the food vacuole does not become more alkaline than approximately pH 7.3 and that neutral red is not an accurate indicator.

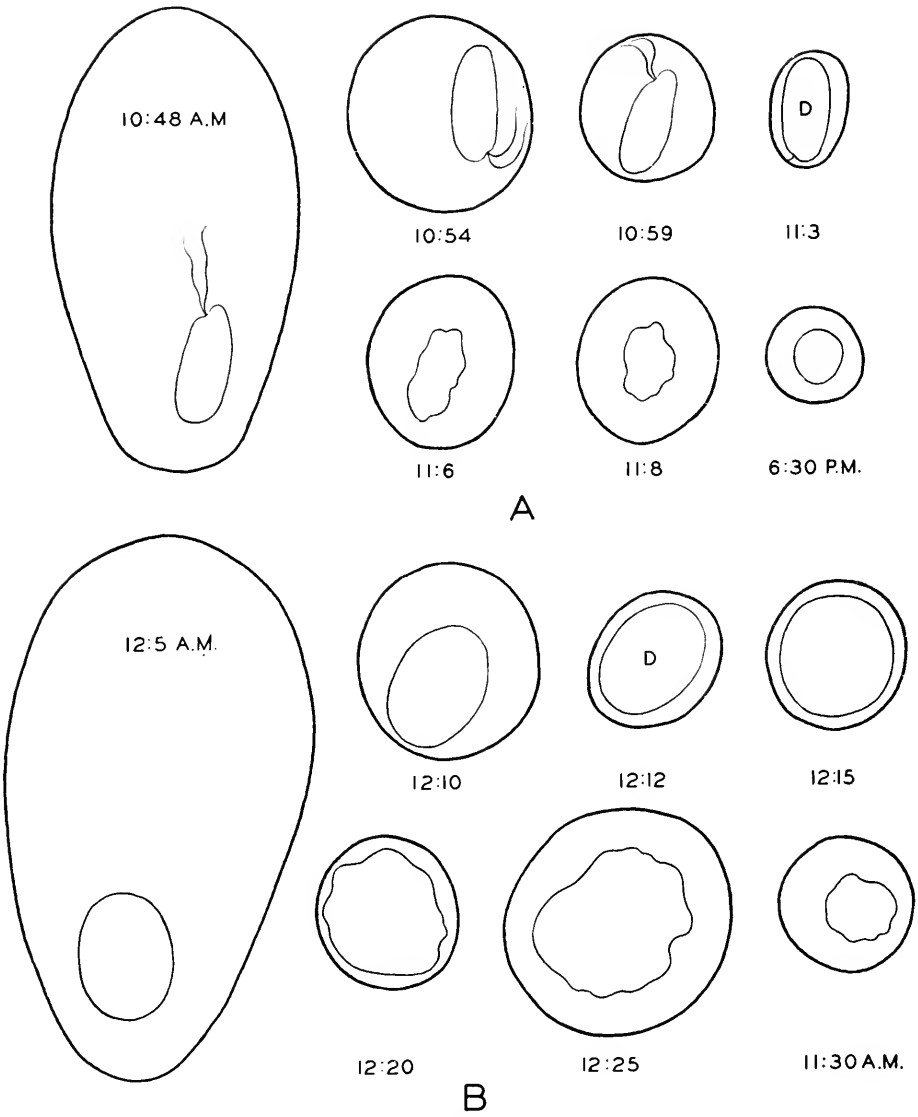

FIG. 1. Camera outlines showing changes in the size of food vacuoles containing organisms ingested by *Amoeba proteus*. *A*, food vacuole containing a chilomonad; *B*, food vacuoles containing a colpidium; numbers, time of day; *D*, approximate time the ingested organisms died.

Note that the vacuoles decreased greatly in size owing to the loss of fluid, then increased rapidly owing to absorption of fluid, and that the organisms died shortly after the fluid content became minimum.

(1:55 P.M.) Colpidium light purple, fluid colorless; (2:00) colpidium denser purple, about like buffer, pH 3.5, fluid colorless; (2:10 and 2:30) no change; (2:50) colpidium pinkish purple, pH 4.5 to 5, fluid colorless; (3:25) colpidium clearly more pinkish, pH 6, fluid colorless; (3:55) colpidium reddish pink, pH 6.6 to 7, fluid colorless; (4:50) colpidium more reddish, pH 7 to 7.5, fluid faintly yellowish, pH 8, clearly more alkaline than colpidium; (6:50) colpidium reddish yellow, pH 8, fluid brownish yellow, pH 8.8 to 9; (8:50) colpidium brownish yellow, fluid about the same.

(2) In another preparation containing chilomonads in place of colpidia the following was observed: (11:24 A.M.) Chilomonad ingested; (11:27) chilomonad dead, faintly purple, pH 3.5, fluid around chilomonad colorless; (11:32) several granules in the chilomonad, deep crimson, rest brilliant purple, pH 3.5, fluid colorless; (11:40) chilomonad definitely pinkish, about pH 7, fluid colorless; (12:30) chilomonad pink with slightly yellowish tint, about pH 7.6, fluid distinctly yellowish, pH 8; (1:30) chilomonad brownish yellow, pH 8+, fluid about the same.

Similar observations were made on many other food vacuoles containing colpidia and chilomonads respectively, some in *Amoeba proteus* and others in *Amoeba dubia*. The results obtained were essentially the same in all. The colpidia and chilomonads in all became distinctly purple almost immediately after they died, then gradually pinkish, then reddish, then brownish yellow. The fluid in the food vacuoles remained colorless until some time after the colpidia and chilomonads in it had become pink, then it became yellow, i.e., the organisms in the vacuoles remained acid for some time after the fluid around them had become alkaline. This shows that the base in the vacuoles originates, not in the ingested organisms but in the cytoplasm around the vacuoles.

The following observations show that the rapid increase in hydrogen ion concentration in organisms in food vacuoles is correlated with death. Colpidia and chilomonads were put into strong solutions of neutral red in Hahnert solution and observed closely. Very soon particles in the food vacuoles in the colpidia and a few granules in the cytoplasm of the chilomonads became red. Shortly after this some of the organisms died and then gradually more, until all were dead. Immediately after death the cytoplasm and the nucleus in all became purple, first very light then rapidly deeper, until the entire body was deep brilliant purple like buffer, pH 3 to 3.5, after which there was no change in color except that under some conditions it gradually faded out. This strongly indicates that the acid in the food vacuoles does not originate in the cytoplasm around them, but in the organisms in them.

The results presented above indicate that shortly after ingestion the organisms in the food vacuoles in *Amoeba* became decidedly acid, but they have no bearing on the question concerning the hydrogen ion concentration of the fluid in the vacuoles at this time. The following experiments concern this.

Neutral red crystals ingested

If a base is added to a neutral aqueous solution of neutral red, the solution becomes yellow and then needle-like brownish yellow crystals form. These crystals are readily soluble in most acid solutions. In oleic acid and probably also in some other fatty acids they round up and become very viscous and deep crimson in color.

The relation between the solubility of the crystals, the change in color and the hydrogen ion concentrations in different solutions was ascertained as follows: Crystals were washed in culture solution (pH 6.8), then a few in a very small amount of this solution were transferred to a number of relatively large quantities of solution differing in hydrogen ion concentration, and the effect noted.

Three different solutions were used: Clark buffers (acetate and phosphate, pH 4, 4.6, 5, 5.6, 6 and 6.6), Hahnert solution plus hydrochloric acid (pH 4, 4.5 and 5) and Hahnert solution plus lactic acid (pH 4, 4.5 and 5).

It was found that the crystals dissolve readily in Clark buffers at pH 5 and lower, and slightly at pH 5.6, and that in Hahnert solution plus lactic or hydrochloric acid they dissolve readily at pH 4 and lower, and slightly at pH 4.5, but somewhat more readily in the former than the latter. Details concerning two experiments follow:

(1) One cc. of each of the Clark buffers was put respectively into Columbia dishes on white paper, and a *very small* amount of Hahnert solution (pH 6.8) containing numerous crystals added. The crystals were then observed with the naked eye, and under low magnification. In buffers pH 4, 4.6 and 5, they became pink and dissolved immediately. In buffer pH 5.6 they became slightly pink at the surface in two minutes, and deep pink in six minutes. Some dissolved in 13 minutes, many in 20, nearly all in 40, and all in 60. In buffer pH 6, the crystals became faintly pink at the surface in 13 minutes, definitely pink in 20, and somewhat more strongly pink in 40. Observations were continued for four hours. Very few if any of the crystals dissolved.

(2) With Hahnert solution plus HCl in place of Clark buffers, pH 4, the crystals became definitely pink in one minute, and strongly pink in four. Nearly all dissolved in 14 minutes, and all in 24. In this solution, pH 4.5, they became slightly pink in four minutes and

definitely pink in 14. A few dissolved in 24 minutes but not all in four hours.

Specimens of *Amoeba dubia* were mounted in culture solution containing neutral red crystals, but no food organisms. The culture solution (Hahnert solution plus a little NaOH) was pH 6.8. The amoebae were active in this solution and appeared normal in all respects. Some of them soon ingested one or more crystals each (one ingested six). Several with crystals were closely followed from the time the crystals were ingested until four hours later. During this time the crystals moved freely with the plasmasol. Most of them appeared to be in close contact with the cytoplasm, not in vacuoles containing fluid. Some were ejected, others not. No change whatever was observed in any of these crystals, either in color or in size or in form.

This experiment was repeated twice with chilomonads added to the culture solution. Several of the amoebae ingested two to 15 chilomonads each, and one or more crystals, but none were found with chilomonads and crystals in the same food vacuoles. All were under close observation for several hours. No changes in any of the crystals were detected.

The experiment was also repeated twice with colpidia in the culture solution in place of chilomonads. In both tests the amoebae ingested colpidia promptly and in the formation of some food vacuoles crystals were taken in with the colpidia. Details concerning two such vacuoles in different amoebae follow:

(1) At 2:55 P.M. a colpidium was discovered violently swimming about in a food vacuole which contained a neutral red crystal. The movement of the colpidium greatly agitated the crystal, proving that it was actually in the fluid in the food vacuole. The food vacuole decreased rapidly in size. At 2:58 the colpidium came to rest and about one minute later it began to become purplish in color. At 3:03 it was very definitely purple (like buffer, pH 3.5) but the fluid in the vacuole was colorless and the crystal had not changed. At 3:20 the colpidium was purplish pink (pH 4.5 to 5) but the fluid was still colorless and no visible changes in the crystal had occurred. At 3:55 the vacuole had considerably increased in size. The colpidium was now pinkish red (pH 6.5) and the fluid around it light brownish yellow (clearly alkaline, about pH 8). The amoeba was lost soon after this, but in other specimens vacuoles were found in which the colpidia gradually changed color from pinkish red to brownish yellow, i.e., they became similar in color to the fluid around them.

(2) An amoeba was discovered forming a food cup over an area on the slide in which there were a colpidium, a large crystal, and an ag-

gregate of small pieces of crystals. As the mouth of the cup closed, the edge on all sides remained in close contact with the slide so that the crystals were taken in with the colpidium. The colpidium swam around in the cup, and the crystal and the aggregate of pieces were violently thrown about by the currents produced by the cilia. After a few moments the large crystal passed out of the vacuole into the cytoplasm and the aggregate became attached to the surface of the colpidium. A few moments later the colpidium became quiet and began to become purplish in color; the vacuole had decreased considerably in size, owing to loss of fluid. About two minutes later the colpidium was purplish pink (pH 3.5 to 4) but the fluid in the vacuole was still colorless and there was no visible change in the crystals. Thirty minutes later the vacuole had distinctly increased in size, the fluid in it was definitely light brownish yellow, but the colpidium was still purplish pink, though considerably darker, and the crystals were still attached to it and intact, with no change in color.

The fact that the neutral red crystals in the food vacuoles did not dissolve or change in color, whereas they became pink and dissolved in Clark buffer, pH 5, and in Hahnert solution, pH 4.5, indicates that the solution in them did not become more acid than pH 4.5. This and the fact that the chilomonads and the colpidia in the food vacuoles became purple (pH 3.5), i.e., much more acid than the fluid around them, indicate that the acid in the food organisms in the food vacuoles in *Amoeba* originates within the vacuoles, not in the cytoplasm around them. The fact that the fluid in the vacuoles becomes brownish yellow and that this occurs when the chilomonads and the colpidia in it are still pink, indicates that this fluid becomes alkaline and that the base in the fluid originates in the cytoplasm around the vacuole, not in the organisms in it. The results obtained in these observations therefore support the conclusions reached in the preceding experiments, and they indicate that the fluid in the food vacuoles does not become more acid than pH 4.5.

Further evidence concerning some of these conclusions was obtained by feeding the amoebae dead organisms.

Dead organisms ingested

Chilomonads and colpidia were respectively put into Hahnert solution containing neutral red and left about one hour, i.e., until the food vacuole in the latter and the neutral red bodies in the former were deeply stained. Then they were killed by rapidly heating the solution, after which enough NaOH was added to make the solution distinctly alkaline and the chilomonads and colpidia brownish yellow. Then

one drop of Hahnert solution containing *Amoeba dubia* was mounted on a slide and one drop of the solution containing the dead, stained chilomonads added, and the preparation observed for several hours. This was repeated with colpidia in place of chilomonads and also with *Amoeba proteus* in place of *Amoeba dubia*. The hydrogen ion concentration of the solution on the slide was measured with glass electrodes in every test. It varied from pH 7.4 to pH 7.8, and the chilomonads and colpidia in it from reddish yellow to brownish yellow. Some of the preparations contained neutral red crystals.

The amoeba ingested the dead chilomonads and colpidia readily, *Amoeba dubia* more readily than *Amoeba proteus*. The changes in the chilomonads and the colpidia in the food vacuoles were essentially the same in both species but they were somewhat more definite in the former than in the latter. After the yellow organisms had been ingested they gradually became pinkish, and in about 15 minutes they were pinkish red, about like neutral red in buffer, pH 7.4 or 7.2. They did not become purplish (pH 3.5 to 4) as they do when they die in solutions containing neutral red. During this time the fluid around the organisms was colorless but shortly became yellow. Then it and the organisms in it gradually became brownish yellow, like buffer pH 8 to 8.8. Then there was no further change in color in either for several hours, after which the color in both usually faded out gradually.

All these characteristics are sometimes seen simultaneously in different vacuoles in an amoeba. This is well brought out in the following description of the food vacuoles seen under high power (20 ocular and 60 objective, oil) in an amoeba which had been in the solution containing stained, dead chilomonads, a little more than three hours. During this time the amoeba had ingested 20 chilomonads. Some of these had much fluid around them, others had very little. In some this fluid was colorless, in others pinkish red (pH 7.2), in still others brownish yellow (pH 8.6). In some the color of the fluid was dense, in others very faint (barely visible). In some vacuoles the color of the chilomonads was the same as that of those in the culture fluid outside; in others it was pinkish red (about pH 7.2); in others yellow to yellowish brown (pH 8 to 8.8).

A considerable number of food vacuoles studied contained neutral red crystals. No changes were observed in any of these crystals. Some of the chilomonads and some of the colpidia studied were digested.

The fact that the neutral red stained chilomonads and colpidia were yellow (pH 7.4 to 7.8) when they were ingested and became pinkish red (pH 7.4 to 7.2) shows that the acidity of the chilomonads increased in the food vacuoles; and the fact that the chilomonads were

dead when they were ingested shows that this change in color was not due to chemical processes involved in metabolism or in dying. The results obtained, therefore, seem to indicate that acid was formed in the cytoplasm and poured into the vacuoles. It is evident, however, that the observed decrease in hydrogen ion concentration could have been due to addition of a weak alkaline solution. All that these results actually show, then, is that a solution which was not more alkaline than approximately pH 7.3 entered the food vacuoles.

Results which are in some respects similar to those presented above were obtained with starch grains in place of dead chilomonads and colpidia. These are presented below.

Starch ingested

Specimens of *Amoeba dubia* which had been without food 24 hours were mounted on slides, in Hahnert solution, containing neutral red and, respectively, rice, wheat and potato starch grains, and examined under low and high magnifications continuously for about 30 minutes and for short periods from time to time for 24 hours or longer. The wheat and rice starch used was taken from the bottom of dishes containing amoebae cultures in which wheat and rice grains had disintegrated. The potato starch was obtained by scraping a piece of potato, mixing the scrapings with water, straining through cheese cloth and washing.

The amoebae ingested the starch grains freely and some soon became well filled with them (Fig. 2). No changes were observed in the ingested grains for about 15 minutes; then they became purple, first faintly and then strongly like buffer pH 2 or less, then very gradually reddish pink (pH 7) and finally brownish yellow (pH 8.5 to 8.7).

The changes in color did not vary with the kind of starch but the colors were definitely more distinct in the potato and rice starch than in the wheat starch and the time required for the changes in color was somewhat less in the former than in the latter, but this varied greatly in all. It required about 15 minutes for the starch grains to become purple, about two hours more to become pinkish and about 12 hours more to become yellowish.

Usually there was but little fluid ingested with the starch grains and much of this soon disappeared, so that by the time the grains had become purple there was often no fluid visible in the food vacuoles. This was especially evident in those which contained potato starch. But in all the vacuoles in which fluid was visible it was invariably colorless until the starch began to become pinkish; then the amount of fluid in the vacuoles increased greatly and as it increased it became

yellow, so that now the vacuoles contained pink starch grains in a distinctly yellow fluid. The starch grains soon became yellow however and later brownish yellow. This indicates that the fluid and the starch grains in the vacuoles became alkaline but that the fluid became alkaline before the starch grains in it.

The results presented seem to indicate that the hydrogen ion concentration of the content of the food vacuoles in *Amoeba* becomes ex-

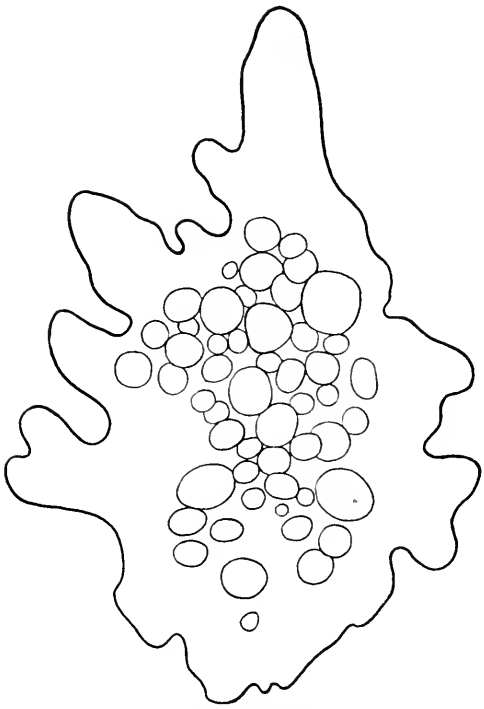

FIG. 2. Camera drawing showing *Amoeba dubia* after having ingested numerous potato starch grains.

traordinarily high, i.e., pH 2 or higher. The fact that neutral red crystals do not dissolve in the vacuoles, however, shows very clearly that it does not become nearly so high. What, then, is the cause of the purple color observed in the starch grains?

Amoebae which contained numerous neutral red stained starch grains varying in color from purple to brownish yellow were mounted in fresh neutral culture fluid, without any neutral red, and crushed by means of pressure on the cover-glass. Nearly all the starch grains flowed out and soon became free in the neutral fluid. Some of these

were crushed by further pressure, so as to expose the interior. The preparation was then carefully studied under 20 ocular and 60 oil immersion objective.

Observations on the crushed starch grains showed very clearly that they were colored throughout (i.e. that the color of the starch grains was not due to accumulation of dye on the surface, but to dye uniformly distributed through them) and they showed that the color in all the starch grains in the neutral fluid gradually faded until it had disappeared entirely, but that before the starch grains became colorless they invariably became purple, i.e. that those which were pink (pH 7) became purple (pH 2) then colorless and that those which were yellow (pH 8) became pink, then purple, then colorless.

The change from yellow (pH 8) to pink (pH 7) was doubtless due to increase in hydrogen ion concentration in the starch grains caused by the neutral fluid around them, but the change from pink to purple obviously could not have been due to this. It was in all probability correlated with the concentration of neutral red in the starch grains for it was repeatedly observed in neutral red solution that starch grains put into them always first became purple regardless of the hydrogen ion concentration, and it was also observed that in very weak solutions the purple color persisted much longer than in stronger solutions. This indicates that the structure of the starch grains is involved in the color, so that if the grain contains only a small amount of neutral red the light which passes through it is purple regardless of the hydrogen ion concentration. Neutral red is consequently under these circumstances not a reliable indicator in the acid range. Results presented below indicate that it is also unreliable as an indicator in the alkaline range.

In the experiments with neutral red it was repeatedly observed that the starch in the solution outside of the amoebae never became colored in the least. The question then arises as to what caused it to take up neutral red in the food vacuoles.

In attempting to answer this question, the following substances in various concentrations were added respectively to Hahnert solution (in small test tubes containing potato starch and neutral red in various concentrations) and left 24 hours or longer: sodium hydrate; hydrochloric, butyric, caprylic, capric, stearic, lactic and oleic acids; fresh, rancid and partially saponified olive oil; tripsin, pepsin and saliva; and lecithin and egg albumin. It was found that none of these substances had any effect on the absorption of neutral red by the starch except oleic and stearic acids and rancid and partially saponified olive oil. Of these, oleic acid and rancid olive oil were most effective but in the solutions containing these, even in very high concentration, the starch

never stained as densely as it did in the food vacuoles. If, however, oleic acid was added to dry starch and the starch then put into Hahnert solution containing neutral red, the starch stained just as deeply as it did in the food facuoles, and this also obtained for all the other acids tested. It would appear then that if starch contains acid it will take up neutral red from an aqueous solution but that oleic acid is the only one of those tested which is capable of replacing water in starch. The action of stearic acid and rancid and partially saponified olive oil is in all probability due to the presence of oleic acid in them.

What then is there in the food vacuoles in *Amoeba* which causes starch saturated with water to take up neutral red? The food vacuoles in *Amoeba*, as is well known, are surrounded by much plasmalemma which is ingested during their formation. This in all probability contains fatty acids which are similar to oleic acid and which, owing to decomposition, go into solution in the fluid in the food vacuole and become considerably concentrated as water in the vacuole is eliminated during the first 15 minutes after ingestion. It is probable that these concentrated fatty acids replace the water in the starch grains and cause them to take up neutral red.

This contention is supported by the fact that starch does not stain with neutral red in the food vacuoles in ciliates. Numerous observations were made in reference to this on *Paramecium caudatum*, *Vorticella similis* and *Colpidium striatum* in solutions containing neutral red and starch. All these ciliates ingested grains of potato and wheat starch very readily and frequently retained them as long as 30 minutes, but none of the starch grains became colored. In some instances, especially in the paramecia, the starch grains in the food vacuoles appeared to be definitely pink, but it was found that if pressure was applied so as to force the grains out, they invariably were colorless. The apparent color in them in the vacuoles was obviously due to numerous red granules in the cytoplasm adjoining the surface of the food vacuoles.

The food vacuoles in the ciliates, as is well known, are not surrounded by a membrane from the surface of the cell (a fatty acid-containing membrane) which decomposes, as in *Amoeba*, hence the lack of fatty acid to replace the water in the starch grains and the inability to absorb neutral red.

The results obtained in observations with neutral red seem to show that the fluid in the food vacuole in *Amoeba* becomes acid during the first 15 minutes after it has been ingested, that the acid probably originates in part from decomposition of some of the plasmalemma taken in during the process of ingestion and is correlated with elimination of fluid from the vacuole, that the content of the vacuole later

becomes alkaline and that this is correlated with entrance of fluid from the cytoplasm and marked increase in the size of the vacuole. These results however give no reliable information concerning the extent of either the acidity or the alkalinity. The following observations have a definite bearing on this problem.

The Maximum Acidity of the Fluid in the Food Vacuoles and Some Observations on Length of Life and Fission of Ingested Organisms

Congo red and brom cresol purple

Active yeast cells and chilomonads in Hahnert solution (pH 6.5) were killed by rapidly increasing the temperature, then congo red was added until the solution was saturated, after which the temperature was raised to boiling and held until the yeast cells and the chilomonads became brilliant orange in color. Amoebae which had been without food for 24 hours or longer were now mounted in Hahnert solution on slides and a little of the congo red solution containing the stained yeast cells and chilomonads added. Living chilomonads were also added to some of the preparations.

The amoebae ingested the yeast cells and the chilomonads freely but *Amoebia dubia* much more freely than *Amoeba proteus*. Some of the food vacuoles formed contained only yeast cells, others only dead chilomonads, others only living chilomonads, and others various combinations of these organisms in reference to number as well as kind. Many of these vacuoles in various amoebae were continuously observed under low and high magnification for an hour and briefly from time to time for 24 hours. No change in color was observed in the stained yeast cells or the stained chilomonads in any of them (no matter if they were alone or in combination with living chilomonads). They remained brilliant orange in color throughout the entire time of observation.[4]

Some of the stained yeast cells were now mounted in buffer solutions containing congo red. Those in buffer, pH 4.5 or higher, remained brilliant orange. Those in buffer, pH 4, became distinctly bluish and those in buffer, pH 3.5 and lower, intensely blue.

These results demonstrate therefore that the acidity of the solution in the food vacuoles in *Amoeba* does not become so high as pH 4, and

[4] Some of the preparations studied contained small amoebae (probably *Amoeba dofleini*) and paramecia. Stained yeast cells were ingested by both. In the former they remained orange, in the latter they became blue, remained so for a short time, then became orange again.

they support the conclusion reached in observations on neutral red crystals, but they do not show the maximum acidity reached.

The methods used in making observations with brom cresol purple were the same as those used in making observations with congo red. The yeast cells became densely sky blue in the hot brom cresol purple solution but the chilomonads did not stain well and were therefore used in only a few of the observations.

The yeast cells in the food vacuoles either alone or with living chilomonads remained sky blue usually for about 15 minutes after they had been ingested, then they gradually became greenish blue and finally greenish yellow, about like buffer, pH 5.6, containing brom cresol purple. Then no further change in color could be observed for some 12 hours, after which they gradually became sky blue again and remained so until they were ejected. There was no indication of any digestive action on the ejected yeast cells.

Stained yeast cells were mounted in buffer solutions containing brom cresol purple and observed under low and high magnification. The yeast cells in buffer, pH 6.4, or over became sky blue, those in buffer, pH 5.4 to 5.8, greenish yellow and those in buffer, pH 5 or less, distinctly yellow, but it was impossible to distinguish between those in any two of the buffers which differed by less than 0.4 pH units, e.g. those in buffer, pH 5.6, could be distinguished from those in buffer, pH 6 or pH 5.2, but not from those in buffer, pH 5.8 or pH 5.4.[5]

The fact, therefore, that the yeast cells in the vacuoles changed in color from sky blue to greenish yellow, strongly indicates that the acidity of the solution in the food vacuoles increased from that of the culture solution to about pH 5.6.

The time required for the changes in the color of the yeast cells in the food vacuoles varied very greatly. In one vacuole observed with two yeast cells and one chilomonad it required three minutes for the yeast cells to become greenish yellow; in another with one yeast cell and one chilomonad it required seven minutes; in another with four yeast cells and no chilomonads, nine minutes; in another with about 20 yeast cells, 35 minutes; and in another with 15 yeast cells and no

[5] Amoebae, chilomonads, paramecia and other organisms thrive for days in extraordinarily strong solutions of brom cresol purple in Hahnert solution, pH 6.5. The cytoplasm in the amoebae becomes distinctly yellowish green but the nucleus remains gray. However, if they die the nucleus becomes strongly blue and the cytoplasm faintly blue. The cause of these changes in color is not clear. The paramecia become very quiet. They ingest quantities of the stained yeast cells. These sky-blue cells (pH 6.5) become distinctly yellow a few seconds after the food vacuoles have left the gullet but later turn to blue again and remain so until they are eliminated. They are not digested.

chilomonads, 45 minutes, but in a large majority it required around 15 minutes. In general, it required longer for the change in color in vacuoles which contained many yeast cells than in those which contained but few, and longer in those which were near the surface than in those which were not. The presence of living chilomonads probably also has some effect although this was not definitely established.

The change from blue to greenish yellow in the yeast cells in the vacuole occurs after it has decreased to minimum in size and the later change to blue again after it has increased considerably. These changes are therefore correlated with changes in the amount of fluid in the vacuoles. The time required to kill the chilomonads after they were ingested varied greatly, but when they died the dead yeast cells ingested with them were almost always still sky blue. This shows that at the time when the chilomonads in the vacuoles die the acidity of the fluid around them is lower than pH 5.6, and consequently that their death is not specifically correlated with acidity for the lethal concentration is much higher than this, i.e. around pH 3.8, as will be demonstrated later.

Some interesting results obtained in observations made on three vacuoles follow:

(1) Several amoebae were mounted (4 P.M.) in Hahnert solution containing chilomonads, stained yeast cells and brom cresol purple, then observed almost continuously under an oil immersion objective.

(4:05 P.M.) An unusually large vacuole forming; contains 15 yeast cells, all sky blue, no chilomonads. (4:25 P.M.) Vacuole closed; amoeba very active, vacuole at the posterior end, no change in color. (4:50 P.M.) Vacuole much smaller, yeast cells slightly greenish. (5:00 P.M.) Yeast cells greenish yellow about pH 5.6. Vacuole continuously near the surface at the posterior end. (5:45 P.M.) No change. (6:00 P.M.) Vacuole broken up into four smaller ones; two with five yeast cells each, one with three and one with two; all near the center of the amoeba moving with the plasmasol; no change in color. (7:00 and 8:00 P.M.) No change. (10:00 P.M.) Yeast cells in the two smaller vacuoles distinctly bluish; one of the larger not found, no change in color in the other. (9:00 A.M.) Two vacuoles found, yeast cells in them sky blue.

(2) Amoebae mounted (3:50 P.M.) in Hahnert solution containing chilomonads, stained yeast cells and brom cresol purple.

(4:05 P.M.) A large vacuole with two yeast cells and one chilomonad formed; chilomonad very active, almost continuously under observation under oil immersion objective; yeast cells sky blue. (4:09

P.M.) Chilomonad very active; yeast cells sky blue. (4:13 P.M.) Chilomonad still active, no appreciable change in size of vacuole, yeast cells distinctly greenish yellow; the vacuole now very near the surface at the posterior end and its content very distinctly visible. During the following 30 minutes the vacuole remained very near the surface of the amoeba, it did not appreciably change in size, there was no visible change in the color of the yeast cells and the chilomonad was almost continuously very active. The chilomonad then suddenly came to rest, and a slight groove around it lengthwise was seen. This rapidly became deeper and in 45 seconds the chilomonad divided.[6] Both daughters soon became active and continued so for 50 minutes, then both suddenly died.

(3) Amoebae were mounted (4:30 P.M.) in Hahnert solution containing chilomonads, stained yeast cells and brom cresol purple and observed intermittently under oil immersion objective.

(4:50 P.M.) An amoeba found with one food vacuole which contained one yeast cell and a living chilomonad and three which contained respectively one, ten and 16 yeast cells but no chilomonads. All the yeast cells in the vacuoles were sky blue. The amoeba was very active and appeared to be in excellent condition. (5:30 P.M.) The yeast cells in the vacuole which contained ten were distinctly greenish yellow, about pH 5.6, those in the other three vacuoles were still sky blue; the chilomonad was still alive, and the vacuole which contained it was still large and at the posterior end very near the surface. (6:00 P.M.) No change; the vacuole containing the chilomonad was still large and very near the surface and the chilomonad active. The chilomonad therefore lived in this vacuole more than an hour and ten minutes and the solution in it did not become more acid than about pH 6.4. (9:00 A.M.) Several vacuoles found containing yeast cells but probably not the ones described above. Some of these contained indigestible remnants of chilomonads and were obviously old. The yeast cells in these were sky blue.

The results obtained in observations with brom cresol purple on yeast cell in food vacuoles in *Amoeba* show then that during about the first 15 minutes after the food vacuoles are formed the hydrogen ion concentration of the fluid in them increases from that of the culture fluid to around pH 5.6 and then decreases, but they give no information as to the extent of the decrease. This is the subject of the following observations.

[6] We studied a great many food vacuoles containing living organisms, but this is the only one in which we observed fission.

The Maximum Alkalinity of the Fluid in the Vacuoles

Cresol red

Two methods were used in the observations made with cresol red. In one the amoebae were fed on living colpidia in a solution of cresol red, in the other they were fed on colpidia which had been killed in a solution of cresol red.

(1) Several specimens of *Amoeba dubia* were put into a weak solution of cresol red in culture solution containing numerous colpidia in a watch-glass and left three days. The amoebae multiplied and became extraordinarily large and well filled with crystals, many of which had globular central portions and two long spine-like projections, extending in opposite directions from the central portion. A few drops of the solution containing four amoebae and a number of colpidia were mounted on a slide and a drop of strong cresol red solution added. This preparation was then covered with a cover-glass and sealed with vaseline. The amoebae moved and fed normally and multiplied extensively.[7] They were examined under low and high magnification (20 ocular and 60 oil immersion objective) from time to time for four days.

During this time colpidia were repeatedly seen in the process of ingestion and in various stages of digestion. They were compared in color with colpidia which had been killed in hot water and mounted in buffer solutions, pH 6 to 8, containing cresol red.

The colpidia in the buffer solutions, pH 7.8 and 8, were purplish (not red); those in the buffer solutions, pH 6.4, 6.2 and 6, were yellow and those in buffer solutions, pH 6.6 to 7.6, were intermediate.

The living colpidia in the food vacuoles were invariably colorless. Those which had recently died in the vacuoles were bright lemon yellow, i.e. acid probably beyond the range of the indicator, but the fluid around them was colorless. Those in all the later stages of digestion were yellow or brownish yellow and the fluid around them was yellow. There was no indication of a purplish tint comparable to that in the colpidia in buffers, pH 7.8 or 8.

This experiment was repeated several times with different concentrations of cresol red. No indication of purplish color as distinct as that in the colpidia in buffer, pH 7.8, was seen in any of the food vacuoles.

These results indicate therefore that the content of the food vacuole in *Amoeba* does not become so alkaline as pH 7.8 and probably not so alkaline as pH 7.6.

[7] *Amoeba* can withstand surprisingly strong solutions of cresol red without any ill effects and in moderate concentrations it appears to be decidedly beneficial.

(2) Colpidia were put into a saturated solution of cresol red and left until they died. Some were then mounted on a slide in a weaker solution of cresol red in Hahnert solution and several specimens of *Amoeba dubia*, which had been without food for 24 hours, added. The dead colpidia were yellow. The amoebae ingested them freely. The following day the amoebae contained numerous food vacuoles with colpidia in various stages of digestion. The content of these vacuoles varied from yellow in the younger ones to brownish yellow in the older ones. There was no indication whatever of a purplish tint (pH 7.8) in the content of any of them. These results therefore support the conclusions reached in the preceding experiments.

Phenol red

Colpidia were put into a saturated solution of phenol red in Hahnert solution and left until they died. Some of them were then put into buffer solutions, pH 6 to 8, containing phenol red and some into a drop of weak solution of phenol red in Hahnert solution on a slide which contained 15 starved specimens of *Amoeba proteus*.

The chilomonads in buffer, pH 7.2 or less, were yellow, those in buffer, pH 8 were red, and those in buffers, pH 7.4, 7.6 and 7.8, were pinkish yellow or yellowish pink.

The amoebae on the slide were examined continuously for two hours and briefly from time to time for two days. During the first two hours nearly half of the amoebae ingested one or more colpidia and at the end of 24 hours several had vacuoles containing colpidia in all stages of digestion. The colpidia were yellow when they were ingested and the fluid around them colorless. They remained yellow and the fluid colorless for several hours (this time varied greatly) then the amount of fluid in the vacuole increased considerably and became distinctly pinkish (pH 7.3). The colpidia in it were however still yellow but they also soon became pinkish.

These observations were repeated several times. *Amoeba dubia* was used in some of these tests. Essentially the same results were obtained in all. Specimens were repeatedly found which at a given time had some vacuoles in which the colpidia in them were yellow and the fluid colorless, some in which they were yellow and the fluid pinkish and some in which both were pinkish, and all these vacuoles intermingled freely in the plasmasol and frequently came in contact with each other.

The results obtained with phenol red seem to show then that the content of the older food vacuoles in *Amoeba* is slightly alkaline (pH 7.3, possibly 7.4), that the fluid in the vacuoles becomes alkaline before

the solid bodies in it and that the alkalinity is correlated with increase in the amount of fluid in the vacuoles. And the results obtained with brom cresol purple seem to show that the fluid in the younger food vacuoles is somewhat acid, probably pH 5.6.

There, however, still remains the question as to whether or not the changes in the color of the content of the food vacuoles in *Amoeba* are specifically correlated with changes in hydrogen ion concentration; for it is well known that the oxygen tension in cytoplasm is very low and that many dyes reduce readily and change greatly in color. This matter will be discussed in some detail in a subsequent paper. Suffice it to say here that results obtained with reducing and oxidizing agents indicate that the color of the dyes in question is probably not appreciably, if at all, affected by the low oxygen tension in the cytoplasm.

The Origin of the Acid and the Base in the Food Vacuoles in *Amoeba*

It is widely held, as previously stated, that the cytoplasm in the protozoa secretes acid and base and "pours" them into the food vacuoles, the former when the vacuoles are young and the latter after they are old.

It is well known that in *Paramecium* and some other ciliates granules which stain deep red with neutral red aggregate at the surface of the food vacuoles. It has been suggested that these contain acid and that they pass into the food vacuole and cause increase in the acidity of its content. There are, however, no such granules in the cytoplasm of *Amoeba* and there is no indication of aggregation of granules of any kind at the surface of the food vacuoles in them. The increase in the acidity of the content of the food vacuoles, therefore, obviously cannot be correlated with granules in the cytoplasm. Moreover, the results presented above show that the increase in the acidity of the fluid in the food vacuoles in *Amoeba* occurs simultaneously with marked decrease in the size of the vacuole, i.e. that it occurs while there is a continuous flow of fluid from the vacuole out into the cytoplasm. It is very difficult to see how acid could, at this time, pass from the cytoplasm into the vacuole against the outward flow of fluid.

These considerations consequently strongly support the conclusion reached above, namely, that the acid which causes the increase in the acidity of the fluid in the food vacuoles in *Amoeba* originates within the vacuoles, not in the cytoplasm around them. But they do not illuminate the processes involved in the production of the acid within the vacuoles.

In the observations on ingested starch it was concluded that some of the acid in the vacuoles probably originates from the decomposition of the plasmalemma ingested during the formation of the vacuoles. Some is, however, produced by metabolism in living organisms in the vacuoles and by the processes involved in the death of these organisms, as is clearly indicated by the great changes observed in the color of absorbed indicators in them. Observations concerning the effect of metabolism on the hydrogen ion concentration of culture fluid follow:

Colpidia from a vigorous culture were concentrated in tap water by means of the centrifuge; then about 0.25 cc. added to each of three 5 cc. vials. The vials were then filled with balanced salt solution (0.001 M) and a little brom thymol blue added to one, brom phenol blue to another and brom cresol green to the third. The vials were then closed air-tight with paraffin impregnated cork stoppers, in such a way that all free air was eliminated. The color of the solution in the vials was then, at five minute intervals, compared with that of standard buffers and the hydrogen ion concentration ascertained. The following results were obtained:

Vials closed 9:20 A.M.; solution in them, pH 7.4; 9:25, pH 7; 9:30, pH 6.8; 9:35, pH 6.4; 9:40, pH 6; 9:45, pH 5.4; 9:50, pH 4.8; 10:00, pH 4.5 (about one-half of the colpidia were now inactive); 10:10, pH 4.4; 10:30, pH 4.4 (still many inactive); 11:30, pH 4.4; 3:30, pH 4.4 (still some active).

This experiment was repeated with colpidia and also with chilomonads and essentially the same results obtained. These results prove conclusively that metabolism in living colpidia and chilomonads in air-tight enclosures produces marked increase in the acidity of enclosed solutions. The rate of increase under these conditions varies, of course, directly with the volumetric ratio of organism to solution and with the activity of the organisms. In the food vacuoles in *Amoeba* this ratio is fairly large and the ingested organisms are extremely active. It is therefore likely that the metabolism of the organisms in the vacuole produces considerable increase in acidity in the fluid in the vacuoles.

There is also considerable evidence (which will be presented in a subsequent paper) that the membrane at the surface of the food vacuoles is not permeable to the acid produced in them. If this evidence is valid, it is obvious that the production of a very small amount of acid in the vacuoles shortly after they are formed would, owing to subsequent great loss of fluid, result in great increase in the acidity of the fluid which remains when the vacuole has decreased to minimum in size.

There is then in the results obtained in observations on the food vacuoles in *Amoeba* no support whatever for the contention that the cytoplasm at the surface of the younger food vacuoles secretes acid and pours it into the vacuoles.

It was demonstrated above that the increase in alkalinity of the content of the food vacuoles in *Amoeba* occurs simultaneously with increase in the size of the vacuoles and entrance of fluid from the adjoining cytoplasm, and that the fluid in the vacuoles becomes alkaline before the solid substance suspended in it. It was consequently concluded that the base which causes this fluid to become alkaline originates in the cytoplasm around the food vacuole. This does not show, however, that the base is secreted by the cytoplasm, for the alkalinity of the fluid, as demonstrated above, is only approximately pH 7.3, and it is highly probable that all the fluid in the cytoplasm is equally alkaline.

Functions of Acid and Base in the Food Vacuoles in *Amoeba*

A number of investigators, as previously stated, maintain that the acid in the food vacuoles in protozoa functions as a killing agent. It was demonstrated in the observations with brom cresol purple, described above, that the fluid in the food vacuoles in *Amoeba* does not become more acid than approximately pH 5.6. It is consequently evident that if the acid in these vacuoles functions as a killing agent this concentration must be lethal for the ingested organisms which die in the vacuoles, notably *Colpidium* and *Chilomonas*. The following experiments concern this problem:

(1) To Hahnert solution in a series of small flasks different quantities of HCl were added and the hydrogen ion concentration of the solution in each measured with glass electrodes. Then 0.1 cc. of each was put respectively into depressions on hollow ground slides and a very small quantity of solution containing numerous chilomonads added and thoroughly stirred. The preparations were then, at short intervals, examined under low and high magnification. The following results were obtained:

In solution, pH 3.5, the chilomonads died immediately; in solution, pH 3.9, about half of them died immediately and the rest in less than one minute; in solution, pH 4.1, about half of them became inactive in two minutes but many of these gradually recovered, so that after 15 minutes nearly all were active; in solution, pH 4.2, about one-fourth of them became inactive and practically all these soon became active again.

This experiment was repeated twice and essentially the same results were obtained. It was also repeated six times with colpidia in place of chilomonads. In three of these lactic acid was added in place of hydrochloric acid.

It was found that the colpidia lived more than four hours in lactic acid (pH 3.5) and that a few lived more than six hours in this acid (pH 3), although many died in less than two minutes. In hydrochloric acid the lethal concentration was slightly lower, but not quite so low as it was for chilomonads.

The results obtained indicate, therefore, that to kill either chilomonads or colpidia with acid, it requires a concentration of at least pH 4. It is consequently evident that since the acidity of the fluid in the food vacuoles in *Amoeba* does not exceed approximately pH 5.6, the death of these organisms in the vacuoles cannot be due to acid. Nirenstein (1905) comes to the same conclusion in reference to bacteria in the food vacuoles in paramecia. Moreover, when colpidia die in weak solutions of acids the contractile vacuole stops pulsating before the cilia stop beating but fluid continues to flow into it and it consequently becomes much enlarged. These phenomena do not occur when the colpidia die in the food vacuoles. Under these conditions the contractile vacuoles continue to pulsate for some time after the cilia stop beating and there is no indication whatever of any enlargement. These observations consequently support the conclusions that death of organisms in the food vacuoles is not caused by acid.

Nirenstein (1905) et al. maintain that digestion of organisms in the food vacuoles does not begin until after their content has become alkaline. The results of observations on *Amoeba* are in full accord with this contention. The evidence in hand seems, therefore, to indicate that the acid does not function in digestion. Nirenstein suggests, however, that it may function in activating "Profermente" and in coagulating proteins, so as to facilitate the action of triptase. This suggestion was probably the result of his conviction that the acid is secreted by the cytoplasm adjoining the food vacuoles and that it consequently must have some function. If, however, the analysis presented above concerning the origin of the acid is correct, there is obviously no need of postulating any function for it. Moreover, the fact that while the content of the vacuole is acid, there is a continuous outward flow of fluid, makes it difficult to see how any digestive enzymes could enter during this time. This fact, therefore, also militates against the view that the acid functions in digestion.

It was demonstrated above that after the acidity in the vacuole has become maximum, fluid rapidly passes from the cytoplasm into the

vacuole. This is doubtless due to increase in the osmotic concentration of the fluid in the vacuole, owing, at least in part, to diffusion from the dead organism in the vacuole; and it may well be that this diffusion and the consequent increase in osmotic concentration is facilitated by action of the acid on the organism. Moreover, the acid may also act on the diffused substance in such a way as to increase the osmotic concentration.

It is generally held that the base in the food vacuole functions in facilitating the action of enzymes on the ingested organisms.

As stated above, the change from acid to alkaline in the food vacuole coincides with a rapid increase in the size of the vacuole owing to rapid inflow of fluid from the cytoplasm. This fluid is doubtless alkaline and it probably contains enzymes which are carried into the vacuole where they act on the ingested organisms.

Factors Involved in the Changes in the Size of the Food Vacuole in *Amoeba*

As previously stated, the food vacuole in *Amoeba*, beginning immediately after it is formed, decreases greatly in size, then rapidly increases considerably and then very gradually decreases until it is eliminated (Fig. 1). The question now arises as to what causes these changes.

Very soon after the vacuole has been formed, sheets of cytoplasm usually pass through it separating off portions. This reduces the size of the vacuole. The fluid in the vacuole is, at this time, essentially like the culture fluid outside. The osmotic concentration of this fluid is lower than that of the fluid in the cytoplasm (Mast, 1923, 1926). There is consequently a flow of fluid out of the vacuole, resulting in reduction in size. After the size has reached minimum, the organisms in the vacuole die and shortly after this fluid passes rapidly from the cytoplasm into the vacuole causing rapid increase in size. This is doubtless due to sudden increase in the osmotic concentration of the fluid in the vacuole, owing to diffusion from the dead organisms in it. Digestion begins at this time and the subsequent gradual reduction in the size of the vacuole is doubtless due to diffusion out into the cytoplasm, of various substances formed during this process.

The Cause of Death of the Organisms in the Food Vacuoles

It was concluded above that death of the organisms in the food vacuoles in *Amoeba* is not due to acid in the vacuoles and it was noted that Nirenstein reached the same conclusion in reference to organisms in the food vacuoles in *Paramecium*. Nirenstein (1905) contends that

death in these vacuoles is caused by some other toxic substance which passes from the surrounding cytoplasm into them. This is, however, in all probability not true in *Amoeba*, for during the entire time between the formation of the food vacuole in this form and death of the organisms in it, there is a continuous flow of fluid out of the vacuoles. What then is it that kills the organisms?

It is well known that the oxygen tension is much lower in active cytoplasm than it is in culture fluid. There is consequently a flow of oxygen from the culture fluid in the vacuoles out into the cytoplasm. This doubtless results in marked reduction in the oxygen tension in this fluid. Moreover, the ingested organisms, as stated above, are extremely active and consequently consume much oxygen in respiration. This doubtless augments the reduction in oxygen tension in the vacuoles considerably. And, furthermore, the amount of fluid in the vacuoles decreases rapidly and this obviously increases the effect of respiration on the reduction in oxygen tension in the fluid remaining in the vacuole. The oxygen tension of this fluid is then doubtless soon reduced nearly, if not quite, to that in the cytoplasm around the vacuoles and it may well be that this is fatal to the ingested organisms. This conclusion is supported by the fact that death in the vacuoles is definitely correlated with reduction in their size (Fig. 1, 3), and by the fact, substantiated in the preceding section on brom cresol purple, that the length of life in the vacuoles is greatly increased if they are near the surface so that oxygen can diffuse into them from the surrounding culture fluid. The results obtained in the following observations on organisms enclosed in air-tight tubes also lend some support to the conclusion.

A number of capillary glass tubes about 2 cm. long and 0.5 mm. in diameter were drawn out to a diameter of about 0.25 mm. at either end. Solution from a vigorous culture of *Chilomonas* was centrifuged until the chilomonads were densely packed in the bottom of the tubes, then the free fluid was poured off. The substance which remained consisted of about four parts of culture fluid to one part of chilomonads. Two of the capillary tubes were entirely filled with this substance and the two ends of each sealed with melted paraffin. A little of the poured-off fluid was then returned to the centrifuge tubes and two more capillary tubes filled and sealed, then a little more fluid was added and two more filled and sealed. The chilomonads in each of the six tubes were then studied at short intervals. This was repeated with colpidia in place of chilomonads and with several more concentrations of organisms.

With chilomonads in the tubes containing ten parts of fluid to one part of organisms, a few died in five minutes and all in 33 minutes; in 40 to one concentration, none died in 20 minutes and all in four hours; in concentration 150 to one, none died in 50 minutes and all in four hours.

With colpidia in the tubes containing four parts of fluid to one part of organisms, a few colpidia died in 13 minutes and all in 47 minutes;

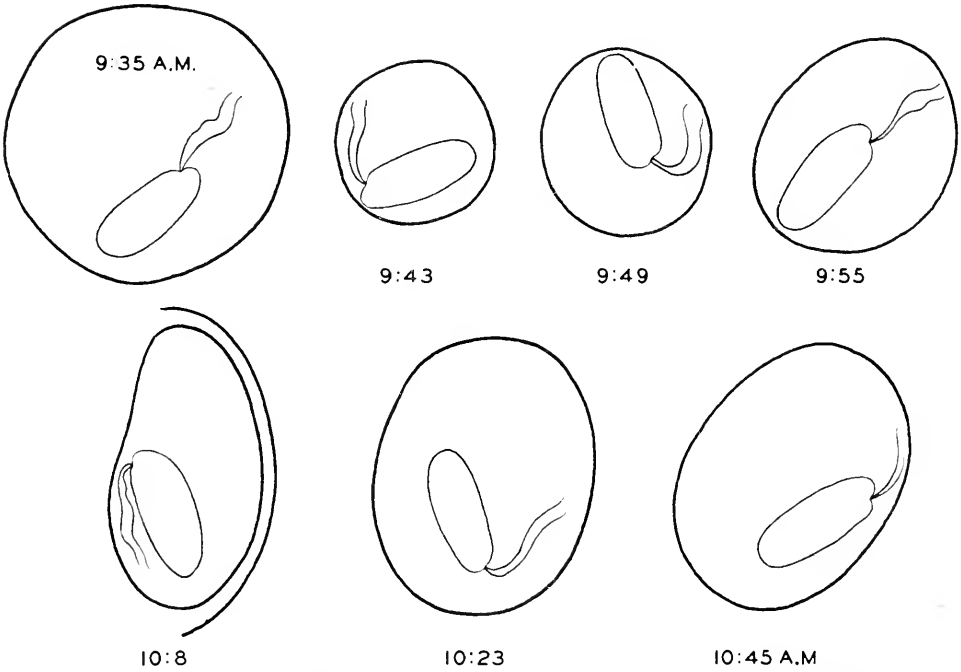

FIG. 3. Camera outlines showing changes in the size of a food vacuole in *Amoeba proteus*, containing an ingested chilomonad. The chilomonad was still active after having been in the vacuole for more than an hour.

Note that there was still much fluid in the vacuole when it had become minimum in size. Compare with Figure 1. This indicates that death of organisms in the food vacuoles is correlated with the extent of the reduction in the amount of fluid they contain.

in concentration six to one, a few died in 16 minutes and all in 55 minutes; in concentration ten to one, a few died in 20 minutes and all in 105 minutes; in concentration 15 to one, a few died in 100 minutes and all in 210 minutes.

These results demonstrate that organisms in fluid in an enclosed tube which simulates a food vacuole, rapidly produce a condition in the fluid (probably due mainly to reduction in oxygen tension) which

is fatal. It is consequently not necessary to postulate production of toxic substance by the surrounding cytoplasm to account for death in the food vacuoles.

Digestion in the Food Vacuoles in *Amoeba*

Greenwood (1886) maintains that proteins are digested in *Amoeba* but that starch is not and that digestion of fat is doubtful. Nirenstein (1905) says there is no evidence which indicates that fat is digested in any of the unicellular organisms. Dawson and Belkin (1928, 1929) conclude that if oil is injected into amoebae some kinds are digested and others are not. This conclusion has been confirmed by Wilber (1942) in observations on various oils injected into *Pelomyxa carolinensis*. Mast and Hahnert (1935) and Mast (1938) proved that the fat, the starch and the protein found in chilomonads are digested in the food vacuoles in *Amoeba*. Since this occurs at room temperature in a weak alkaline solution these food vacuoles must contain enzymes.

It is generally assumed that the enzymes found in the food vacuoles are formed in the cytoplasm of the amoebae and pass from it into the vacuoles. Mast and Doyle (1935) suggested that the beta granules (mitochondria) are involved in this transfer and others have postulated other carriers. There is, however, very little evidence in support of these suggestions. Moreover, there is no necessity for the postulation of special carriers, for, as stated above, much fluid passes from the cytoplasm into the vacuoles shortly before digestion begins and enzymes could be carried in with it. Furthermore, there is evidence which strongly indicates that at least some of the enzymes do not originate in the cytoplasm of the amoebae, and consequently are not carried into the food vacuoles.

Greenwood used wheat starch in her observation on digestion in *Amoeba* and found that the grains were often retained for several days without any indication whatever of digestion. I obtained similar results with potato and rice starch as well as with wheat starch. The starch grains were freely ingested. The food vacuoles changed in size normally and fluid passed from the cytoplasm into them. The starch grains were retained for hours and sometimes for days without the slightest indication of etching (digestion) on any of them. This seems to show that there was no amylase in these food vacuoles and none in the cytoplasm in the amoebae. How then can the digestion of the starch in chilomonads observed by Mast and Hahnert be explained?

Mast and Pace (1933) found that if chilomonads are starved, the starch in them soon disappears, owing to hydrolysis. This shows

that the chilomonads contain amylase. The food vacuoles which contain chilomonads therefore obviously also contain amylase, and the digestion of the starch in them is doubtless due to the action of this amylase. There is, therefore, no need for the postulation of amylase in the cytoplasm of amoebae to account for the digestion of the starch in the ingested chilomonads.

Holter and Doyle (1938) in some very accurately controlled experiments found considerable amylase in amoebae. If there is none in the cytoplasm of these organisms, how can this be explained? In their experiments they took about 1000 amoebae which had been feeding on chilomonads, reduced them to a "homogeneous suspension in M/120 phosphate buffer" and found amylase in this suspension. The amoebae used doubtless contained chilomonads in food vacuoles. It is consequently obvious that the amylase found may have had its origin in these chilomonads and not in the cytoplasm. The results obtained by Holter and Doyle do not, therefore, militate against the contention that the cytoplasm of *Amoeba* contains no amylase.

The fact that fat injected into *Amoeba* is digested seems to show that the cytoplasm contains lipase, but at least some of that found in the food vacuoles probably also originates in the ingested organisms. And this can also be said concerning the origin of the pepdidase found there.

The Hydrogen Ion Concentration of the Fluid in the Cytoplasm in *Amoeba*

Pantin (1923) maintains that the hydrogen ion concentration of the cytoplasm in a small marine amoeba is pH 7.6–7.8 in the plasmasol, pH 7.2 in the plasmagel and pH 6.8 in the protruding pseudopods. Needham and Needham (1925) conclude that in *Amoeba proteus* it is pH 7.6 throughout, and Chambers, Pollack and Hiller (1927) contend that in *Amoeba proteus* and *Amoeba dubia* it is pH 6.9 $\pm$ 0.1.

The results presented above show that the maximum alkalinity of the fluid in the food vacuoles in *Amoeba proteus* is approximately pH 7.3 and that this obtains immediately after a relatively large quantity of fluid has passed from the cytoplasm into the vacuole. They show that before this the fluid in it is slightly acid. The fluid which passes in from the cytoplasm must therefore be somewhat more alkaline than pH 7.3. The quantity that is in the vacuole at this time is however so small compared with the quantity which enters, that the effect of the former on the alkalinity of the latter must be slight. The fluid in the cytoplasm is therefore probably approximately pH 7.4, unless that which passes from the cytoplasm into the food vacuoles differs in

acidity from that which is ordinarily found in it. This, however, is not at all likely. Moreover, it has been known for some time that the fluid in the crystal vacuoles in *Amoeba* is definitely alkaline (Mast 1923, 1926). This supports the conclusion that the fluid in the cytoplasm is alkaline.

The conclusion of Chambers et al. that the acidity of the cytoplasm in *Amoeba* is pH 6.9 $\pm$ 0.1 is based upon changes in color of several different injected dyes. It is well known that injury causes marked increase in the acidity of the cytoplasm and it is, obviously, impossible to inject dyes without injuring the cytoplasm. It is, therefore, highly probable that the values obtained by Chambers et al. are somewhat too low. Their results therefore do not militate against the contention that the hydrogen ion concentration of the fluid in the cytoplasm in general is the same as that of the fluid which passes from the cytoplasm into the food vacuoles, i.e. that it is approximately pH 7.4.

Pantin's conclusion, stated above, is based upon observations on marine amoebae stained with neutral red. He does not say what it is that stains in these amoebae. In *Amoeba proteus* staining with neutral red is confined to various substances in the food vacuoles, the refractive bodies and the fluid in the vacuoles which contain crystals. The refractive bodies become crimson. They are clearly acid in reaction, owing doubtless to the presence of fatty acids. The fluid in the crystal vacuoles becomes brownish yellow, showing that it is definitely alkaline in reaction. The content of some of the food vacuoles is distinctly acid, that of others distinctly alkaline, and that of still others partly acid and partly alkaline. There are, then, in the cytoplasm of *Amoeba proteus*, at any given time localized acid and alkaline substances, and in specimens which are stained with neutral red, localized red and yellow regions. Under moderate magnification these colored regions make the entire cytoplasm appear to be stained either red or yellow or intermediate, depending upon the relative number of red and yellow regions. I have found that if observations are made with a first class optical system containing an oil immersion objective there is no indication of any color in the cytoplasm between the localized colored regions. The color seen in these regions under moderate magnification is doubtless due to the action of the colored regions on the light which passes through the cytoplasm. It is consequently obvious that the conclusions based upon the appearance under moderate magnification of different regions in an amoeba stained with neutral red are not reliable.

Pantin does not describe the optical system used, but his statements imply that the amoebae studied contained numerous granules in the form of a network and that these granules stained with neutral red.

It may well be, then, that all the color observed originated in these granules and that the different shades depended upon their concentration and distribution, and not upon the hydrogen ion concentration of the fluid in the cytoplasm *per se*.

Needham and Needham base their conclusions concerning the hydrogen ion concentration of the cytoplasm of *Amoeba* on observations made under moderate magnification ("$\frac{1}{4}$ inch objective and a Watson eyepiece giving a magnification of 15") on some specimens stained by immersion in a solution of neutral red, and others injected respectively with neutral red, brom thymol blue and phenol red. They say they used *Amoeba proteus*, but give no description. They maintain that the cytoplasm in all the tests assumed a color like that of Clark buffer, pH 7.6.

If they actually had *Amoeba proteus*, the color observed in the cytoplasm of specimens stained by immersion in a solution of neutral red was undoubtedly due to the color of the content of the food vacuoles, the refractive bodies and the fluid in the crystal vacuoles, and not to color in the fluid in the cytoplasm. Whether or not this obtains for injected specimens is not known, but it does throw considerable doubt on the validity of the conclusions reached, namely, that the hydrogen ion concentration of the cytoplasm in *Amoeba proteus* is pH 7.6.

Summary

1. In *Amoeba* new food vacuoles which contain chilomonads or colpidia decrease greatly in size and the organisms in them die soon after they have decreased to minimum; then the size increases rapidly and digestion begins, after which the size decreases very gradually.

2. If the culture fluid contains neutral red, the organisms in the vacuoles become purple (like buffer solution, pH 3.5, containing neutral red) immediately after they die, but the fluid around them remains colorless until after the vacuoles have increased in size, then it becomes yellow like buffer, pH 8, but the organisms in it remain purple for some time after this, then become brownish yellow.

3. The color of the organisms immediately after death in the food vacuoles is the same as that of organisms which have died in a solution of neutral red outside. This and the fact that the fluid in the food vacuole does not, at this time, become colored indicates that the acid originates in the vacuole, not in the cytoplasm around it. The fact that the fluid in the vacuole later becomes yellow before the organisms in it do, indicates that the base originates in the cytoplasm around the vacuole, not in the organisms in it.

4. Potato or wheat starch grains ingested by amoebae in culture fluid containing neutral red become purple like buffer, pH 2. This color is, however, largely a physical phenomenon, and therefore does not accurately indicate hydrogen ion concentration.

5. Neutral red crystals become pink and dissolve in buffer, pH 5, but they do not become pink and do not dissolve in the solution in the food vacuoles, either if they are alone or if they are in the vacuoles with organisms. This solution, therefore, does not become as strongly acid as pH 5.

6. Chilomonads killed in alkaline solution containing congo red are brilliant orange in color. They become bluish in buffer, pH 4 to 3.5. Amoebae ingest them readily. They remain orange in color in the food vacuoles. This shows that the acidity of the fluid in the vacuoles does not reach pH 4.

7. Chilomonads killed in alkaline solution of brom cresol purple are strongly purple. They become yellowish in buffer solution, pH 5.8 to 5.6. In the food vacuole they become slightly yellowish. This indicates that the acidity of the solution in the vacuoles reaches at least pH 5.6.

8. Results obtained with chilomonads killed respectively in cresol red and phenol red indicate that the maximum alkalinity of fluid in the food vacuoles is approximately pH 7.3.

9. The hydrogen ion concentration in the food vacuoles in *Amoeba* changes from about pH 5.6 to about pH 7.3, i.e. not nearly so much as indicated by the changes in the color of the content of food vacuoles which contain neutral red.

10. The increase in the acidity of the fluid in the food vacuoles probably is due to respiration in the ingested organisms, chemical changes associated with their death, disintegration of the ingested plasmalemma, impermeability to acids of the membrane around the vacuoles and diffusion of fluid from the vacuoles. The decrease in acidity is due to diffusion of alkaline fluid from the cytoplasm into the vacuoles. The cytoplasm secretes neither acid nor base.

11. The acid in the food vacuoles probably facilitates increase in the osmotic concentration of the fluid in the vacuole, resulting in diffusion of fluid containing enzymes from the cytoplasm into it. The base promotes digestion.

12. Death of the organisms in the food vacuoles is not caused by acid. It probably is caused by decrease in oxygen in the vacuoles, owing to respiration of the organisms in them, diffusion of oxygen out into the cytoplasm and decrease in the volume of fluid in the vacuoles.

13. The changes in the size of the food vacuoles are probably due to differences between internal and external osmotic concentrations.

14. The hydrogen ion concentration of the fluid in the cytoplasm is approximately pH 7.4.

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A SEROLOGICAL STUDY OF SOME AVIAN RELATIONSHIPS[1]

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Introduction

Serological techniques have played and will continue to play an important role in the construction of a better system of classification. As an independent, and more objective, source of information, serological tests have in general corroborated the broad outlines of animal classification.

Boyden (1942) in an extensive survey of the literature on systematic serology, in which is embodied a critical analysis of the theory and fact of systematic serology, clearly indicates the complementary nature of serological data to morphologically established facts. There are, however, certain instances in which the systematic position of the forms concerned is admittedly "temporary" or purely speculative. This is either the result of conflicting views among morphologists, or disagreement between adult anatomical and developmental data. Such conflicts are bound to occur when the data are largely subjective. A recent publication by Wilhelmi (1942) demonstrates how well the relatively objective precipitin reaction lends itself to the "testing" of theories concerning the origin of vertebrates. Confirming the belief of some zoologists, Wilhelmi finds that the relationship between the echinoderms and prochordates is far greater than that indicated between annelids or arthropods and prochordates. There is good reason to believe, therefore, that serological techniques may eventually give us the answer to the "phyla of uncertain systematic position." The selection of the proper antigens (tissues, fluids, etc.) to be compared will play the important role in such investigations.

Most of the serological phylogenetic information thus far has come to us through the use of the ring test. In this test, when properly performed, comparable dilutions of several antigens are permitted to react with a constant amount of an antiserum made to one of the antigens. The highest dilution of each antigen giving a reaction with the antiserum is noted. These end-points are compared and the degree of

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similarity of the antigens is evaluated on the basis of the amount of antigen needed to produce the "final ring." Actually only one point, however, in the entire reaction is compared (Boyden, 1942). Other methods, Boyden and Baier (1929), Baier (1933), Wolfe and Baier (1938) and Baier and Wolfe (1942), have been employed to compare antigens and determine relationships; most of these are more sound, statistically, because of the relatively smaller errors involved. These investigators have recorded the volume of precipitate formed when several dilutions of the antigen were tested with homologous and heterologous antisera. This procedure has enabled them to differentiate between the sera of very closely related forms.

The invention of the photronreflectometer, a photoelectric nephelometer, by Libby (1938), and its first application in relationship studies (crustacean and mammalian) by Boyden (1939) have served as the impetus for the extension of a problem which had been confined to ring test technique. This instrument has provided the means of procuring a rapid, accurate, and more nearly quantitative determination of the degree of chemical similarity of antigens than is made possible by the widely used ring test method. The successful use of the instrument with such heterogeneous groups as crustaceans and mammals made it desirable to test the instrument with a homogeneous group such as birds. It was also considered important to compare the values obtained by photronreflectometric tests with those obtained by ring test technique as previously suggested by DeFalco (1941).

Erhardt's (1930) tabulated survey of the literature and the results of his own researches indicate the possibility of distinguishing some of the birds from others. The experiments of Sasaki (1928) have demonstrated that it is also possible to distinguish between the species of the same genus; and those of Cumley and Irwin (1940) show that it is possible to distinguish between the serum of a hybrid dove and that of its parents. Some of the work by other investigators (Graham-Smith, 1904; Cohen, 1939) has made it possible to link the birds with the reptiles (Crocodilia and Chelonia) with considerable success.

Materials and Methods

While the tables and figures which follow indicate the use of one sample of antigen in each test, actually each sample represents the pooled sera of from four to 95 birds, with the exception of turkey buzzard which is the serum from one individual. These samples are probably typical of the species they represent because duplicate and triplicate pooled samples (with the one exception noted above) from entirely different sources were tested against a common antiserum

before one was chosen for this series of experiments. In all cases the birds of the same breed were killed at one time and their bloods permitted to clot in a common container. After a period of from seven to 15 hours the expressed serum was centrifuged until free of cells, then filtered sterile with a Seitz filter.

The crystalline egg albumins of leghorn, turkey, duck, and goose were prepared according to the method of Hopkins and Pinkus (1898). Each albumin was precipitated three times to free it from as much of the other proteins as possible.

The hemoglobins were prepared by the method of Welker and Williamson (1920). Spectroscopic examination by Dr. James B. Allison, of the Department of Physiology and Biochemistry, Rutgers University, revealed that these antigens were in the methemoglobin form.

The lens proteins were prepared by grinding clean, washed lenses in a mortar. The resulting pasty material was then dissolved in buffered saline, filtered sterile, and preserved in the refrigerator as were all other antigens.

To study the possible effect of the lipids on the serological reactions of antigens, a portion of each antigen was extracted with petroleum-ether (Merck's Benzin B. P. 30°–60° C.) for a period of 24 hours. The antigens were extracted in a Friedrichs extracting chamber in which ether, condensing, drops to the bottom of a side arm tube containing the antigen. As the ether collects on the surface of the antigen it overflows through the side-arm and into an Erlenmeyer flask only to be reheated and recondensed to continue the process. The condenser and side-arm tube are cooled continuously with running tap water. In the tables and figures which follow, these extracted antigens are tested with precipitating antisera made to extracted antigens; only native antigens are tested with antisera made to native antigens.

The total and non-protein nitrogen of all native and extracted antigens were determined by the Folin-Wright method (1919), a modified Kjeldahl, and the protein nitrogen calculated and converted to grams of protein per hundred milliliters of serum. These values are shown in Table I.

All antisera were made in a similar manner, i.e., four injections of doubling doses of antigen on alternate days, the initial dose being 5 mgs. per kg. of body weight of rabbit. The rabbits were bled 14 days after the last injection.

The biochemical similarity of the antigens was determined by the precipitin ring test as described by Boyden and Noble (1933), in which .5 ml. of doubling dilutions of buffered (pH 7.0) antigen and .1 ml. of

antiserum are incubated at 37.5° C. for one hour. These results were then compared with those obtained by a new method employing the Libby (1938) photronreflectometer, a photoelectric nephelometer.

This instrument provides a constant source of parallel rays of light from an electric bulb which are permitted to pass through the turbid systems produced by the interaction of antigens and antibodies. Light rays reflected from the suspended particles fall upon a photoelectric cell and generate a current of electricity which causes a deflection on a galvanometer. In this manner the instrument was used to indicate the relative turbidities formed at 37.5° C. when 1.2 ml. of doubling dilutions of buffered (pH 7.0) antigen and .2 ml. of antiserum were incubated for 20 minutes. In all tests dilutions are in terms of protein rather than parts of serum. The galvanometric readings furnish the

TABLE I

Serum antigens used in experiments

Scientific name	Common name	Grams protein per 100 ml.
Gallus domesticus	leghorn	3.90
Gallus domesticus	plymouth rock	3.55
Gallus domesticus	buff orpington	4.20
Numida meleagris	guinea hen	2.82
Phasianus c. colchicus	pheasant	2.80
Meleagris gallopavo	turkey	3.95
Anser domesticus	domestic goose	3.94
Anas domesticus	domestic duck	3.50
Cath. a. septentrionalis	turkey buzzard	2.94
Pelecanus erythrorhynchos	pelican	3.20
Larus californicus	western gull	3.76
Drom. n. novae-hollandiae	emu	2.30

points for a curve, the area under which is used to determine the reaction of an antigen with an antiserum over a wide range of antigen dilutions. Comparison of the areas under the curves constructed from homologous and heterologous reactions furnishes evidence of the degree of chemical similarity between any two antigens.

The ring test, consisting of a series of tubes with doubling dilutions of antigen, is a very delicate and sensitive test. The slightest jarring or convection currents of the water bath may disturb the fine ring present in the titer tube, giving a false reading. All ring tests were run at least three times, many were tested six or more times. In only a few cases did a series vary by more than one tube on repetition. Considering that a variation of one tube, because of the doubling dilutions, represents a large error, the need for at least three readings is obvious. With the photronreflectometer, the variability of repeated determinations of the total areas was found to be within three per cent.

Results

The following tables indicate some of the results obtained when the native and extracted antigens were tested with precipitating antisera by ring and photronreflectometric tests. The homologous titer indicated over each antiserum is in terms of protein rather than nitrogen content.

In the body of the table the homologous titers and areas are valued at 100 per cent. The heterologous titers and areas are expressed in per cent values which indicate their relation to the homologous values. A dash indicates that no test was made.

Figure 1 is a graphic representation of the total area of reaction between a diluted (1 + 2) (one part serum plus two parts of buffered saline) anti-leghorn serum and each of several antigens. While the even-numbered tube numbers have been omitted from the figure to prevent over-crowding, the turbidity values obtained with the even-numbered tubes will be found on the graph. In all figures, tube number 1 is a dilution of one part of protein to 31.75 parts of saline; tube number 2 is a dilution of one part of protein to 62.5 of saline; tube number 3 a dilution of one part to 125; each succeeding tube possessing one-half of the concentration of protein of its predecessor.

In a similar manner the crystalline egg albumins, hemoglobins, and lens-proteins were used to determine relationships. Six antisera made to the hemoglobins of leghorn, duck, and turkey could not distinguish between the heterologous and homologous antigens by ring test. A similar condition was experienced with five antisera made to the lens proteins of leghorn, duck, and turkey. With the photronreflectometer, however, the same antigens and antisera reacted to give values comparable to those obtained with the serum-proteins. The following table shows some of the values indicated by ring and photronreflectometric tests.

Discussion

The results tabulated here represent a portion of a problem which has been in progress for a period of over four years. During this time a total of over 4,000 ring tests and several hundred turbidity curves have been completed. It is from this larger number of tests, and not only from the typical ones illustrated, that materials for the discussion and conclusion have been obtained.

Just as in a typical taxonomic study based on morphological data, similarities and differences of varying degrees of conservatism are revealed, so serological studies indicate the presence of substances

common to all of the birds but possessing sufficient differences to permit their differentiation. The fact that the sera of members of the various orders of birds reacted with most of the precipitating antisera is a

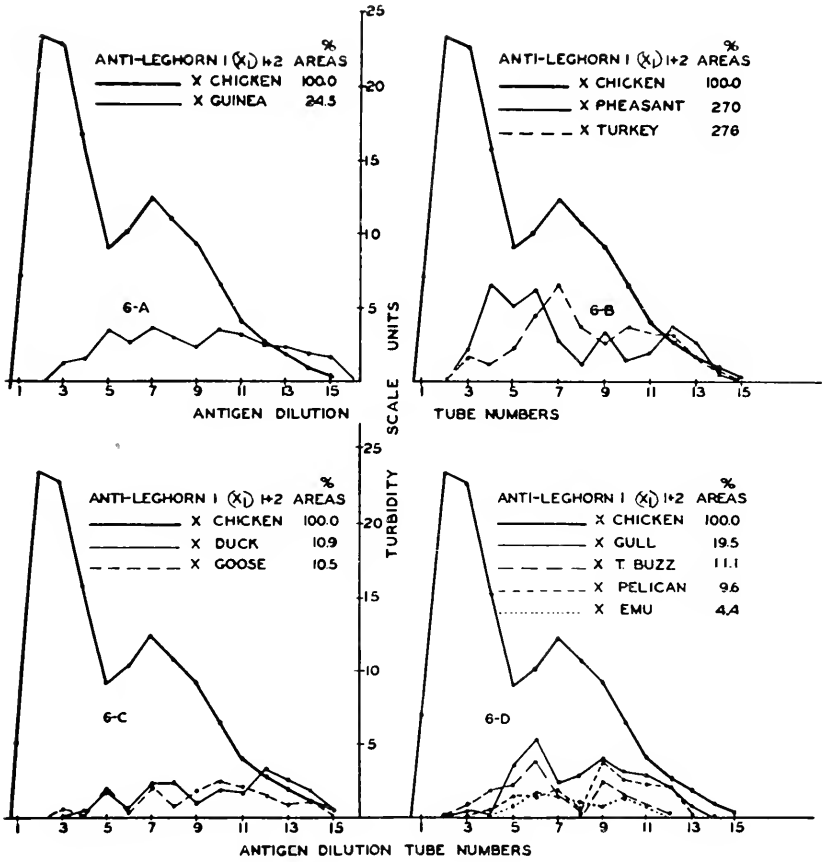

FIG. 1. Graphic representation of areas described by the interaction of a single antiserum (leghorn) with each of several antigens. The turbidity readings for the varying dilutions are plotted along the abscissa. Tube number 1 is a dilution of one part of protein to 31.75 parts of saline; tube number 2 is a dilution of one part of protein to 62.5 of saline; tube number 3 a dilution of one part to 125; each succeeding tube possessing one-half of the concentration of protein of its predecessor. Note the bimodality, or polymodality, of these curves which is typical of reactions in which sera serve as antigens. (Reprinted by permission from DeFalco, 1941, *Proc. Soc. Exper. Biol. and Med.*, **46**: 500.)

strong indication of the chemical similarity of all the birds, especially since the antisera were produced to a single series of injections. Similar single-series all inclusive precipitating antisera for other classes of

vertebrates are rare, if at all possible. Unpublished data of Dr. D. G. Gemeroy of this department reveal the difficulties encountered in demonstrating a link between "closely related" fishes even with very strong precipitating antisera. Results obtained with multiple series antisera made to amphibian sera by Boyden and Noble (1933) and to mammalian sera by Wolfe (1939) have indicated the limited range of reactivity of these antisera. This is most probably due to the greater degree of evolutionary divergence in the chemical constituents of these sera. Thus it seems clear that birds are the most homogeneous class of vertebrates both morphologically and serologically. The tables and figures indicate, however, that chemical differences among birds may be readily demonstrated by the precipitin reaction.

With some exceptions there is a general agreement between the relative systematic order indicated by the more objective serological techniques and the relative systematic order obtained by the more subjective morphological comparisons of the taxonomist. One very striking exception in this series of tests is the relationship of the guinea hen to the other domestic chickens. It may be noted in Table II that the relationship of the guinea hen to leghorn is 1.6 per cent. Approximately the same value was obtained with four other antisera. In a study with the lens and hemoglobins of these animals, however, a much greater degree of relationship was indicated. An attempt to ascertain the cause of the discrepancy between these two sets of data revealed that the ratios of serum proteins (basis for ring test) were quite different.

Leghorn	0.74 (albumin) to 1.0 (globulin)
Guinea hen	3.60 (albumin) to 1.0 (globulin)

Thus at comparable dilutions the guinea hen serum possesses about one-fifth as much globulin as the leghorn. Add to this the fact that the guinea hen globulin is different qualitatively and we may have the cause for the unexpected low value of 1.6 per cent. With the reciprocal test the fact that more but yet different globulin is present in the leghorn serum may account for the higher value of 25 per cent. Studies in this laboratory have shown that the globulin fraction is usually a better stimulant of antibody production than is albumin. If this is true, then the serological relationship between guinea hen and leghorn should be based on comparable amounts of active antigen rather than comparable amounts of total protein. A more correct basis for comparison would, therefore, be provided by tests of antisera made to purified fractions and tested with equivalent amounts of the same kind of protein in all the species compared.

TABLE II

Sera (native and extracted)	Titer 512,000 Antileghorn serum (to native) × native sera		Titer 2,048,000 Antileghorn serum (to extracted) × extracted sera		Titer 128 000 Antiturkey serum (to native) × native sera		Titer 2,048,000 Antiturkey serum (to extracted) × extracted sera		Titer 1,024,000 Antiduck serum (to native) × native sera		Titer 2,048,000 Antiduck serum (to extracted) × extracted sera	
	RN*	PN*	RE*	PE*	RN*	PN*	RE*	PE*	RN*	PN*	RE*	PE*
Leghorn	100.0	100.0	100.0	100.0	25.0	53.2	50.0	—	0.8	—	6.3	7.8
Plymouth	100.0	102.1	100.0	—	25.0	53.0	25.0	48.8	0.8	28.7	3.1	—
Buff Orpington	100.0	97.9	100.0	—	25.0	—	50.0	—	0.8	—	6.3	—
Guinea Hen	1.6	24.5	1.6	7.0	25.0	18.2	25.0	—	0.8	—	0.8	—
Pheasant	50.0	27.0	25.0	41.8	12.5	37.8	50.0	—	0.4	—	1.6	—
Turkey	50.0	27.6	50.0	63.7	100.0	100.0	100.0	100.0	0.8	20.7	6.3	—
Goose	3.1	10.5	1.6	—	6.3	7.2	25.0	4.4	50.0	70.0	50.0	37.1
Duck	3.1	10.9	1.6	5.5	3.1	—	25.0	3.9	100.0	100.0	100.0	100.0
Turkey Buzzard	3.1	11.1	0.8	—	0.4	7.8	12.5	—	6.3	—	1.6	—
Pelican	3.1	9.6	3.1	—	0.0	13.6	6.3	—	1.6	10.3	3.1	1.7
Gull	0.0	19.5	0.8	11.1	1.6	—	12.5	—	0.4	—	0.8	—
Emu	0.4	4.4	0.8	—	0.0	—	6.3	—	0.4	—	0.8	—

* RN = Ring test values obtained using native sera against an antiserum made to native leghorn sera.
PN = Photronreflectometer values using same sera and antiserum as in RN.
RE = Ring test values obtained using extracted sera against anti-extracted leghorn sera.
PE = Photronreflectometer values using same sera and antiserum as in RE.

In the body of Table II may be noted the ring test value of 0.0 per cent, for gull serum when tested with an anti-leghorn sera failed to produce rings yet gave a value of 19.5 per cent when tested with one of these antisera by the photronreflectometric method. At present this cannot be explained.

The bimodality exhibited by the interaction of complex antigens, such as sera, with antisera (as illustrated by Figure 1) is typical of these antigens. Simple antigens, such as hemoglobins, lens-proteins and crystalline egg albumins have quite consistently exhibited unimodal curves indicating the probability of the presence of a single anti-

TABLE III

Sera (native and extracted)	Titer 2,048,000 Antiguinea serum (to native) × native sera		Titer 1,024,000 Antiguinea serum (to extracted) × extracted sera		Titer 1,024,000 Antipelican serum × native sera	
	RN*	PN*	RE*	PE*	RN*	PN*
Leghorn	25.0	32.5	25.0	—	0.8	30.9
Plymouth	25.0	—	25.0	—	0.8	—
Buff Orpington	25.0	—	25.0	—	0.8	—
Guinea Hen	100.0	100.0	100.0	—	1.6	19.7
Pheasant	50.0	34.7	25.0	—	0.4	—
Turkey	25.0	22.0	25.0	—	0.8	—
Goose	25.0	9.8	6.3	—	0.8	—
Duck	12.5	6.8	3.1	—	3.1	3.7
Turkey Buzzard	12.5	5.1	0.0	—	6.3	18.3
Pelican	12.5	2.4	3.1	—	100.0	100.0
Gull	25.0	5.8	0.8	—	1.6	26.5
Emu	12.5	0.1	0.0	—	1.6	—

* RN = Ring test values obtained using native sera against an antiserum made to native leghorn sera.
PN = Photronreflectometer values using same sera and antiserum as in RN.
RE = Ring test values obtained using extracted sera against anti-extracted leghorn sera.
PE = Photronreflectometer values using same sera and antiserum as in RE.

genic substance. It may be of particular interest to note that, contrary to popular belief, lens-proteins may be readily distinguished. This separation, however, is not easily afforded by the ring test but is accomplished with comparative ease with the photronreflectometer.

The injection of a mixture of antigens, such as serum, may result in the production of: (a) antibodies to one fraction alone; (b) antibodies to several or all fractions, with more to one fraction than to another. This may be easily demonstrated by testing several antisera made to a mixed antigen with its purified constituent parts. In sera, the various protein fractions are rarely present in equal amounts, so the ring test

endpoints may, in some cases, be the result of one fraction and its antibodies, and in other cases the result of another fraction with its antibodies. Since in relationship studies the antiserum is kept constant, a small but strongly antigenic fraction spends the greater part of its reaction within the first few tubes, whereas a larger, but less antigenic, fraction yields a wider prozone and positive reactions occur in the higher dilutions of antigen.

In view of the variability in the response of the rabbit (the usual antibody-producer), and because three antisera made in exactly the same manner may differ greatly in specificity, it is very unlikely that

TABLE IV

Source of Antigen	Titer 2,048,000 Anti-leghorn Crystalline Egg Albumins Serum		Titer 2,048,000 Anti-turkey Hb Serum		Titer 1,024,000 Anti-turkey lens Serum	
	RN*	PN*	RN*	PN*	RE*	PE*
Leghorn	100.0	100.0	100.0	52.0	100.0	80.4
Guinea Hen	—	—	100.0	30.9	—	40.5
Pheasant	—	—	100.0	74.8	—	—
Turkey	100.0	34.0	100.0	100.0	100.0	100.0
Goose	50.0	16.0	100.0	—	100.0	11.8
Duck	50.0	26.4	100.0	—	100.0	11.4
Turkey Buzzard	—	—	100.0	34.0	—	—

* RN = Ring test values obtained using native sera against an antiserum made to native leghorn sera.
PN = Photronreflectometer values using same sera and antiserum as in RN.
RE = Ring test values obtained using extracted sera against anti-extracted leghorn sera.
PE = Photronreflectometer values using same sera and antiserum as in RE.

reciprocal tests would often confirm the original tests. In those cases in which antigens are ring-tested with pooled antisera the internal compensation of the mixture might conceivably make for better agreement between original and reciprocal tests. Possibly because of the more adequate data obtained by photronreflectometric methods, in contrast to the ring-test, the reciprocal tests show better agreement with the original tests than is seen by ring tests.

There is considerable evidence to indicate that the antibodies responsible for the sensitivity, or titer, of an antiserum may not be the same antibodies responsible for the greater part of the precipitating capacity of that antiserum. Several antisera, made in the course of this study, possessed titers of 128,000 or more, and yet, photronreflectometrically the turbidity at various dilutions of antigen was zero.

Other antisera with a titer of 10,000,000 or more produced less precipitate than others with a titer of 512,000. Wolfe and Baier (1938) have had a similar experience as indicated by the statement in their summary, "Indications are that at least two types of antibodies are present: (1) a 'titer'-producing antibody and (2) a 'precipitate'-forming antibody."

While the correlation between the precipitating capacities and the sensitivities of antisera appears to be weak and irregular, there is, nevertheless, an almost perfect parallelism in the systematic position of the birds as revealed by relationship values derived from ring and turbidity tests.

It would be difficult at this time to state with any degree of certainty what the effect of petroleum-ether extraction was upon the antigens. The differences between relationship values obtained by use of unextracted (native) materials and those obtained with extracted antigens are of the same magnitude as might be expected by injecting two rabbits with the same material; the rabbit being the greatest variable. In experiments with antisera made to extracted sera it has been noted repeatedly that while the sensitivity of these antisera is just as high as those made to the unextracted sera, the precipitating capacity of the former is in all cases decidedly smaller than the latter. This is not true of extracted lens proteins and extracted crystalline egg albumins.

Table V shows the ring test values obtained when both native and extracted antigens are tested with antisera made to each of them. Antisera made to native antigens react more strongly with native than with extracted antigens. Also, antisera made to extracted antigens react more strongly with extracted antigens than do native antigens. This would imply that extraction with petroleum-ether leaves behind a protein molecule whose surface configuration is somewhat different from that before extraction. Apparently, the portion of the molecule least affected is that responsible for the antigenic specificity, for the greater losses are found with the heterologous reactions. In some cases (such as plymouth rock $\times$ anti-native guinea hen$_{\times 2}$) there must have been considerable denaturation of the antigen to give such a low value. This could not be detected, however, as precipitated material or by nitrogen determination which accounted for all the protein.

The fact that almost all photronreflectometric curves were unimodal when extracted antigens were used, and that all anti-extracted sera were poorer precipitating sera, might well indicate the inactivation of a portion of the antigen. Fortunately, it appears that extraction is not necessary in avian studies. Should it become a necessary procedure

in the study of closely related forms, attempts should be made with extracting agents less destructive to protein than petroleum ether, possibly sodium ricinoleate.

The close relationship of gallinaceous birds as determined by morphological methods is corroborated by both ring and photronreflectometric tests. The close relationship of the Anseres (duck and goose) is likewise confirmed. The distant relationship of the flightless birds (primitive), represented by the emu, to the gallinaceous birds, is con-

TABLE V

The effect of extracting antigens with petroleum-ether on relationship values

Antigens	Anti-native guinea hen sera		Anti-native leghorn sera		Anti-extracted leghorn serum	Anti-extracted turkey serum
	× 1	× 2	× 1	× 2	× 1	× 2
Native guinea	100.0	100.0	1.6	1.6	0.0	12.5
Extracted guinea	50.0	100.0	0.8	0.8	1.6	25.0
Native plymouth	25.0	50.0	100.0	100.0	12.5	50.0
Extracted plymouth	12.5	0.4	100.0	100.0	100.0	50.0
Native buff orp.	25.0	50.0	100.0	100.0	12.5	50.0
Extracted buff orp.	25.0	12.5	100.0	50.0	100.0	50.0
Native turkey	12.5	25.0	25.0	50.0	12.5	100.0
Extracted turkey	3.1	1.6	6.3	25.0	50.0	100.0
Native duck	12.5	12.5	6.3	1.6	0.0	6.3
Extracted duck	0.8	0.8	0.0	0.4	1.6	25.0
Native gull	25.0	3.1	0.0	0.0	0.0	3.1
Extracted gull	3.1	0.0	0.0	0.0	0.8	12.5

firmed by these tests. That the birds as a class constitute a homogeneous group chemically is clearly indicated throughout these tests.

SUMMARY AND CONCLUSIONS

1. Chemical resemblances and differences between avian sera are demonstrated by precipitating antisera.

2. Two techniques are employed to determine the interaction between antigen and antibody. The ring test, though revealing only the end point, has the advantage of greater sensitivity. The photronreflectometer measures the degree of reaction between antigen and antibody over the entire range of dilutions within which a demonstrable reaction occurs.

3. Tests with antisera made to extracted sera show that extraction of the antigens causes the production of poorer precipitating antisera, that is, few antibodies, or weaker ones.

4. All antisera non-specific by ring test were specific by photron-reflectometric test.

5. The systematic position of the birds is generally confirmed by these tests.

6. Birds are serologically an essentially homogeneous group.

7. Extraction of antigens with petroleum-ether is not necessary in avian serological studies. If found necessary in studies with closely related forms it is suggested that a less destructive method, or reagent, or both, be found.

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THE RESISTANCE AND ACCLIMATIZATION OF MARINE FISHES TO TEMPERATURE CHANGES. I. EXPERIMENTS WITH GIRELLA NIGRICANS (AYRES) [1]

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Introduction

Fishes occur in nature over a wide range of temperature, from the freezing point of sea water (about −2°) to at least 37° C. (Sumner and Sargent, 1940) or higher. The range of tolerance of most species apparently does not include both of these extremes. Differences of resistance to extreme temperatures shown by different species, both under experimental conditions (Huntsman and Sparks, 1924; Huntsman, 1926; Hathaway, 1927) and in nature (Storey, 1937), have been found to correspond to differences of distribution (geographic or vertical) in relation to temperature.

Quantitative studies of acclimatization, as a factor in the resistance of fishes to high temperatures, have been undertaken repeatedly (Loeb and Wasteneys, 1912; Hathaway, 1927; Binet and Morin, 1934; Sumner and Doudoroff, 1938). Detailed studies of acclimatization to low temperatures are lacking. This oversight is not surprising. The view that low temperatures are less injurious than equally extreme high temperatures, at least when freezing is not involved, is not uncommon. Heretofore, Maurel and Lagriffe (1899a, b, c) have attempted to compare experimentally the upper and lower limits of tolerance of some, chiefly fresh-water, fishes. They concluded, "Qu'ils semblent mieux organisés pour résister aux températures extrêmes du froid qu'aux températures extrêmes de la chaleur." It is not clear what criterion was used in judging whether a given temperature was more or less "extreme" than another. Aside from the fact that the methods used were not entirely satisfactory, it appears that the above conclusion was probably based upon some faulty line of reasoning, and is not an obvious outcome of the experimental results.

In general, the sensitivity of fishes to chilling has received little attention from experimental physiologists, who have most frequently

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used relatively cold-hardy fresh-water and estuarine marine fishes as laboratory material. The survival of fishes which had been frozen for a long time in water, or frozen solid throughout in air, has been reported by a number of early observers (for references see Borodin, 1934). This has not been confirmed by the more reliable and recent investigations, but it has been shown that some cold-hardy fishes can survive super-cooling (i.e., without freezing) to temperatures below the freezing point of their body fluids, and also brief and partial freezing of their superficial tissues (Regnard, 1895; Britton, 1924; Weigmann, 1930, 1936; Borodin, 1934; Schmidt, Platonov and Person, 1936; Luyet, 1938). The phenomenon or possibility of death after more prolonged exposure to moderately low (above-zero) temperatures has attracted much less attention. Indeed, Luyet and Gehenio (1938), in the "compendium" of their extensive review dealing with the lower limit of vital temperatures, state: "The third group, namely that of organisms which are killed at above-zero temperatures, includes only the homoiotherms and some of the higher plants." A number of observations on the death of poikilotherms from chilling are, however, on record (e.g., Weigmann, 1929).

Changes of cold-tolerance, resulting from continued exposure to high and low temperatures, have been observed in fishes by Wells (1935) and Sumner and Doudoroff (1938), but accurate quantitative studies of their magnitude were not undertaken. There is also surprisingly little evidence available for an influence of acclimatization upon susceptibility to chilling in other animals.

The term acclimatization will be used here to designate only gradual changes in the effect upon an organism of a given environmental factor, which can be produced experimentally in the individual organism by altering its environment with respect to this particular factor. Heilbrunn (1937) introduces his interesting chapter on acclimatization with a fairly comprehensive discussion of the modern meaning of the term. However, Heilbrunn cites as examples of temperature acclimatization certain seemingly adaptive differences between individuals of the same species from different latitudes, as well as seasonal variations of resistance to extreme temperatures, etc. (e.g., Hörstadius, 1925; Bodenheimer and Klein, 1930). This is not entirely consistent with his own definition. Such variations may be inherent, i.e., sub-specific (resulting from mutation and selection) or inherently periodic, or they may be related to differences of diet or to environmental differences other than those of temperature. The use of the more general term "adaptation" (Bělehrádek, 1935; Fox, 1939) is

preferable in referring to such variations, which have not been shown to be due to acclimatization alone, as defined here.

True acclimatization to cold, as indicated by changes of resistance to freezing (sub-zero temperatures), has been studied in insects by Payne (1926a, b) and by Mellanby (1939). Mellanby (1939, 1940) also investigated the influence of acclimatization upon resistance to immobilization by cold at non-freezing temperatures (chill-coma) in insects and amphibia. The influence upon survival at such temperatures was not studied in detail. Ogle and Mills (1933) produced changes of cold-tolerance in rabbits. The mechanisms of acclimatization in cases in which death is associated with a thermoregulative breakdown (in strict homoiotherms), or with the freezing of body fluids, are probably quite different from that involved in changes of resistance of poikilotherms to chilling.

The acclimatization of animals to heat is well known. Studies with fishes have already been cited. Heilbrunn (1937) cites other references.

The present paper deals with quantitative experiments on the resistance of the marine fish *Girella nigricans* to extreme temperatures and on the phenomena of acclimatization to heat and especially to cold, and with their ecological significance. Experiments with other species and a discussion of some possible causes of death at extreme low temperatures will be presented in a later publication.

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Material

The young (immature) greenfish, *Girella nigricans* (Ayres), used in the experiments were taken in the intertidal area near the Scripps Institution, La Jolla, California. This marine shore-fish is an active and fairly hardy inhabitant of the littoral zone, occurring in open water and in tide-pools along the open coast.

For proper evaluation of the results, with respect to their ecological implications, information relative to temperature conditions to which the animals are normally exposed in their natural habitat is necessary. The following data are based on daily records of water temperatures at the end of the Scripps Institution's 1000-foot pier. The average surface temperature for 23 years (1916 to 1938) was 17.0° C. The average for January and February, the coldest months, was 14.0°, and that for July and August, the warmest months, was 20.6°. The lowest

and highest monthly averages on record were 11.8° and 23.6°, respectively. Corresponding weekly averages were 11.7° and 25.1°. More significant as indices of the normal temperature range are the mean yearly minimum and maximum, that is, means of the lowest and highest temperatures, respectively, occurring during each summer and each winter. Since the daily records were not available to the writer, means of the lowest and highest weekly averages were calculated. These values were 13.3° and 22.0°. The average bottom temperature at a depth of 5 meters was 0.8° lower than at the surface. This vertical gradient averaged 1.9° in 5 meters during the two warmest months. The range of daily temperatures at a given level within a single week sometimes exceeds 5° or even 7°.

In tide-pools in which some Girella remain during low tide the temperature generally does not differ greatly from that in open water, but occasionally temperatures above 26° have been observed. According to Jordan, Evermann and Clark (1930), the range of distribution of *G. nigricans* extends along the "coast of California from Monterey to Cape San Lucas," i.e., further southward than northward from La Jolla.[3] This probably does not represent the extent of the normal habitat of the species. Since different physiological races or subspecies may be involved, experimental results obtained with specimens from one locality are not strictly applicable to those from other localities.

Methods

In comparing the resistance of fishes to extreme temperatures, some workers (e.g., Loeb and Wasteneys, 1912) determined the average duration of survival at constant lethal temperatures. Others (Maurel and Lagriffe, 1899a, b; Huntsman and Sparks, 1924; Sumner and Doudoroff, 1938; et al.) determined the temperature at which death or coma occurred when the water was warmed or cooled fairly rapidly. These methods are satisfactory for some purposes, but the results are not readily applicable to many ecological problems. The ecologist is interested in knowing and comparing the most extreme temperatures at which organisms can survive indefinitely, or at least for equal and long periods of exposure. That the limit of tolerance varies with the duration of exposure involved is well known, and one may not assume that closely parallel relations always hold between temperatures just tolerated for brief periods and ones tolerated for long periods, especially by different species.

More suitable for present purposes was the method used by Hathaway (1927) in experiments on heat-tolerance. It involved the use of

[3] For data on surface temperatures in this range see U. S. Weather Bureau (1938)

a series of constant test temperatures and fixed periods of exposure. Hathaway's calculated "average tolerance limits," do not, however, actually represent what the term implies, being lower than the true values. For any given period of exposure he regarded a given temperature as being the limit of tolerance for a percentage of the experimental animals obtained by taking the difference between the percentages surviving at this temperature and at the next higher (more extreme) test temperature. The formula used yielded the mean of the "tolerance limits" thus obtained. Yet the interval between the two test temperatures corresponds to a statistical "class-interval," into which fall the individual tolerance limits of the above percentage of specimens. The usual statistical procedure is to assume uniform distribution over the class-interval, and in computing the mean, the mid-value (not the lower limit) of each class is taken as the average value for all items in that class.

In the present experiments, instead of the mean, the *median tolerance limit* was adopted as a measure of resistance. The upper or lower median tolerance limit (T_m) for any given period of exposure will be defined as a temperature at which just 50 per cent of the specimens under consideration are able to survive for this period of time.[4] This value is more easily estimated than the mean tolerance limit, and is not affected by occasional extreme variants, such as unhealthy individuals.

The fishes were held in large tanks supplied with running sea water. Constant temperatures were maintained by thermostatic control or otherwise, and did not deviate from the stated values by more than 0.5° C. (usually less). When temperatures above 20° were maintained. the removal of excess air (supersaturation) was provided for by bubbling a stream of compressed air through the heated water before it entered the aquaria.

In testing their heat-tolerance, the fishes were transferred to an aquarium supplied with an ample flow (about one liter per minute) of heated and air-equilibrated water. The temperature was kept constant by thermostatic control and generally did not deviate from the stated values by more than 0.1°, the maximal deviation allowed being 0.2°. The same degree of accuracy was maintained in cold-tolerance tests. These were performed in two ways. In early (long-time) experiments the aquarium was supplied with cooled (but not aerated) running water. In later experiments, in which the periods of exposure were limited to 24 hours, the tests were made in about nine liters of standing water in glass battery jars immersed in a constant-

[4] Obviously, equal numbers of specimens have tolerance limits above and below this value; hence the designation "median."

temperature tank. The water in this case was well aerated with compressed air. In a given series of experiments the same method was used throughout.

The determination of the upper or lower median tolerance limit of a group of fishes for any desired period of exposure was made in the following manner. A number of specimens (usually ten or more) were transferred directly to the test tank, maintained at a temperature which was believed to be close to the limit of tolerance. At the end of the desired time interval the number of specimens surviving was noted. If exactly 50 per cent of those tested survived, this temperature was taken as the median tolerance limit. If more or less than 50 per cent survived, similar tests were performed at other temperatures 1° C. (or, in a few instances 0.5°) apart, until two temperatures were found at one of which more than half and at the other less than half of the specimens tested survived. The median tolerance limit was then estimated by graphical interpolation. The percentage surviving at each of the two temperatures was plotted against the temperature on arithmetic co-ordinate paper, and the two points connected by a straight line. The temperature which corresponded to 50 per cent survival on this graph was taken as the median tolerance limit.[5] What is meant by survival will be explained later.

The maximal error in the estimation of the median which may result from the assumption of a straight-line relationship between the experimental points is, for present purposes, negligible, not exceeding a few tenths of one degree. The differences of tolerance in which the writer was interested were so clear-cut that the computation of the probability of their significance would be superfluous.

Results

Effects of Extreme Temperatures and the Criterion of Survival Adopted

At rapidly lethal high temperatures great stimulation, loss of equilibrium, and finally permanent and almost simultaneous cessation of respiration and of other movements (heat-coma) were observed. At slowly lethal high temperatures stimulation, disturbances of equilibrium and other signs of distress (initial shock effects) were frequently observed directly after transfer from low temperatures. This was followed by temporary recovery and more or less normal behavior. Specimens conditioned to high temperatures began to show distress only a few hours or minutes before death. Thus, neither recovery from the initial effects, nor failure to show any distress after transfer to a

[5] The upper median tolerance limit is identical with the "thermal index" used by Whitney (1939) in comparing the heat-resistance of mayfly nymphs.

high temperature, is an indication that the fish will survive the change. Recovery from heat-coma after return to a normal temperature was never observed in Girella.

At lethal low temperatures the initial shock effect was generally more pronounced than at high temperatures. It ranged from mild distress and disturbance of equilibrium to a violent, convulsive paroxysm, followed by cessation of respiratory and all other movements. I shall refer to the latter state as "primary chill-coma." At very low temperatures the fishes died without showing any further visible signs of life, except, in some cases, a brief quivering of the opercles and fins. At less extreme temperatures the fishes recovered more or less from the initial shock. Regular respiratory movements, if they had ceased, were resumed, and frequently the fishes righted themselves and regained normal appearance and behavior. After some hours or days, however, they again showed increasing distress, and finally ceased to respire and to respond to stimulation. This stage, to which I shall refer as "secondary chill-coma," was soon followed by death.

The initial shock was not manifest until several seconds after transfer to the low temperature, and apparently was not due to stimulation of the cutaneous sense organs, but was produced only when the low temperature had penetrated internally, probably to the central nervous system. Accordingly, it was more delayed in large specimens than in small ones. By cooling the animals gradually the shock was sometimes apparently prevented or reduced without appreciably delaying the onset of secondary chill-coma.

If it occurred at all, spontaneous temporary recovery from primary chill-coma (i.e., resumption of respiratory and other movements, occurring at the low temperature at which the coma was produced) began within a few minutes or, at most, within half an hour. Thus, in Girella primary chill-coma was not a very pronounced phenomenon, except at very rapidly lethal temperatures. In most cases the initial shock effect, although intense, did not involve complete coma of appreciable duration. In other species, however, primary chill-coma, followed by temporary recovery, may be quite prolonged. If returned to a normal temperature within one hour, Girella in a state of primary chill-coma were frequently capable of rapid and permanent recovery. After the onset of secondary chill-coma, spontaneous recovery was never observed, but the fishes sometimes showed brief and partial or even complete recovery on warming, provided that they were returned to the normal temperature within a few minutes. The more slowly lethal the temperature and, accordingly, the more delayed the onset of

secondary coma, the less likely were the fishes to recover on warming. The administration of artificial respiration, by passing a stream of water through the mouth and over the gills, favored recovery.

The accurate quantitative comparison of relative tolerances requires the adoption of a uniform and convenient "criterion of survival," that is an end-point after which the organism is regarded as having succumbed to the effects of the lethal agent. It is best to adopt as an end-point the permanent (not spontaneously reversible) cessation of some important function which is among the first to be arrested. The time required for the attainment of an advanced stage of necrobiosis is not as accurately indicative of relative rates of injury, and may be greatly influenced by temperature and other factors independently of the rate of injury. The permanent cessation of respiratory and other movements, either spontaneous or induced by mechanical stimulation, presumably resulting from the functional inactivation of the central nervous system, apparently satisfies the above requirements.

In the determination of heat-tolerance the application of the end-point adopted (i.e., cessation of all spontaneous and reflex movements) presented no difficulty. The occurrence of two types of chill-coma introduced some difficulty. Primary chill-coma differs from chill-coma which has been described in insects (Mellanby, 1939) and other animals in that the fishes were capable of rapid adaptation to the low temperature, with consequent temporary recovery. In this respect it differs also from heat-coma. On the other hand, the onset of secondary chill-coma is comparable with that of heat-coma and a satisfactory end-point. Neither was ever followed by spontaneous recovery, and both apparently represented the onset of death, as the fishes failed to recover on return to normal temperatures or lost the ability to do so within a very short time. At very extreme low temperatures, at which no spontaneous recovery from primary coma was noted, an entirely comparable end-point was lacking. In practice, Girella were regarded as having succumbed if they had failed to resume movements at the end of one hour after transfer. Thereafter, as after the onset of secondary coma, spontaneous recovery was apparently impossible, and recovery on warming was only rarely observed.

By way of summary, it can be stated that a specimen was regarded as having succumbed if no movements, either spontaneous or induced by mechanical stimulation, could be detected, but no determinations of lower tolerance limits were made for periods of exposure shorter than one hour. In this way any uncertainty as to whether movements had ceased permanently at the time of observation or had been suspended temporarily was eliminated.

Time-Temperature Relations of Heat and Cold Tolerance and the Influence of Acclimatization

The specimens used in the first series of quantitative experiments were collected in the fall of 1939, and held at 20° for three and a half months.[6] On March 14, 1940, they were divided into three lots, which were acclimatized thereafter to 12°, 20° and 28°, respectively, for nearly two months before tolerance determinations were begun. Evidence to be presented later indicates that the fishes were then completely acclimatized to the new temperatures, that is, their tolerance had reached a constant value and would not change appreciably with more prolonged acclimatization. Therefore, the results of subsequent tests may be regarded as comparable, in spite of unavoidable differences in the duration of acclimatization previous to testing.

Groups of eight to 14 specimens of similar history were tested in running water at a series of low or high temperatures, usually 1° C. apart. The number surviving was recorded at fixed intervals of time, namely, after 1, 3, 6, 12, 24, 48 and 72 hours of exposure to the test temperatures, and sometimes also after other periods.[7] The lengths of the specimens ranged from 6.0 cm. to 9.2 cm. The mean lengths in the different groups under comparison were approximately equal, although those of lots tested at high temperatures were slightly greater than those of lots tested at low temperatures. Since no obvious correlation was noted between the duration of survival and the size of the individual specimens, the smaller differences between the mean lengths of the test lots could be of no great consequence.

The experimental data are presented in Tables I and II. From these data the lower and upper median tolerance limits for the various periods of exposure after which observations were made were estimated by the method described earlier and plotted against the duration of exposure in Figures 1 and 2. The resulting curves will be referred to as the time-temperature curves of cold-tolerance and of heat-tolerance, respectively.

From Tables I and II it may be deduced that among specimens of similar history the variability of the individual tolerance limits (i.e., of the lowest and highest temperatures which individual specimens are

[6] The appearance in the stock-tank of a fatal, seemingly infectious disease (dermatitis), which was later brought completely under control, necessitated this delay.

[7] Signs of external disease frequently appeared after prolonged exposure to extreme low temperatures, especially in specimens which had been conditioned to the higher acclimatization temperatures. Since it was desirable to avoid disease as a complicating lethal factor, and to deal only with the direct lethal effects of cold, determinations of lower limits of tolerance for periods longer than three days were not attempted with such specimens.

just able to withstand) for any given period of exposure is small. The difference between temperatures at which 100 per cent and 0 per cent survival was obtained averaged slightly over 2°, and in no case exceeded 3°. In contrast with this, marked differences of tolerance were shown by specimens conditioned to different temperatures. The lower median tolerance limits for the various fixed periods of exposure adopted (one to 72 hours) of fishes conditioned to 28° and to 20° differed, on the average, by 4.3°, and those of fishes conditioned to 20° and 12° by 4.0°. The corresponding average difference between the upper median tolerance limits of 20°- and 12°-conditioned specimens was 3.2°. There can be no doubt that these differences are significant. Only the heat-tolerance of specimens conditioned to 20° and to 28° did not differ appreciably.

The time-temperature curves of cold-tolerance (Figure 1) are dissimilar (not parallel). After 24 hours the lower median tolerance limits of 28°-conditioned specimens remained constant at 13°. This temperature may apparently be regarded as the "ultimate" or "true" lower median tolerance limit for these specimens, that is, the least extreme low temperature at which 50 per cent of the specimens are killed by cold, regardless of the duration of exposure. The ultimate limits of tolerance for specimens conditioned to 20° and to 12° cannot be stated, but can be judged by extrapolation to be slightly above 8° for the former, and somewhat above 5° for the latter.

It is noteworthy that, whereas for brief periods of exposure (one or three hours) the differences between the lower tolerance limits of fishes conditioned to 20° and 28° was considerably smaller than the difference between those of specimens conditioned to 20° and 12°, the reverse is true for longer periods of exposure (12 hours or more).[8] Short-time experiments obviously do not yield an entirely satisfactory measure of the changes of relative tolerance produced by acclimatization.

The time-temperature curves of heat-tolerance (Figure 2) tend to become parallel with the time axis sooner than the cold-tolerance curves. The ultimate or true upper median tolerance limits were apparently 31.4° for both the 28°-conditioned and 20°-conditioned specimens and 28.7° for the 12°-conditioned specimens. The absence of any difference between the 72-hour upper limits of tolerance of specimens conditioned to 28° and 20°, in contrast with a difference of

[8] The ratio (R) of the former difference to the latter $\left(R = \frac{T_m 28° - T_m 20°}{T_m 20° - T_m 12°}\right)$ increases with increasing time of exposure from 0.56 for one hour to 1.47 for 72 hours of exposure. When plotted against the logarithm of the duration of exposure to the test temperatures in hours ($\log t$), the values of R tend to fall roughly in a straight line ($R = 0.56 + 0.49 \log t$).

TABLE I

Per cent survival of Girella at various low temperatures and the estimated lower median tolerance limits (T_m) in relation to temperature of previous acclimatization and to time (duration) of exposure to the test temperatures

Date 1940	Acclim. temp. (° C.)	Test temp. (° C.)	No. of fish	Mean length (cm.)	Per cent surviving after							
					1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.
5/8	28°	13°	10	7.0	100%	100%	100%	100%	90%	50%	50%	
5/13	"	12°	11	7.1	100	100	91	45	0	0	0	
5/11	"	11°	10	7.1	100	100	90	30	0	0	0	
5/14	"	10°	10	7.2	100	100	0	0	0	0	0	
5/15	"	9°	10	7.2	100	90	0	0	0	0	0	
5/16	"	8°	10	7.2	80	10	0	0	0	0	0	
5/17	"	7°	10	7.0	20	0	0	0	0	0	0	
5/18	"	6°	8	7.1	0	0	0	0	0	0	0	
				T_m:	7.5°	8.5°	10.6°	12.1°	12.6°	13.0°	13.0°	
5/3	20°	8°	14	7.3	100%	100%	100%	100%	93%	64%	50%	
5/1	"	7°	12	7.2	100	100	100	58	17	0	0	
5/6	"	6°	14	7.3	100	93	36	0	0	0	0	
5/7	"	5.5°	12	7.2	75	42	0	0	0	0	0	
5/7	"	5°	14	7.3	0	0	0	0	0	0	0	
				T_m:	5.3°	5.6°	6.2°	6.9°	7.4°	7.8°	8.0°	
5/28	12°	5°	10	7.1	100%	100%	100%	100%	100%	100%	90%	50%
5/21	"	4°	11	7.1	100	100	100	100	100	45	0	0
5/25	"	3°	11	7.1	100	100	100	100	55	0	0	0
6/3	"	2°	10	7.2	90	70	60	10	0	0	0	0
6/4	"	1°	10	7.1	20	0	0	0	0	0	0	0
				T_m:	1.4°	1.7°	1.8°	2.4°	2.9°	4.1°	4.6°	5.0°

TABLE II

Per cent survival of Girella at various high temperatures and the estimated upper median tolerance limits (T_m) in relation to temperature of previous acclimatization and to time (duration) of exposure to the test temperatures.

Date 1940	Acclim. temp. (° C.)	Test temp. (° C.)	No. of fish	Mean length (cm.)	Per cent surviving after								
					1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.	Other periods
6/19	28°	33°	8	7.6	100%	100%	88%	0%	0%	0%	0%	0%	
6/21	"	32°	9	7.7	100	100	100	100	44	0	0	0	
6/24	"	31°	9	7.7	100	100	100	100	100	100	89	89	168 hrs.: 89%
			T_m:					32.5°	31.9°	31.5°	31.4°	31.4°	31.4° (168 hr.)
6/18	20°	35°	10	7.8	0%	0%	0%	0%	0%	0%	0%	0%	0.5 hr.: 50%
6/18	"	34°	12	7.9	100	0	0	0	0	0	0	0	
6/17	"	33°	12	7.8	100	100	25	0	0	0	0	0	
6/15	"	32°	12	7.7	100	100	100	75	0	0	0	0	
6/8	"	31°	12	7.8	100	100	100	100	100	83	83	83	
			T_m:		34.5°	33.5°	32.7°	32.3°	31.5°	31.4°	31.4°	31.4°	35.0° (0.5 hr.)
6/29	12°	31°	8	8.0	25%	0%	0%	0%	0%	0%	0%	0%	
6/28	"	30°	8	8.0	100	0	0	0	0	0	0	0	2 hrs.: 50%
6/22	"	29°	12	7.9	100	83	50	50	33	33	33	33	
6/19	"	28°	10	7.9	100	100	100	90	90	90	90	90	
6/14	"	27°	10	7.8	100	100	100	100	100	100	100	100	
			T_m:		30.7°	29.4°	29.0°	29.0°	28.7°	28.7°	28.7°	28.7°	30.0° (2 hr.)

5° between the corresponding lower limits, is of interest. A change of cold-tolerance produced by acclimatization is not necessarily accompanied by a corresponding change of heat-tolerance.

The lower the temperature of acclimatization, the earlier does the time-temperature curve of heat-tolerance tend to become parallel with

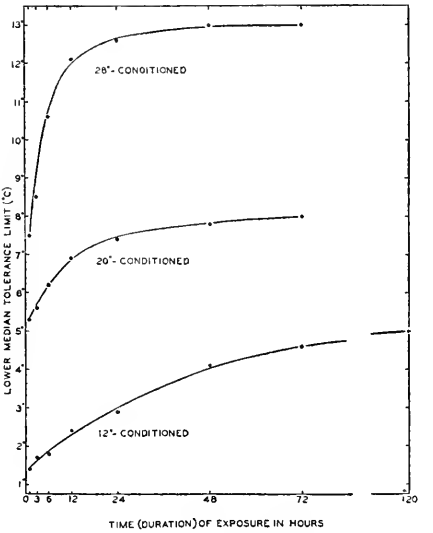

FIG. 1. Time-temperature curves of cold-tolerance: Lower median tolerance limits in relation to the time (duration) of exposure to the test temperatures. The specimens were previously acclimatized to three different temperatures (12°, 20° and 28°), indicated on each curve. Data from Table I.

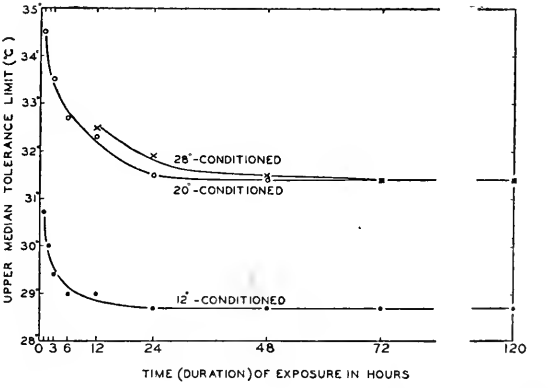

FIG. 2. Time-temperature curves of heat-tolerance: Upper median tolerance limits in relation to the time (duration) of exposure to the test temperatures. The specimens were previously acclimatized to three different temperatures (12°, 20° and 28°), indicated on each curve. Data from Table II.

the time axis. This is the reverse of the relationship between the corresponding curves of cold-tolerance (Figure 1). At slowly lethal temperatures the fishes probably undergo some acclimatization, the extent of which must vary with the magnitude of the change involved in the transfer to the lethal temperature. The differences noted above between the curves obtained with fishes acclimatized to different temperatures can be explained, at least in part, on the basis of this assumption.

The Rate of Acclimatization

Data already available relative to the rate of change of heat-tolerance of some marine fishes with acclimatization (Loeb and Wasteneys, 1912; Sumner and Doudoroff, 1938) indicate that increased heat-resistance is acquired rapidly after transfer to higher temperatures. While this change is reversible, heat resistance is apparently lost after transfer to lower temperatures much more slowly than it is gained. No detailed data were available on the corresponding rates of change of cold-tolerance, although a few observations bearing on this question have been made by Wells (1935) and by Sumner and Doudoroff (1938). The latter problem was now investigated in greater detail.

Limitations of material, time and facilities necessitated the adoption of some single test-period of exposure, for which tolerance limits were to be determined after varying periods of acclimatization to new temperatures. Within certain limits, the longer the period of exposure the more nearly should the results indicate the rate of change of the ultimate or true median tolerance limit. On the other hand, certain practical considerations made the use of a period longer than 24 hours undesirable, and this period was adopted.

Each test lot consisted of ten specimens, carefully selected with regard to size. The specimens varied in length between 5.6 cm. and 7.5 cm., and the mean length in each lot was 6.3 to 6.4 cm. Determinations of heat-tolerance were made in running water, and those of cold-tolerance in standing (aerated) water. The acclimatization temperatures used were 26°, 14° and, in a few experiments, 20°. The specimens were collected in the fall of 1940 and held at current sea-water temperatures. Some were transferred to 26° on October 7, some to 14° on November 16, and some to 20° on December 3.[9] After having been held at these respective (first) acclimatization temperatures for at least 42 days, lots of these specimens were transferred at convenient intervals of time to new (second) acclimatization temperatures. They were held in wire-screen cages immersed in the constant-temperature

[9] The sea-water temperatures on these dates were 19.7°, 17.3° and 16.5°, respectively.

TABLE III

*Per cent survival after 24 hours at various test temperatures and the estimated 24-hour median tolerance limits (T_m) of Girella, which had been acclimatized to various initial (first) temperatures and subsequently subjected for varying periods to other (second) acclimatization temperatures. The percentages are based on 10 specimens, except those marked *, which are based on 20 specimens. Where no second temperature is indicated, the fishes were transferred to the test temperature directly from the first acclimatization temperature. Tolerance limits marked (u) are upper, while the rest are lower median tolerance limits.*

Date of test	First acclim. temp.	Days at first temp.	Second acclim. temp.	Days at second temp.	Test temp.	Per cent surviving 24 hours	T_m (24-hour)
11/18/40	26°	42	—	0	11°	90	10.2°
11/20/40	"	44	—	0	10°	40	
11/25/40	"	49	14°	0.42	10°	80	9.3°
11/28/40	"	52	"	0.42	9°	40	
11/21/40	"	44	"	1	9°	100	8.4°
11/23/40	"	46	"	1	8°	20	
11/27/40	"	48	"	3	8°	90	7.2°
11/24/40	"	44	"	3	7°	40	
12/ 2/40	"	50	"	6	7°	100	6.4°
11/30/40	"	48	"	6	6°	20	
12/10/40	"	52	"	12	6°	90	5.5°
12/ 8/40	"	50	"	12	5°	10	
12/16/40	"	50	"	20	5°	80	4.4°
12/18/40	"	52	"	20	4°	30	
12/20/40	"	42	"	32	5°	90	4.3°
12/18/40	"	42	"	30	4°	30	
1/ 7/41	"	42	"	50	5°	100	4.2°
1/ 5/41	"	42	"	48	4°	40*	
12/11/40	"	65	—	0	11°	100	10.2°
12/ 9/40	"	63	—	0	10°	40	
12/13/40	"	65	20°	2	9°	90	8.2°
12/14/40	"	66	20°	2	8°	40	
12/31/40	14°	45	—	0	4°	60	3.8°
1/ 2/41	"	47	—	0	3°	0	
1/13/41	"	58	26°	0.15	5°	70	4.7°
1/14/41	"	59	"	0.15	4°	0	
1/10/41	"	55	"	0.42	6°	70	5.7°
1/12/41	"	57	"	0.42	5°	10	
1/ 7/41	"	51	"	1	7°	80	6.6°
1/ 4/41	"	48	"	1	6°	0	
1/ 8/41	"	51	"	2	8°	100	7.3°
1/ 8/41	"	51	"	2	7°	30	
1/ 9/41	"	51	"	3	8°	60	7.8°
1/ 7/41	"	49	"	3	7°	0	
1/12/41	"	51	"	6	9°	90	8.5°
1/14/41	"	53	"	6	8°	10	
1/20/41	"	53	"	12	10°	90	9.4°
1/18/41	"	51	"	12	9°	20	
1/28/41	"	53	"	20	10°	55*	9.9°
1/29/41	"	54	"	20	9°	0	

TABLE III—*Continued*

Date of test	First acclim. temp.	Days at first temp.	Second acclim. temp.	Days at second temp.	Test temp.	Per cent surviving 24 hours	T_m (24-hour)
1/31/41	14°	45	26°	31	10°	65*	9.8°
2/ 1/41	"	45	"	32	9°	0	
2/11/41	"	45	"	42	10°	50	10.0°
1/18/41	"	63	—	0	5°	100	4.2°
1/16/41	"	61	—	0	4°	40	
2/ 4/41	"	80	20°	0.42	5°	80	4.6°
2/ 6/41	"	82	"	0.42	4°	0	
1/20/41	"	63	"	2	6°	100	5.5°
1/22/41	"	65	"	2	5°	0	
2/ 1/41	"	69	"	8	7°	100	6.3°
1/31/41	"	68	"	8	6°	30	
1/16/41	20°	44	—	0	7°	65*	6.8°
1/20/41	"	48	—	0	6°	0	
2/ 1/41	"	60	14°	0.42	7°	100	6.2°
1/30/41	"	58	"	0.42	6°	40	
1/20/41	"	46	"	2	6°	100	5.4°
1/23/41	"	49	"	2	5°	10	
2/ 4/41	"	55	"	8	5°	100	4.5°
2/ 6/41	"	57	"	8	4°	0	
1/25/41	"	51	26°	2	9°	100	8.5°
1/27/41	"	53	26°	2	8°	0	
2/ 2/41	"	61	—	0	7°	50	7.0°
11/20/40	26°	44	—	0	31°	100	31.8° (*u*)
11/18/40	"	42	—	0	32°	40	
11/22/40	"	45	14°	1	32°	80	32.4° (*u*)
11/26/40	"	49	"	1	33°	0	
11/24/40	"	45	"	3	32°	80	32.4° (*u*)
11/27/40	"	48	"	3	33°	0	
12/23/40	"	42	"	35	30°	90	30.8° (*u*)
12/21/40	"	42	"	33	31°	40	
12/ 1/40	"	55	—	0	32°	50	32.0° (*u*)
1/ 1/41	14°	46	—	0	30°	70	30.5° (*u*)
1/ 6/41	"	51	—	0	31°	30	
1/ 8/41	"	52	26°	1	31°	100	31.7° (*u*)
1/10/41	"	54	"	1	32°	30	
1 15/41	"	57	"	3	31°	100	31.8° (*u*)
1/13/41	"	55	"	3	32°	40	
2/ 4/41	"	45	"	35	31°	100	31.7° (*u*)
2/ 2/41	"	45	"	33	32°	30	

tanks. Determinations of upper and lower 24-hour median tolerance limits were made with specimens taken directly from the first acclimatization temperatures, and with specimens which had been held for varying periods at the second temperatures. The experimental data and the estimated 24-hour median tolerance limits are presented in Table III. The tolerance limits are plotted against the duration of acclimatization to the second temperatures in Figures 3 and 4.

The difference between the upper limits of tolerance of specimens conditioned to 14° and to 26° was small (1.4°). With respect to the rate of change of heat-tolerance, the results obtained with Girella (Figure 3) are essentially in agreement with those which had been obtained with other species by other methods in earlier investigations. The 14°-conditioned fishes gained heat-tolerance rapidly after transfer to 26°. Acclimatization was apparently complete after about one day, the upper tolerance limit remaining constant thereafter.[10] On the other hand, the 26°-conditioned fishes showed no such rapid loss of heat-tolerance at 14°. Indeed, after one day they appeared to be somewhat more resistant than before, and this apparently increased resistance persisted for three days.[11] After 34 days at 14°, however, their resistance was considerably lower and did not differ significantly from that of fishes initially acclimatized to this temperature.

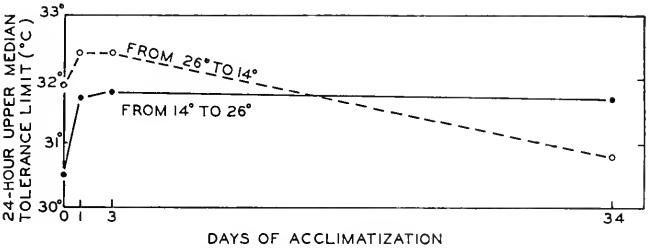

FIG. 3. Rate of change of heat-tolerance with acclimatization: The 24-hour upper median tolerance limits of Girella, initially acclimatized to 14° (solid circles) and to 26° (empty circles), in relation to the duration of acclimatization to new temperatures. The initial (first) and the new (second) acclimatization temperatures are indicated on each curve. Days of acclimatization (abscissas) refer to time at the second (new) temperatures only. The initial tolerance limit (0 days) of 26°-conditioned specimens is the mean of two determinations. Data from Table III.

The data on the rate of change of cold-tolerance are more detailed. The smooth curves which it was possible to fit to the experimental points (Figure 4) will be referred to as "acclimatization curves" of cold-tolerance. Although the curves are not identical in form, no very striking difference is to be noted between the rates of change of cold-tolerance following a corresponding rise and fall of temperature, such as was observed in the case of heat-tolerance. The tolerance limits of fishes transferred from 14° to 26° for acclimatization periods of 0.42 to 20 days may be shown to fall very nearly in a straight line when plotted

[10] Later experiments, which need not be presented in detail, indicated, however, that in two hours complete or even pronounced acclimatization did not occur.

[11] An indication of a similar slight gain of resistance was observed by Sumner and Doudoroff (1938) in Gillichthys. Its significance is too uncertain to warrant discussion.

against the logarithm of the duration of acclimatization. After 20 days at 26° complete acclimatization (i.e., a constant 24-hour lower median tolerance limit) was apparently achieved. The converse acclimatization curve of fishes transferred from 26° to 14° does not show the above logarithmic relationship. Acclimatization was somewhat slower during the first day, but, the curve being somewhat flatter, complete acclimatization again appears to be achieved in 20 days.

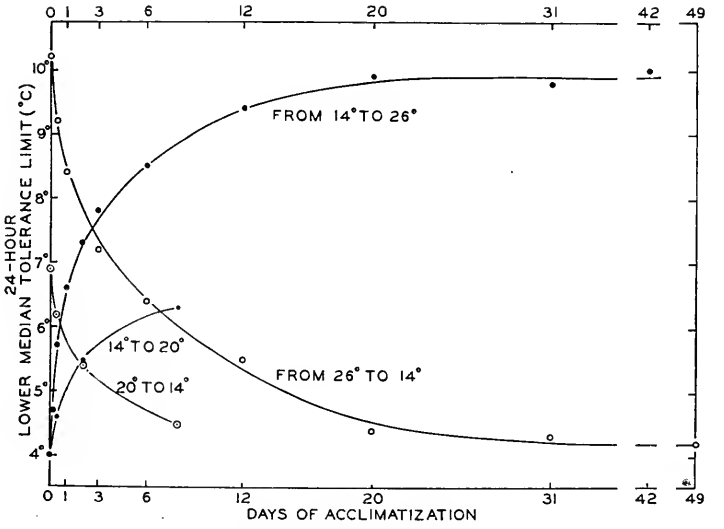

FIG. 4. Rate of change of cold-tolerance with acclimatization (acclimatization curves): The 24-hour lower median tolerance limits of Girella, initially acclimatized to 14° (solid circles), 20° (dotted circles) and 26° (empty circles) in relation to the duration of acclimatization to new temperatures. The initial (first) and the new (second) acclimatization temperatures are indicated on each curve. Points obtained with fishes transferred from 14° to 26° and to 20° are indicated by large and by small solid circles, respectively. Days of acclimatization (abscissas) refer to time at the second (new) temperatures only. The initial tolerance limit (0 days of acclimatization) is in each case a mean of two determinations. Data from Table III. The single determinations with fishes transferred for two days from 20° to 26° and vice versa are not plotted.

To what extent are the above acclimatization curves applicable to and descriptive of changes of cold-tolerance which result from smaller changes of temperature, such as may occur in nature? Although the available data for smaller changes (i.e., 14° to 20° and vice versa, etc.) are not complete, since complete acclimatization has not in any case been achieved, a satisfactory comparison can be made with corresponding portions of the curves obtained with larger temperature changes. The relationships can be illustrated by computing the fraction (per-

centage) of complete acclimatization achieved in each case after given periods (0.4, 2 and 8 days) of acclimatization to the second temperatures. The 24-hour lower median tolerance limits for fishes initially acclimatized to 26°, 20° and 14° and transferred directly to the test temperatures were 10.2°, 6.9° and 4.0° respectively. These values are means of the results of two experiments with each. They may, presumably, be taken to represent the tolerance limits after complete acclimatization. The percentages given in Table IV were calculated

TABLE IV

*Extent of acclimatization, expressed as per cent of total change of cold-tolerance, achieved in varying periods of acclimatization to new (second) temperatures after various changes of acclimatization temperature. (Values marked * were obtained by interpolation.)*

Acclim. temperatures		Per cent acclimatization achieved in:		
Initial (first)	New (second)	10 hours	2 days	8 days
14°	20°	21%	52%	79%
20°	14°	24%	52%	83%
14°	26°	27%	53%	79%*
26°	14°	16%	40%*	69%*
20°	26°		48%	
26°	20°		61%	

on this basis. The corresponding values in this table, that is, values for the same period of acclimatization, show no very striking differences and most show close agreement. In other words, the fraction of the total change of cold-tolerance (complete acclimatization) which occurs in any given time does not vary greatly with the direction or the magnitude of the total change. The somewhat low values obtained consistently with fishes transferred from 26° to 14° may be due to a certain amount of cold-injury, resulting from the sudden cooling to a temperature only slightly above their limit of tolerance. Such injury, while insufficient to cause visible distress, may account for the observed delay in the increase of resistance to further cooling. Gradual recovery, with adjustment to the new temperature, would account for the fact that complete acclimatization was not appreciably delayed, having been achieved in about 20 days, as in the case of the reverse change. Nevertheless, it appears that, at least for any moderate temperature change within the normal temperature range, the course of the acclimatization process (change of cold tolerance) can be described with reasonable accuracy as follows: about 50 per cent of the acclimatization

occurs in two days, and complete acclimatization is achieved in about 20 days, regardless of whether the temperature is raised or lowered.

This rate differs markedly from that reported by Mellanby (1939) for the change of chill-coma temperature in insects. This author usually obtained complete acclimatization in less than 20 hours, and at most in two or three days.

Discussion

It is uncertain to what extent temperatures may determine the distribution of species by virtue of their direct lethality. Non-lethal temperatures may be unsuitable for normal development, growth and activity, or for the activation of reproductive processes, or they may increase susceptibility to disease (e.g., see Staff, 1926). Furthermore, temperature may be a guiding stimulus in the movements of migratory fishes (Danois, 1924; Eggvin, 1937; Ward, 1921). Temperature selection by active fishes has also been demonstrated experimentally (Doudoroff, 1938), but the correspondence of these reactions to those under natural conditions is questionable. Since the distribution of many fishes may be confined to a narrower temperature range than that which is compatible with life, and they are not necessarily ever called upon to withstand temperatures near both their upper and lower limits of tolerance, it is interesting to compare the relative importance of lethal high and low temperatures as potential sources of danger to fishes in their natural habitats and, accordingly, as factors which may limit dispersal. While the opinion that fishes are more sensitive to warming than to cooling has sometimes been expressed or implied, it will be shown that injury by chilling deserves at least as much attention, as a limiting factor in the distribution of marine fishes, as heat-injury. This may not apply to fishes which are normally exposed in their habitat to temperatures at or near the freezing-point of their medium and are adapted to withstand them. Highest possible environmental temperatures are not so sharply defined, although in the open ocean 32° appears to be approximately the upper limit.

The destruction of marine fishes in large numbers in their natural habitat, apparently ascribable directly to unusual temperatures, has been reported repeatedly (Verrill, 1901; Storey and Gudger, 1936; and citations; Miller, 1940; Gunter, 1941). All such reports which have come to the writer's attention deal with destruction by extreme cold and not by heat. This circumstance already suggests a greater importance of lethal cold as a real hazard and as a factor limiting dispersal. Furthermore, of marine fishes which normally live at or near the surface, where temperatures are most variable, the more active and

sensitive ones may be able to escape abnormally high surface temperatures, but not low temperatures,[12] by means of a relatively short vertical migration.

In considering the experimental evidence the previous history of the experimental animals and the temperatures which are normal in their habitat must be taken into account. Maurel and Lagriffe (1899c) apparently failed to consider these factors, and hence their conclusions are of little value. Whether any given temperature should be regarded as more or less extreme than another depends upon its relationship to temperatures at which the fishes in question normally live. The mean surface temperature in the habitat from which the Girella were obtained is about 17°. The mid-value of the average yearly range (i.e., 13.3° to 22.0°), which for present purposes is also a significant point of reference, is 17.7°. Thus a temperature of 17.5° may be regarded as more or less typical of the habitat of young Girella in this locality. Specimens acclimatized to this temperature may be regarded as "normal," and temperatures equally removed from it as equally extreme or atypical. The limits of tolerance of normal Girella can be estimated by interpolation. Referring to Figure 5, the upper

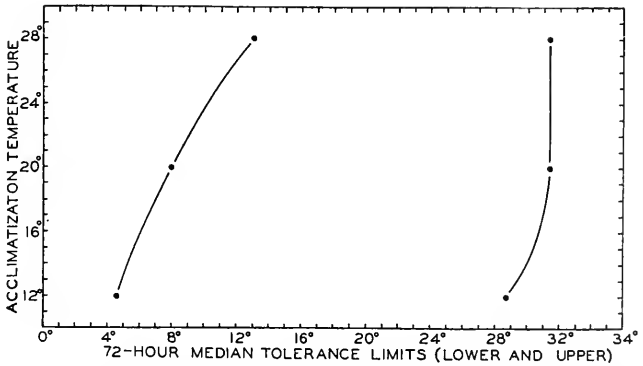

FIG 5. Relation between the temperature of previous acclimatization and the upper and lower 72-hour median tolerance limits. Curves fitted for approximate interpolation. Data from Tables I and II.

and lower median tolerance limits for 72 hours of exposure (the longest period for which complete data are available) corresponding to an acclimatization temperature of 17.5° are found to be about 31° and 6.8°, respectively. Thus the maximal rise of temperature tolerated for three days is by 2.8° greater than the maximal drop, and the lower limit of tolerance is, therefore, less extreme than the upper limit. With

[12] Unlike that of fresh water, the density of sea-water increases with decreasing temperature down to the freezing point.

longer periods of exposure the difference would be still greater. It seems safe to conclude that the fishes are no more resistant to cooling or to extreme low temperatures than they are to warming or to extreme high temperatures.

In nature fishes are not subject to such sudden changes of temperature as have just been considered. That changes of resistance to cold as well as to heat occur with gradual acclimatization has been amply demonstrated. In evaluating their significance, both their magnitude and their rate must be considered. The magnitude of the observed difference of tolerance resulting from a given difference between acclimatization temperatures was variable. The change of heat-tolerance was small in comparison with that of cold tolerance, especially because Girella, unlike other species which have been studied, showed no appreciable increase of heat-tolerance with acclimatization to temperatures above 20°. Possibly Girella could with gradual acclimatization survive cooling to as extreme a temperature as that to which it can be warmed. This, however, is uncertain, and the rise of temperature tolerated by specimens conditioned to the average yearly maximum (22°) is still greater than the drop tolerated by specimens acclimatized to the average yearly minimum (13.3°). This can be deduced from Figure 5. While few generalizations can be made it appears that the change of tolerance, both to heat and to cold, produced by a given change of acclimatization temperature is always considerably smaller than the latter change, and when the results obtained with long periods of exposure only are considered, it tends to decrease as the acclimatization temperature approaches the limit of tolerance.

The range of temperatures tolerated by "normal" Girella is greater than that to which fishes are exposed in the locality in which the specimens were taken. Therefore, the survival value of acclimatization is not obvious in this case. However, acclimatization may make possible the dispersal of a species over a wider range. Furthermore, much greater seasonal variations of surface temperature (i.e., as great as 20° to 28° C.) occur in some localities. Finally, the observed changes of resistance may be only more or less incidental manifestations of more fundamental adjustments of organisms to changes of environmental temperature, involving a general increase of vitality (resistance to disease, etc.) at non-lethal temperatures.

The physiological mechanism of the changes of resistance to heating and to chilling is not known. A change in the degree of saturation and, accordingly, of the melting and solidification points of protoplasmic lipoids has been suggested as a possible factor (Heilbrunn, 1937; Bělehrádek, 1935). The observations that in Girella a large change

of cold-tolerance could be obtained without a corresponding change of heat-tolerance, and that the rates of change of resistance to heat and to cold are quite different, indicate that these two changes are more or less independent of one another. Further investigation of the relative rates of physiological changes which occur with acclimatization (see also Sumner and Doudoroff, 1938) should prove to be of value in establishing which of these may be closely related and perhaps in revealing their mechanisms.

The ecological significance of the rate of acclimatization obviously depends upon the rate of fluctuation of environmental temperatures. In Girella even the relatively slow acclimatization to cold was complete after about 20 days. If this rate is typical, acclimatization should keep pace with seasonal temperature variations. However, large temperature fluctuations in marine environments resulting from the displacement of water masses, etc., may be quite rapid.[13] The gain of heat-tolerance of fishes apparently occurs very rapidly, while its loss on cooling is slow. The survival value of this relationship is obvious. Heat acclimatization will keep pace with a fairly rapid rise of temperature, and in an unstable (eurythermal) environment repeated brief sojourns at high temperatures may result in an increase of tolerance. On the other hand, acclimatization to cold in Girella was relatively slow, and the gain of cold-tolerance no more rapid than its loss. If this relationship is typical,[14] cold acclimatization should be relatively ineffective as a protective mechanism against occasional extreme temperatures in an environment in which fluctuations are rapid. These relationships can be advanced as further evidence that cold as a lethal agent may be more important as a factor limiting distribution than lethal heat.

The great sensitiveness of Girella and of other marine fishes to low temperatures or to cooling was further indicated by their reactions to temperature gradients (Doudoroff, 1938), in which the avoidance of low temperatures was much more pronounced than that of high temperatures. The temperatures most frequently selected by Girella which had been held at normal seasonal temperatures were 26° to 27°, i.e., much nearer their upper than their lower limits of tolerance. The fact that the reactions, just as heat-tolerance, were altered much more by acclimatization to very low temperatures than to high temperatures (Doudoroff, 1938, Figure 4) indicates a possible relationship between heat-resistance and selection in the gradient. However, the relative

[13] E.g., see Parr (1933), who reports changes of surface temperature in open water as great as 11° C. in two days.

[14] Some results of Sumner and Doudoroff (1938) indicate that it probably holds also for Gillichthys (see alternation experiments, loc. cit., pp. 421–422).

rates of acclimatization to higher and lower temperatures, as indicated by the reactions (Doudoroff, 1938, Figure 5), were more similar to the corresponding rates of change of cold-resistance than of heat-resistance.

Summary

The resistance of young *Girella nigricans* (Ayres) to low and to high temperatures and the changes of tolerance produced by acclimatization are examined in relation to temperatures in the natural habitat of the fishes.

The fishes were killed by moderately low temperatures well above 0° C., the lower limits of temperature tolerance being no more extreme, in comparison with normal environmental temperatures, than the corresponding upper limits.

The relationship between the time (duration) of exposure to the test temperatures and the highest or lowest temperatures tolerated by 50 per cent of the experimental animals (upper or lower "median tolerance limits") is representable by smooth time-temperature curves of tolerance.

Acclimatization to different temperatures has a pronounced influence upon subsequent resistance to cold (chilling), as well as to heat, and upon the form of the respective time-temperature curves of tolerance.

Experiments on the rate of acclimatization corroborate the conclusion, based on earlier observations, that heat-resistance in fishes is gained rapidly after a rise of temperature and lost slowly after cooling.

Acclimatization to cold (i.e., increase of resistance to chilling) is relatively slow, and cold-resistance is lost no more slowly than it is acquired. After any given rise or fall of environmental temperature within the normal range, about 50 per cent of the total resulting change of cold-tolerance occurs in two days, and complete acclimatization (i.e., constant cold-tolerance) is apparently achieved in about twenty days.

Injury by chilling is no less important as a possible limiting factor in the distribution of marine fishes than heat-injury.

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SOME EFFECTS OF HOMOTYPIC EXTRACTS ON THE RATE OF CLEAVAGE OF ARBACIA EGGS

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In a series of papers in 1937, Allee and Evans reported that under a variety of experimental conditions eggs of the purple sea urchin, *Arbacia punctulata*, show an accelerated cleavage rate when in relatively dense, as contrasted with relatively sparse, populations. Frank and Kurepina (1930) had obtained similar indications with a sea urchin from the Murmansk coast of Russia, and Maxia (1933), with *Paracentrotus lividus*, when tested at or near the beginning of swimming, had found most rapid development with 40 to 65 eggs in seven drops of sea water. These European workers interpreted the observed results in terms of mitogenetic rays.

Sugiyama (1938), with eggs of still other sea urchins, *Anthocidaris crassipina*, *Pseudocentrotus depressus*, and *Strongylocentrotus pulcherrimus*, also found an optimum density for most rapid cleavage. In his work this optimum came with 20 to 30 eggs in five to ten drops of sea water. He does not record his method of testing for causal factors but he does say that mitogenetic rays are not involved.

This paper deals with a number of possible underlying causes for the acceleration of early cleavage in Arbacia eggs; in this connection the role, conjectural at best, of mitogenetic rays has been ignored. In a preliminary survey, Allee and Evans (1937*b*) found that the observed acceleration of cleavage rate in the denser populations of Arbacia eggs was not to be ascribed to differential temperatures externally imposed, nor to differential hyper- or hypotonicity, nor to contamination with coelomic fluid or fragmented eggs. Further, while the denser populations lowered the pH significantly, an indication of a possible role of carbon dioxide, appropriate tests with varying amounts of carbon dioxide up to that sufficient to produce the same pH observed in dense populations, had no stimulating effects on the rate of cleavage. In this connection Smith and Clowes (1924) and Haywood and Root (1930) found that slight increases in carbon dioxide tension retarded cleavage

[1] The first two authors were actively engaged in these and related experiments for three summers or more; the last three each worked for a single season.

of Arbacia eggs. Cleavage was also retarded rather than accelerated by placing fertilized eggs which had been well washed, in water in which other eggs had stood, whether these were unfertilized, recently fertilized, or undergoing cleavage. This last set of results taken with those of Springer (1922) and Peebles (1929), indicates that the presence of accelerating substances or conditions shown by the more rapid cleavage in the denser populations is masked or inhibited when the entire conditioned medium is present but the developing eggs are lacking.

A series of experiments was carried out at the Marine Biological Laboratory at Woods Hole during the summers of 1939, 1940, and 1941 in an effort to examine this problem of possible cleavage-promoting substances, and to this end a number of extracts of developing Arbacia eggs were prepared.

Preliminary Extracts

Peebles (1929) made an extract of Arbacia plutei by putting them in 90 per cent alcohol or 50 per cent acetone; "then the mixture without grinding the plutei was evaporated to dryness, and to a given quantity sea water was added" (p. 183). Such preparations retarded the growth of plutei. "If however, the fats are removed from the acetone extract, and a water solution made from the residue there is evidence that the inhibiting effect is removed."

We have made acetone extracts as follows: Eggs were washed four times, fertilized, allowed to pass third cleavage, and centrifuged lightly to concentrate. The eggs were shaken up in 50 per cent reagent grade acetone and let stand in different preparations from one to 22 hours. They were then centrifuged and the supernatant acetone was removed. In one preparation 0.7 cc. of eggs was treated with 10 cc. of 50 per cent acetone. For this extract an extract-control was made by adding 10 cc. of such acetone to 0.2 cc. of sea-water; the resulting solution was treated as was the egg extract. In both, one fraction was dried to constant weight immediately and the other after having been treated with ether to remove the ether soluble materials was similarly dried. Each fraction was taken up in sea water before being used in the experiments.

The procedure followed in assaying the extract was essentially the same as that used by Allee and Evans (1937*b*). With appropriate modifications of which the important ones will be mentioned later the same general technique was used in all the assays reported in this paper. All experiments were carried on in a room with north exposure; the windows were closed to avoid excess evaporation and sudden

changes in temperature. The customary precautions regarding the handling of the sea urchins, their sexual products, and the glassware were scrupulously observed. As a matter of course, within any experiment, all the eggs came from one female. Freshly filtered sea water was used throughout. The eggs were fertilized in about 150 cc. of sea water in a finger-bowl. After five minutes, portions of the fertilized stock were transferred by means of a dropping pipette to Syracuse watch dishes in which extracts of the appropriate dilutions had been previously placed. Adjacent dishes contained the extract or the sea-water controls. After three minutes in the Syracuse dishes, the eggs were transferred, in mildly sparse suspensions, to small shell vials by means of a haemocytometer pipette. These vials were placed in rows and columns in a brass holder. When the transfer was complete, each vial contained 20 cu. mm. of sea water, treated or untreated, plus the eggs contained in this volume. The time occupied by the transfer from Syracuse dishes to the vials ranged from 8.5 to 17.3 minutes, and averaged 11.7 minutes; the entire period from fertilization to completion of transfers averaged 19.6 minutes. In most experiments 48 vials were used.

Precautions were taken in the design of the experimental set-up to prevent prejudice in favor of either the control or the extract insofar as position in the holder or, more important, the order in which the eggs were transferred to the vials was concerned. The metal holder containing the vials was placed in a moist chamber which in turn was placed in a cold cabinet where the temperature was kept at 19° C. in order to retard the progress of cleavage so that the eggs might be in contact with the extract for a longer time.

Rows of eggs were killed during the progress of second cleavage by the addition to the vials of one per cent formalin in sea water at 0.5, 1, 1.5, or 2 minute intervals, depending on the progress of cleavage as observed through a binocular microscope. Great care was taken by means of elaborate washing procedures in cleaning out all possible traces of formalin from the vials before they were used again in an experiment. Allee and Evans (1937*a*) showed that the presence of traces of toxic material produced retardation of cleavage in sparse populations of eggs as contrasted with a reduced effect with denser populations. In the present experiments comparable numbers of eggs were used in experimental and control suspensions and in any event throughout all experiments the presence of toxic material, other than that which may have been an intrinsic part of the accelerating extract itself, was scrupulously avoided.

After this serial killing of all the rows in a given experiment, vials of eggs were washed successively into large depression slides and the eggs were counted under the low power of a compound microscope for one-, two-, and four-celled stages. Throughout the work results were not used when the cleavage fell below 90 per cent; usually it was 95 per cent or more.

Twelve experiments were made with a series of different solutions of the acetone extracts. When the results at or near 50 per cent second cleavage were studied, six per cent more eggs had cleaved in the whole acetone extract than in the accompanying sea water controls. This mean apparent acceleration, when examined for statistical significance by "Student"'s method for paired comparisons, had a P-value of 0.0984.[2] The fat-free portion gave a mean apparent acceleration of 12.1 per cent, but with much wider variation, and the P-value was 0.0941. Since P was greater than 0.05 in both instances, the seeming accelerations lacked statistical validity. When the rate of cleavage of eggs in the acetone extracts was compared with that of those in the extract-controls, the apparent acceleration of 8.3 per cent also lacked statistical significance with $P = 0.1096$. It is evident, then, that such extracts failed to stimulate the rate of cleavage.

Another extract was more elaborately prepared (Figure 1), and the potency of various fractions tested. The extract was made from a large number of eggs which had passed third cleavage. These were centrifuged down to 1.2 cc. and were ground with clean, ignited sea-sand and distilled water; they were again centrifuged to throw down the sand and the larger particles. The resulting red, watery, supernatant liquid was divided into three fractions: A, B, and C. Fraction A was treated with three times its volume of 99.5 per cent acetone and again centrifuged; the supernatant portion was evaporated to dryness at low temperature and was soon thereafter taken up in 10 cc. sea water and tested for its effect on the rate of cleavage in various dilutions. The precipitate was washed twice with distilled water, twice with absolute ethyl alcohol; the precipitate was mixed with 5 cc. ethyl alcohol and the suspension was divided. Half the material was evaporated to dryness in a desiccator, and the dried material was taken up in 10 cc. 0.05 N NaOH at room temperature for eight hours, and then stored in the refrigerator. The other half was washed with ether, dried, washed with 10 cc. distilled water, and then taken up in 10 cc. 0.01 N

[2] Statistical significance is given by the P-value which is calculated by the method of "Student" (1925). This method is designed for the analysis of situations where a small number of cases is involved. A P-value under 0.05 is customarily considered as indicating statistical significance; it is roughly equivalent to two times the standard error.

NaOH and stored in the refrigerator. Both of these fractions were assayed promptly in various concentrations against sea water as well as against comparable NaOH controls.

Fraction *B* had 2.5 times its volume of 95 per cent ethyl alcohol added. After centrifuging, the supernatant liquid was stored in the icebox (for two weeks) after which it was dried in a desiccator, and was later taken up in sea water for assay; this fraction, called by us "*Ba*," will be reported on at length later. The precipitate from fraction *B* was treated essentially as was the precipitate from fraction *A* except

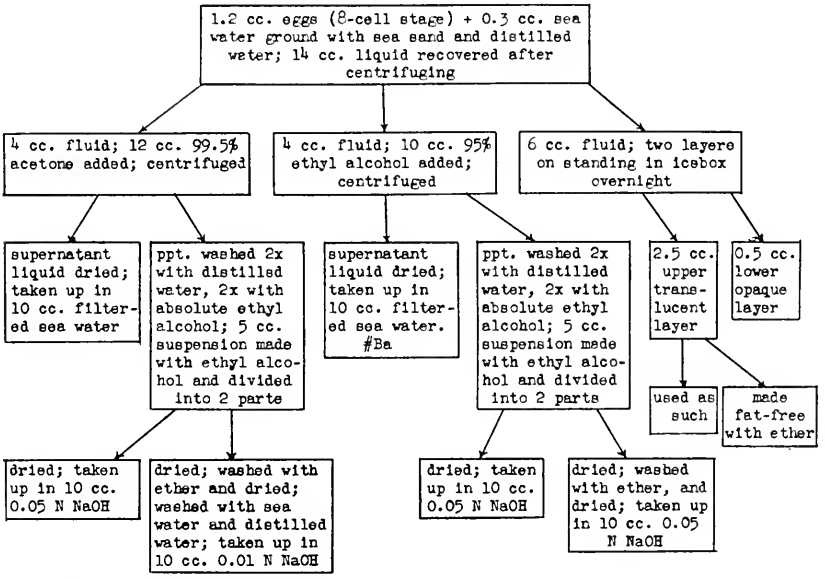

FIG. 1. Fractionation of extracts of Arbacia eggs. This paper is largely based on work with extract No. *Ba*. The other fractions failed to show acceleration; see text.

that this time both sub-fractions were taken up in 0.05 N NaOH for assay.

Fraction *C* of the original extract separated into two layers on standing in the icebox overnight. The upper translucent layer was (1) tested as such, and (2) was made fat-free with ether and tested. The lower, opaque layer was diluted in sea water and when tested produced a marked retardation.

The only one of these sub-fractions which gave an indication of producing fairly steady acceleration of cleavage when tested against appropriate controls was the alcohol extract called "*Ba*"; further experimentation centered on this extract.

Alcohol extract

Our problem was not concerned with assaying the effects of various homotypic extracts upon the rate of cleavage of Arbacia eggs; rather it was to find some possible factors which might produce the observed acceleration when populations of sea urchin eggs are relatively crowded as compared with well-scattered populations. We therefore felt justified in focussing attention on that part of the extract which produced acceleration most regularly in the early assays.

The first batches of this extract were made according to the directions given in the preceding pages. Later, a simplified procedure was used. The eggs were prepared for extraction as usual and then a small amount of distilled water was added; sand was no longer used in view of the experience of Grave (1935). The mixture was allowed to stand with occasional stirring for about an hour; by this time the eggs were fairly well broken. Then 95 per cent or absolute ethyl alcohol was added, and after a few minutes the mixture was centrifuged and the supernatant liquid was decanted into a crucible for drying to constant weight, which was accomplished by gentle heat, 40° C. or less.

Small amounts of the dried extract were taken up in sea water to make stock solutions which were stored in the refrigerator. Experience soon showed that even a well-chilled stock solution changed in potency after relatively few hours, and that consistent results could not be expected by the third day. Successive hundredfold dilutions were made from a stock solution, and from each of these dilutions 1.0 cc. was added to a measured amount of sea water which had been placed in a Syracuse dish, and to which was then added a measured amount of suspension of fertilized eggs. In different experiments the latter quantity varied from one to 3 cc.; the total amount in the Syracuse dish ranged from 7 to 10 cc. That portion of the extract which proved to be insoluble was subsequently recovered from the original stock, and was washed, dried and weighed to determine the amount which had presumably gone into solution. The average amount of the dissolved material was .125 mgm. per cc. in the original stock solution. The range of the assay concentrations for the three years was: 8.4×10^{-5} to 8.4×10^{-14} mgm. per cc. Since the spacing of the concentrations within these limits varied somewhat, and since the exact values of dissolved portions were not determined for all the batches of extracts used, the concentrations given in Table I are approximate only and serve to show orders of magnitude rather than precise quantities.

The method of assay during 1939 and a part of 1940 has already been described. For the remainder of the tests cleavage was followed in Syracuse dishes rather than in small vials. The former were set on

a clean towel on the laboratory bench in two or three rows of five each: one row for the experimental extracts and the others for the controls. The latter contained the same amount of sea water and eggs as did the experimental dishes. One control always contained untreated sea water; the other, when present, was a check on the possibility that some foreign material in the solvent, or traces of the solvent itself, might be causing the observed accelerations. This latter control was prepared by making use of the same quantities of all the ingredients and the same treatments used in making the regular alcohol extracts with the important exception that no eggs were used. It was, in fact, an alcohol extract of approximately 0.3 cc. of sea water, plus whatever residues might have been left from treatment with double-distilled water and the laboratory alcohol. An analysis of all the data collected on this point showed no significant difference in the rate of cleavage in the two types of controls.

TABLE I

Summary of analysis of assays of the alcohol extract during the summers of 1939, 1940 and 1941

Approximate concentrations of extract in mgm. per cc.	Mean difference in per cent cleaved between extract and sea-water control at or near 50 per cent second cleavage	Number of experiments	*P* values
8.4×10^{-5}, 10^{-6}	7.56	48	0.0007
8.4×10^{-7}, 10^{-8}	6.1	43	0.0093
8.4×10^{-9}, 10^{-10}	4.97	43	0.0029
8.4×10^{-11}	5.04	32	0.0357
8.4×10^{-13}, 10^{-14}	−1.34	23	not significant

In the more recent assays, the eggs were fertilized as usual in a finger bowl. After five minutes, 3 cc. of egg suspension were added simultaneously to an experimental dish and to its controls. The whole process of distributing eggs into the Syracuse dishes took about half a minute. The eggs were then undisturbed except for occasional examination under a binocular microscope until the time of second cleavage, 65 to 85 minutes later.

Sample lots of the cleaving eggs were killed several times during second cleavage by dropping a few drops of each egg suspension into separate vials which already contained a small amount of 2 per cent formalin in sea water. This method of killing requires due care lest the pipettes become contaminated. The formalin stopped development immediately and the percentage of cleavage at the time of killing was determined by sample counts made during the subsequent few hours.

The results of a typical experiment from 1939 are illustrated in Figure 2. The solid lines show the ascertained segment of the cleavage

curve for two concentrations of the extract: 5×10^{-5} mgm. per cc. (triangles) and 5×10^{-6} mgm. per cc. (squares). The broken lines show the appropriate controls in sea water and their mean. Cleavage in this experiment was 96 per cent. The mean difference in percentage cleaved when the accelerated experimentals were at mid-cleavage was 21 per cent.

During the 1939 season this extract was tested in dilutions that ranged from 5×10^{-5} to 5×10^{-10} mgm. per cc. Acceleration was

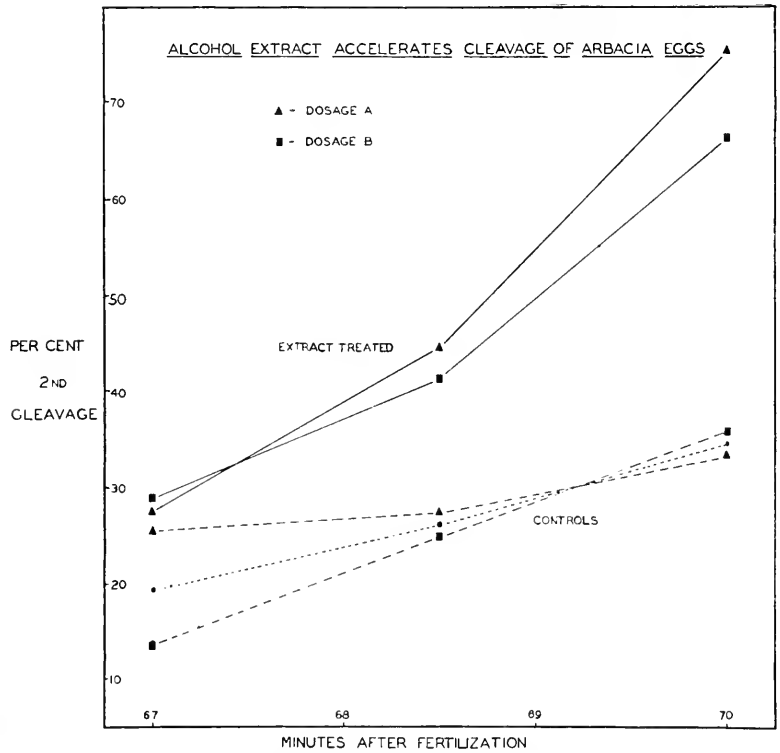

FIG. 2. Acceleration of second cleavage of Arbacia eggs by treatment with homotypic extracts. See text for details.

most effective in the relatively greater concentrations although dilutions as great as 5×10^{-9} mgm. per cc. seemed to show an accelerative influence. In the more effective dosages, in the dilutions just noted, in 21 paired experiments, 99 paired comparisons, of which six are shown in Figure 2, indicated that the treated eggs were ahead of their controls. The differences between the percentages of the eggs which achieved second cleavage averaged 10.59. An analysis for the 99 pairs gave a P-value of less than 0.0000001, a highly significant figure.

These data were further analyzed by another method, one of which was used in the previous work by Allee and Evans. In each experiment the percentage of cleavage nearest the 50 per cent mark for the treated eggs was compared with the value for the corresponding untreated eggs; again, the value nearest 50 per cent for the controls was compared with that for their comparable experimental eggs. The average of these two differences was used in the analysis by paired experiments. The formula, then, for the value of the difference is

$$\frac{(50\%\ \text{Exp.—its Control}) + (50\%\ \text{Control—its Exp.})}{2}.$$

Twenty-one such paired experiments of mid-cleavage values showed that the mean percentage difference between extract-treated eggs and their controls was 10.36, with a significant *P*-value of 0.0025.

Although the results in 1939 seemed fairly conclusive and were made the basis for a preliminary report (Allee and Finkel, 1939), we spent parts of two more summers in further work on the subject, and have made some refinement in procedure. Thus, for 1941, cleavage curves were constructed from the data. Where possible, percentage differences were read from the 50 per cent point of second cleavage on the control line vertically to the experimental line, and similarly from the 50 per cent point on the latter line vertically to the control line. The mean of these values was taken to indicate percentage difference at 50 per cent cleavage.

All available results for the three seasons are summarized briefly in Tables I and II. Table I shows mean acceleration for different levels

TABLE II

Comparison of treatments by summing all concentrations of alcohol extracts

Comparison	Mean difference in per cent	*P* values
Extract *vs.* sea-water control	5.952	0.0010
Extract *vs.* extract control	8.218	0.0008
Extract control *vs.* sea-water control	−2.605	0.1050

of dilution of the extract. It will be noted that there is the largest mean difference at the strongest concentrations of the extract and that there is also the greatest statistical significance for these results. Also, in general there is a suggestion of a dilution effect which becomes evident in the weakest concentrations. The differences, while small, are highly significant statistically for the three stronger concentrations. Should we, therefore, have tried still stronger concentrations? The indications are that we should not. The strongest evidence comes from the work of 1940 when the greatest percentage difference (5.8) was found with those concentrations shown in the second and third lines of

Table I, and the greatest statistical significance ($P = 0.0176$) was shown with the concentrations summarized in the third line.

Since the mean percentage difference in acceleration shown by the combined data for the three summers' work varies relatively little between extract strengths of 8.4×10^{-5} and 8.4×10^{-11} mgm. per cc., it is interesting and probably fair to combine all these data and examine their statistical significance. These results are given in Table II. The mean acceleration shown by the eggs in the extracts as compared with others in the sea-water controls is seen to be a trifle under 6 per cent with good statistical likelihood of being significant. Further, the acceleration is over 8 per cent when the extract-treated eggs are compared with others in accompanying extract-controls, with still better statistical significance. The slight retarding tendency of the extract-controls does not approach statistical validity when compared with the results in sea water.

For some purposes it is interesting to examine the results in a different way. If one studies the cleavage curves for the different concentrations of the 59 experiments which met our standards, there are in all 356 parts of these experiments in which it is possible to get valid comparisons between the trends of the partial cleavage curves for experimental and control eggs. Many of these fragments of cleavage curves were too far removed from the 50 per cent level to be in the comparisons given in Table I. Of these 356 cleavage curves, 63.2 per cent showed acceleration of eggs in the extract as compared with those in the accompanying sea-water controls. This percentage is about the same for each of the three summers during which these experiments were made, and for the extracts prepared by each of the three workers who attempted to make them. This percentage of positive experiments is not out of line with the mean acceleration and the statistical significance which we have observed.

Echinochrome

The alcohol extract with which acceleration was obtained was red in color; it had about the same color as that shown by a concentrated mass of eggs. This coloring matter in sea urchin eggs was found by McClendon (1912) to be identical with the previously described pigment echinochrome. Since some echinochrome was obviously present in our extracts, it seemed advisable, during the summer of 1941, to determine just how much was present. It was found that about half the echinochrome in the eggs was carried into the alcohol extracts, and that these extracts when dry were about 25 per cent echinochrome. The next step was to purify some echinochrome and determine whether it had

any effect on the rate of development. The method of purification was essentially that of Ball (1934) and was done by repeated precipitation by ammonium hydroxide from an acid alcohol solution. The final alcohol solution was evaporated and extracted with distilled water and petroleum ether. It was then dried to constant weight before solution in sea water for use in the experiments. A total of 16 experiments conducted in the same manner as those with the alcohol extracts failed to show either acceleration or inhibition by echinochrome in a wide range of concentrations, from approximately 5×10^{-3} mgm. per cc. to 5×10^{-11} mgm. per cc. Hence, the accelerating effects of the alcohol extracts are not a result of the echinochrome they contain.

Discussion

It should be remembered that the present paper records the results of experiments which were made in an attempt to analyze for possible causal factors that produce more rapid cleavage in dense populations of Arbacia eggs as compared with those in sparse populations. It has already been recalled that earlier work (Allee and Evans, 1937*a*, *b*) showed that neither externally imposed temperature nor effects of hyper- or hypotonicity are involved. All of the work of the senior author and his associates supports the statements in the literature that contamination with coelomic fluid, the presence of numbers of broken eggs, the use of water in which unfertilized eggs have stood ("egg water"), or in which fertilized eggs have cleaved ("cleavage water"), and the presence of even slight increases in CO_2 tension, all retard rather than accelerate cleavage of Arbacia eggs introduced into such conditions. Evidently the explanation of the increased rate of cleavage in denser populations is not to be in terms of the action of such contaminations. Neither is there any indication that this density effect can be interpreted in terms of reduced calcium in the sea water, a condition which has been shown to accelerate Arbacia cleavage (Shapiro, 1941).

The amount of acceleration for which explanation is needed was shown by Allee and Evans (1937*a*, p. 224) to average about three minutes at 50 per cent second cleavage in one extended set of experiments in which the conditions were not quite comparable with those used in the present work. Under the conditions then tested the sparse populations of eggs reached the mid-point of second cleavage in 97.67 minutes while the denser populations took 94.72 minutes. This difference of 2.95 minutes is statistically significant ($P = 0.0016$) and represents an acceleration of 3.2 per cent on the horizontal axis of the cleavage curve. The maximum acceleration observed in the work which was reported

in 1937 was on the order of ten minutes in 90 minutes elapsed time after fertilization. In another set of experiments in which the assays were made by counting eggs killed at spaced intervals, as in the present experiments, the mean difference in per cent cleaved at approximately the mid-point of second cleavage was 18 per cent ($P < 0.00001$) (Allee and Evans, 1937*b*, p. 19). By interpolation with tests of the action of copper, to be discussed a little later, in which there was a difference, at the middle of second cleavage, of 18.6 per cent and for which cleavage curves showed a mean difference of 60.8 seconds, we can assume that the time difference observed by Allee and Evans was of the same order. Hence we have to find some accelerating mechanism which will account for a hastening of at least one minute at 50 per cent second cleavage or of at least 18 per cent difference in percentage cleaved, since this was the amount observed in experiments similar to those in which cleavage was hastened by the presence of relatively dense populations of cleaving eggs.

The mean increase in rate of cleavage to the same end point which was produced by the alcohol extract, with which we have been mainly concerned in the present work, was between 6 and 8 per cent (Table II); this means that the mean difference in elapsed time was approximately 20 to 25 seconds at the middle of second cleavage. The extract then produces an acceleration which is 30 to 45 per cent of that observed in the most nearly comparable basic dense-sparse experiments. We have no ground for assuming, however, that any part of the acceleration in the basic experiments was in fact a result of the action of a substance comparable to our extract. The experiments do show that there is in fertilized eggs a readily prepared substance, or substances, which, under appropriate conditions, will produce at least one-third of the increase in the rate of cleavage as does optimal crowding.

While the differences we have observed between extract-treated and control groups of eggs are small, they are highly significant statistically. This significance is primarily a consequence of a fair degree of consistency of the values of these differences throughout a large number of experiments. As might be expected under the circumstances, not all of the experiments yielded positive results, and there were even some runs of experiments which, when averaged together, show no statistically suggestive acceleration. This means either that the accelerating principle in our extract is not very potent or that its activity has been offset in part by the presence of contaminants. The whole situation is complicated by the known variability of Arbacia eggs both as to season and from female to female. We know that the extract as

used is a mixture of unidentified parts and our data give no indication of its potency if purified.

In another series of experiments (Allee, Finkel, and Garner, 1941) it was found that copper, added as $CuCl_2$ to sea water, produced at the optimum concentration of 10^{-13} M a mean acceleration at 50 per cent second cleavage of 69.4 seconds ($P = 0.0004$). In order to check the possibility that copper as a contaminant may have been responsible for the accelerating effects of the extracts, these were analyzed for the presence of copper. The analysis was done by the standard method of colorimetric determination with sodium diethyl-dithio-carbamate. The details of the analysis are described in another paper in greater detail (Finkel, Allee, and Garner, 1942). Suffice it to say at this point that the extracts analyzed showed a mean copper content of 11.29×10^{-6} mgm. per mgm. of dried extract material. Since our records show that an average of 10.68 mgm. of extract, soluble and insoluble, was taken up in the basic stock solutions, such stocks of 10 cc. would accordingly contain 12.05×10^{-6} gm. of copper per liter; i.e., each stock would be about 1.9×10^{-7} molar. Now the experiments with the effect of copper on the rate of cleavage of Arbacia eggs indicated that copper was largely ineffective beyond an assay concentration of 10^{-13} M. This would mean that in our extracts any effective copper would have been diluted out beyond the extract concentrations of 8.4×10^{-8} mgm. extract per cc. Since Table I shows that effective acceleration of cleavage was obtained with dilutions of extract beyond this point, the active principle in the extract, it can be said fairly safely, is something other than mere copper contamination.

There is another factor which is frequently suggested as being possibly responsible for the increased rate of cleavage in dense as contrasted with sparse populations of eggs: this is the matter of a temperature differential internally imposed. Allee and Evans (1937*b*) briefly discussed this possibility and decided that it was probably not important since the dense-sparse differential is as large when populations of the same density are contrasted whether they are in 20 cu. mm. or in 220 cu. mm. of sea water, and while the difference was somewhat reduced in 2020 cu. mm., those in the denser populations still cleaved significantly faster than did the accompanying sparse populations.

If we had data from the smallest drops only, and if we made the assumption, contrary to fact, that glass conducts no heat and that no heat is lost to the air, then basing our calculations on the observations of Rogers and Cole (1925), and with the additional assumption that the specific heat of sea water plus about 5000 eggs is unity, we can account for about 30 seconds acceleration by the middle of second

cleavage. This is approximately one-half the observed difference in comparable experiments (Allee and Evans, 1937*b*). When, however, increasing the volume 11 times does not alter the time relations between dense and sparse cleavage rates, and when there is still a significant difference in 2020 cu. mm., there is no real reason for considering that the heat produced within the cleaving population is actually an important factor in speeding the cleavage in the denser populations.

The present work might be considered an extension of the field of population physiology to the field of early echinoderm development. It is, however, difficult to conceive of Arbacia development at the early stages of cleavage in terms of utilization of nutrient materials. Indeed, what we are dealing with here is essentially a unique type of population: one in which the factor of nutrition in the external environment is absent. And inasmuch as this factor of food is not involved, the environment of an experimental study of a population of this kind is more readily controlled, at least in this one respect.

From another point of view, the present work is part of a more general program of investigation, for some time under way at the University of Chicago, dealing with the phenomena of the effect of population density upon various biological processes, both in nature and in the laboratory. The demonstration of the existence of optimum densities for various processes and organisms, widely spread throughout the living world, has indicated that undercrowding is as real in its effects as is overcrowding. The present work is an extension of an attempt to gain a better understanding of the complexities of one of these situations: the population aspect of Arbacia egg cleavage. The more general implications of this work are discussed by the senior author in his "Animal Aggregations" (1931) and more recently in his 1938 book on "The Social Life of Animals."

Summary

1. Fertilized Arbacia eggs contain a readily extracted substance (or substances) which, in our tests, accelerated the rate of cleavage of such eggs to a statistically significant degree.

2. The acceleration produced by such an extract is not a result of the echinochrome it may contain nor of possible copper contamination.

3. The results are discussed against a background of population physiology, and the problems of over- and undercrowding of organisms.

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A TIME STUDY OF EVENTS IN THE LIFE SPAN OF DAPHNIA MAGNA[1]

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Cladocera have been used by many biologists as experimental animals in various types of investigations. Their use is advantageous in many respects. They are small, have a relatively short life span, and are easy to culture. They mature early and reproduce rapidly so that they are readily available in large numbers. Since reproduction can be limited to the production of females by diploid parthenogenesis, genetic constancy can be maintained. Their size and transparency allow microscopic observation of the activity of many organs and systems without resorting to operative techniques.

In spite of the fact that Cladocera have been widely used, many workers have paid little or no attention to the past history of the individual experimental animals and the stage of the life history in which they have been employed. The past history of the individual is of considerable importance in determining the results of experiments inasmuch as Ingle, Wood, and Banta (1937) have shown that well fed animals grow more rapidly, reproduce in greater numbers, and have a higher heart beat frequency than those reared on limited food. The stage in the life history is significant since heart beat frequency, oxygen consumption, and resistance to toxic materials vary with the age of the animal (Ingle, Wood, and Banta, 1937; Obreshkove, 1930; Terao, 1931; MacArthur and Baillie, 1929b; Terao and Tanaka, 1929 and 1930; and Breukelman, 1932). The time within the instar is not to be ignored since a new layer of chitin forms during the latter part of each instar (Anderson and Brown, 1930) and this may modify rates of permeability of various substances. Again their reactions may vary depending on the time within the instar although we do not know of any studies with reference to this particular point. The matter of sex should not be overlooked. Males are more susceptible to poisons than females (Breukelman, 1932). In general the metabolic activity of the males is higher than that for females (MacArthur and Baillie, 1929a). Again members of different clones show striking physiological differ-

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ences (Obreshkove and Banta, 1930, and Banta, 1939). These considerations show that in order to secure reproducible results the individual experimental animals must be reared under adequately controlled conditions and selected with respect to age, time within the instar, sex, and clone.

Individual rearing of experimental animals offers a means whereby a complete check may be made on age, time within the instar, and their general physiological condition. For animals from mass cultures as have been used by many investigators this is only partly possible. Time studies of the events in the life span of several varieties of only one species, *Daphnia longispina*, have been made (Ingle, Wood, and Banta, 1937, and Banta, 1939). No such data are available for the most used species, *Daphnia magna*. We have, therefore, undertaken this time study of events in the life span of individually reared female *Daphnia magna* together with analyses of criteria for determining their physiological condition at the various stages in their life history.

Materials and Methods

A first consideration was culture medium. Numerous media have been employed by various investigators. One of the simplest is the manure-soil medium of Banta (1921a). We have used a modification of this with considerable success for over a decade. Air dried horse manure was used instead of fresh as stipulated by Banta. This together with soil was allowed to stand in tap water for two days after which the infusion was filtered through fine silk bolting cloth. Before using, the filtrate was permitted to stand until the turbidity disappeared, which usually occurred within two days. Medium over five days old from the time of filtering was never used.

Within an hour after their release from the brood chambers of the mothers individual females were placed in vials of 20 to 25 cubic centimeters capacity. The medium was changed at the end of the second day. On the fourth day the animals were transferred to larger vials containing 50 to 60 cubic centimeters of culture medium and every third day thereafter they were transferred to vials containing fresh medium. The smaller vials were used during the first four days merely to facilitate finding the small cast carapaces of the early instars.

Another consideration was temperature. Twenty-five degrees Centigrade was decided upon since it is easily maintained throughout most of the year with the simplest of constant temperature equipment. It is well under the lethal temperature for *Daphnia magna* (Brown, 1929) and also below the highest temperature at which normal life functions occur (MacArthur and Baillie, 1929a).

Observations were made hourly from 8 A.M. to 11 P.M. each day from the time the animals passed as eggs into the brood chamber of the mother until about five-sixths of the animals had died. Daily observations were then made until all were dead. Records were kept of the time each individual passed as an egg into the brood chamber of the mother, the time of release from the brood chamber as a free living young, the time of each ecdysis, the time of release of its young, and the number of young produced in each brood.

Number of Pre-adult Instars

One hundred fifty-nine female *Daphnia magna* Straus were observed from the time of their passage as eggs into the brood chambers of their mothers throughout their whole life span. Of these, 48 were primiparous during the fifth instar, 86 during the sixth, and 24 in the seventh, while one died in the fourth. The number of pre-adult instars, therefore, varied from four to six. This is the first time that less than five pre-adult instars have been reported for this species.[3] The variation in the number of pre-adult instars might conceivably be due to either or both hereditary and environmental factors. Inasmuch as the animals were all of one clone they were genetically identical, but mutations do arise occasionally (Banta, 1939) and this might account for the variation. However, this seems unlikely since individuals from the same brood varied. On the other hand differences in the culture medium may have been the cause. Twenty animals were set out each day over a period of eight days simply as a matter of convenience. As a consequence the animals were started out on three different batches of culture medium. We found that a particular number of pre-adult instars predominated for the animals set out on any one day. For these reasons we believe that the variation in the number of pre-adult instars was due to environmental factors.

In order to facilitate further discussion of our results the animals have been divided into three groups on the basis of the number of pre-adult instars.

Longevity

Figure 1 shows the survival curves for the animals observed. The animals primiparous in the fifth instar had a mean life span of 944 $\pm$ 32 hours ($\bar{x} \pm \sigma_{\bar{x}}$); those primiparous in the sixth, 966 $\pm$ 26 hours; and those primiparous in the seventh, 990 $\pm$ 43 hours. In terms of instars, they lived 16.5 $\pm$ 0.4; 17.2 $\pm$ 0.4; and 18.3 $\pm$ 0.6, respectively.

[3] Anderson (1932) erroneously interpreted Rammner (1930) as having found eggs produced during the fifth instar.

The differences between any two groups either in terms of hours or instars are not statistically significant. However, it is interesting to note that the average number of adult instars is the same for each group and the differences in longevity correspond to the differences in the accumulated time at the end of the pre-adult instar period.

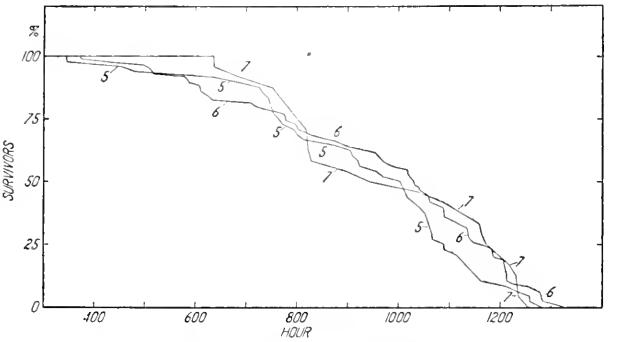

FIG. 1. Survival curves for female *Daphnia magna* primiparous during the fifth, sixth, and seventh instars as indicated by the numerals.

MacArthur and Baillie (1929a) in their experiments on the effect of temperature on longevity found that female *Daphnia magna* lived 29.24 ± 0.44 days (701.8 ± 10.6 hours) at 28° C. and 44.73 ± 0.47 days (1073.5 ± 11.3 hours) at 18° C. Our animals lived longer than would be expected from their results. In all probability our culture conditions were more favorable since, as will be brought out later, the number of young produced was greater.

DURATION OF THE INSTARS

The duration of each instar for each pre-adult instar group is given in Table I. This is also shown graphically in Figures 2, 3, and 4. In general the duration of the instars increases with age. This agrees with the results secured by Ingle, Wood, and Banta (1937) for *Daphnia longispina*. The duration of any one pre-adult instar for animals primiparous during either the fifth or sixth instars is significantly different from that of any other pre-adult instar. This does not hold true for the animals primiparous in the seventh instar. The first adult instar, the instar during which the females are primiparous is distinctly longer than the longest pre-adult instar. While the duration of adult instars varied considerably the differences are not often significant. In a few cases, notably in the eleventh and twelfth instars of the group primiparous in the fifth instar, the twelfth and thirteenth

of those primiparous in the sixth, and the thirteenth and fourteenth of those primiparous in the seventh, the instars are definitely longer than those preceding and on the average longer than those which follow. This we believe was due to a deficiency in food during those instars. This point will be discussed more fully in connection with young production in the section which follows.

TABLE I

Duration of instars in hours for animals primiparous in

Instar	Fifth Instar		Sixth Instar		Seventh Instar	
	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations
B*	49.5±0.1	48	48.9±0.2	86	47.8±0.2	24
1	20.3±0.2	48	20.4±0.2	86	20.2±0.2	24
2	21.3±0.1	43	21.3±0.2	68	24.7±0.5	18
3	24.8±0.1	43	24.1±0.2	60	37.5±1.8	19
4	32.5±0.2	34	25.5±0.1	60	24.4±0.4	15
5	50.1±0.3	16	32.5±0.4	33	23.1±0.4	17
6	52.8±1.1	5	50.8±0.4	29	30.5±0.3	21
7	57.9±0.2	13	55.0±0.5	31	48.8±0.3	23
8	59.8±0.7	22	59.3±0.8	15	52.8±0.6	15
9	58.9±1.1	18	58.1±0.5	18	54.0±2.1	3
10	62.6±1.0	19	61.0±0.8	25	55.6±1.3	8
11	70.1±1.3	14	66.1±0.8	22	57.7±0.7	13
12	68.9±1.5	11	71.3±1.3	21	62.4±0.4	11
13	66.0±0.7	11	68.0±0.8	23	76.4±1.1	14
14	66.1±0.9	12	67.9±0.9	26	70.7±2.2	10
15	66.3±1.0	6	65.8±0.9	24	64.6±0.9	9
16	67.0±0.6	15	65.2±0.5	18	69.6±0.6	7
17	70.3±0.9	17	67.0±0.8	18	65.8±1.3	5
18	72.0±1.2	9	70.2±2.0	17	66.0±0.6	5
19	67.6±0.8	5	66.2±0.9	15	66.0±0.7	2
20			74.0±0.5	6	66.3±2.9	3
21			72.5±0.6	4	66.0±2.1	2
22			82.0±7.8	2		

* Brooding period.

Here also it is interesting to compare our results with those of MacArthur and Baillie (1929a) who state that the time between broods (the equivalent of duration of the instar) was three days (72 hours) at 28° C. In very few instances did the mean durations exceed 72 hours in our observations. As we did with respect to longevity, we would interpret this to mean that our culture conditions were more satisfactory.

The accumulated time at the end of each instar is given in Table II and is graphically portrayed in Figures 2, 3, and 4. It should be noted

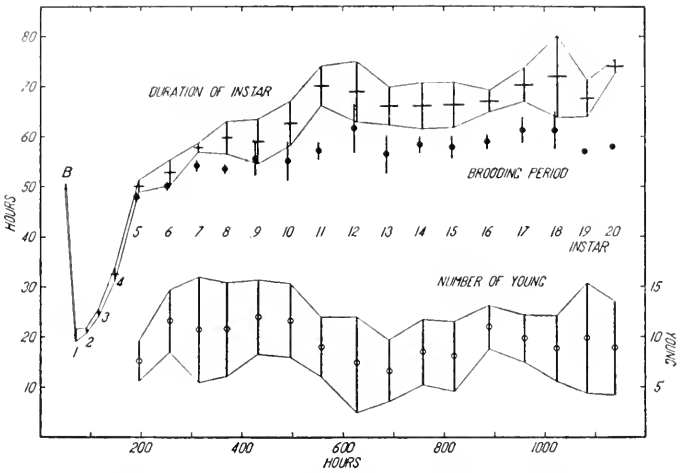

FIG. 2. Duration of individual instars, accumulated time at the end of instars, duration of the brooding periods, and the number of young released during each instar for the animals primiparous during the fifth instar. The duration of each instar is given with respect to the ordinate in hours. The average duration is the point where the bars intersect while the length of the vertical bar represents two standard deviations, one on each side of the mean. The point of intersection also represents the accumulated time with reference to the abscissa and the length of the horizontal bar represents two standard deviations from the mean. The duration of the brooding periods is in the same terms as the duration of the instars with the solid circles denoting the means. The number of young is given with respect to the right ordinate. Here also the vertical length represents two standard deviations and the open circles the means.

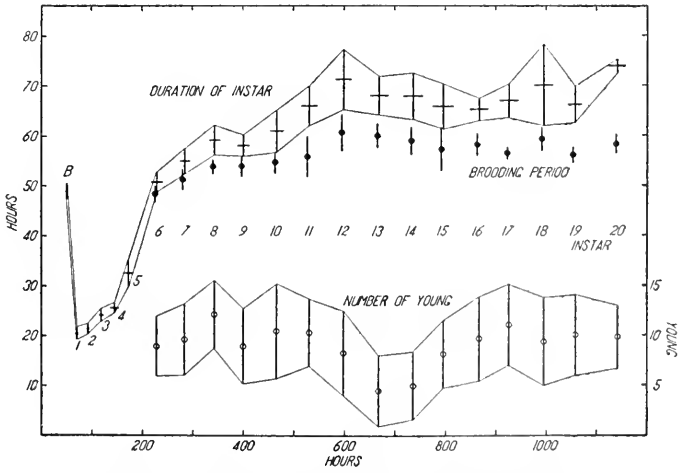

FIG. 3. Duration of instars, accumulated time at the end of instars, duration of the brooding periods, and the number of young released during each instar for the animals primiparous during the six instar. See Figure 2 for further explanation.

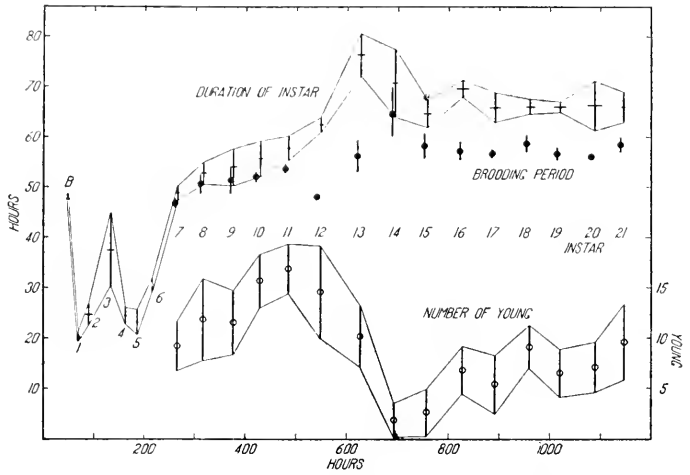

FIG. 4. Duration of instars, accumulated time at the end of instars, duration of the brooding periods, and the number of young released during each instar for the animals primiparous during the seventh instar. See Figure 2 for further explanation.

that after the third instar the accumulated time at the end of any instar for any one group was significantly different from that for the corresponding instar of any other group until the twentieth instar was reached.

REPRODUCTION

The number of young produced during each instar for each group is given in Table III and also in Figures 2, 3, and 4. In the main, the number of young produced increased for the first few adult instars after which it decreased. In two of the three groups the peak was reached in the fifth adult instar. This is similar to the course followed in *Daphnia longispina* (Ingle, Wood, and Banta, 1937; and Banta, 1939) and in *Daphnia pulex* (Anderson, Lumer, and Zupancic, 1937). Striking reductions in young production occurred during the middle of the reproductive period for each group. This we believe was due to an unsatisfactory batch of culture medium since the reduction appeared simultaneously for all the animals in the experiment. Dunham (1938) has shown that the number of young produced decreases considerably when well fed *Daphnia longispina* are placed on limited food.

MacArthur and Baillie (1929a) found that the average total young production for individual females raised at 28° C. was 15, at 18° C. 49, and at 8° C. 36. In comparison our animals produced three or more times as many. This tends to demonstrate further that our

cultural conditions were better. An alternative explanation would be that their stock was genetically different since Banta (1939) has pointed out that genetic differences often become apparent in this way.

In the preceding section we brought out the fact that certain instars were of markedly longer duration than those immediately before and

TABLE II

Accumulated time at the end of instars in hours for animals primiparous in

Instar	Fifth Instar		Sixth Instar		Seventh Instar	
	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations
B*	49.5± 0.1	48	48.9± 0.2	86	47.8±0.2	24
1	69.8± 0.3	48	69.3± 0.3	86	67.9±0.3	24
2	91.4± 0.3	43	91.7± 0.2	68	94.8±1.7	20
3	116.0± 0.3	46	116.8± 0.6	62	133.3±1.5	23
4	147.6± 0.3	35	144.2± 1.1	65	162.4±0.8	18
5	195.6± 0.4	16	171.8± 1.4	53	185.8±0.5	21
6	256.7± 1.1	19	228.4± 1.6	54	215.6±0.6	24
7	312.2± 0.5	40	283.8± 1.3	53	264.4±0.7	23
8	370.4± 1.5	30	342.0± 1.7	41	316.0±1.2	15
9	431.0± 1.6	30	398.8± 1.6	61	374.5±1.9	8
10	495.2± 1.7	36	464.7± 1.9	48	428.5±0.8	24
11	556.8± 2.7	19	530.2± 2.4	41	484.2±0.9	13
12	627.1± 2.5	22	597.4± 2.4	41	548.5±0.9	21
13	691.2± 2.2	28	668.8± 2.6	49	626.0±2.0	16
14	757.3± 3.6	20	735.7± 2.9	43	694.1±1.3	17
15	819.7± 2.7	19	795.1± 3.1	44	759.0±2.4	12
16	889.6± 1.7	22	866.2± 3.4	30	827.4±3.9	10
17	959.2± 2.3	22	924.6± 3.2	32	891.8±3.8	9
18	1025.4± 3.7	13	994.5± 3.9	29	961.6±2.8	8
19	1084.4± 7.7	5	1056.3± 3.0	21	1019.8±5.2	6
20	1178.0±19.2	2	1141.6± 4.8	11	1088.5±5.3	6
21	1206.0±	1	1206.0± 6.9	6	1145.0±2.1	2
22			1285.0±17.0	2	1249.0±	1

* Brooding period.

following. This increase in duration coincides with the decrease in number of young produced as is revealed by inspection of Figures 2, 3, and 4. Wood and Banta (1936) have pointed out that growth during any instar is negatively correlated with the duration of that instar and is directly correlated with the number of young produced in *Daphnia longispina*. Young production and the duration of an instar should, therefore, be negatively correlated. To test this point we have determined the coefficients of correlation for the number of young released during several instars and the duration of the instars

immediately preceding, during which the eggs are developed. For the animals primiparous in the fifth instar we found a coefficient of correlation of $+ .14 \pm .24$ ($r \pm \sigma_r$) for the young released in instar six and the duration of instar five; $- .68 \pm .12$ for the number of young in instar nine and the duration of instar eight; and $- .24 \pm .25$ for the young in instar 12 and the duration of the eleventh. For animals primiparous in the sixth we found $- .36 \pm .16$ for the young in the seventh instar and the duration of the sixth instar; $- .55 \pm .14$ for the young in the eleventh instar and the duration of the tenth; and

TABLE III

Number of young produced by animals primparous in

Instar	Fifth Instar		Sixth Instar		Seventh Instar	
	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations
5	7.6±0.3	48				
6	11.5±0.5	48	8.9±0.3	86		
7	10.7±0.8	48	9.6±0.4	86	9.2±0.5	24
8	10.8±0.7	47	12.1±0.4	86	11.8±0.8	24
9	11.9±0.5	47	8.9±0.4	85	11.5±0.7	24
10	11.6±0.6	46	10.5±0.5	84	15.6±0.5	24
11	9.0±0.4	45	10.3±0.4	83	16.8±0.5	24
12	7.4±0.8	45	8.3±0.5	79	14.5±0.9	24
13	6.6±0.5	44	4.5±0.4	71	10.1±0.6	24
14	8.4±0.5	40	5.0±0.4	70	1.8±0.4	22
15	8.0±0.6	32	8.1±0.4	63	2.6±0.5	22
16	10.9±0.4	32	9.7±0.6	58	6.8±0.5	20
17	9.8±0.5	28	11.0±0.5	57	5.4±0.8	14
18	8.8±0.7	21	9.4±0.7	46	9.1±0.6	12
19	9.9±1.5	13	10.0±0.6	40	6.5±0.7	12
20	8.9±1.6	8	9.8±0.6	32	7.1±0.7	11
21	3.5±0.4	2	5.7±0.7	14	9.6±1.2	10
22			5.4±1.4	5	7.8±1.8	3

$- .79 \pm .09$ for the young in instar 13 and the duration of the twelfth. For animals primiparous in the seventh instar we found $- .18 \pm .20$ for the number of young in the eigth instar and the duration of the seventh; $- .18 \pm .27$ for the number of young released in the twelfth and the duration of the eleventh; and $- .78 \pm .12$ for the number of young in the fifteenth and the duration of the fourteenth. It is thus apparent that the number of young produced and the duration of an instar are correlated negatively or not at all. Lack of correlation is to be had when there is relatively little variation in the duration of the instars (see Table I and Figures 2, 3, and 4) and whenever con-

siderable variation occurs, a high negative correlation is secured. We believe, therefore, that we are justified in concluding that the number of young produced during any instar varies inversely with the duration of that instar.

Inasmuch as the rate of reproduction is directly correlated with the growth increment (Wood and Banta, 1936; and Anderson, Lumer, and Zupancic, 1937) and negatively correlated with instar duration, the use of the rate of reproduction as an index of the general metabolic condition of a daphnid, as employed by Banta (1921b) and Anderson (1933), is more fully justified. Indeed, it is perhaps the simplest criterion that might be used since a single observation on the number of young released or on the number of eggs or embryos in the brood chamber can be made rapidly and accurately.

Duration of the Brooding Periods

We wish here to introduce the term *brooding period* to designate the time that the developing individual spends in the brood chamber of the mother, i.e. the time from the individual's passage as an egg into the brood chamber of the mother until its release as a free swimming young. Ordinarily eggs are deposited in the brood chamber within a few minutes after ecdysis and the young which develop are released shortly before the next ecdysis. In these experiments we did not check specifically whether or not eggs were deposited immediately after molting since in our experience we have found no instances of retarded egg laying. The beginning of the instar, therefore, is taken as the beginning of the brooding period. Careful observation was made to note the time of release of the young. The duration of the brooding periods is given in Table IV and in Figures 2, 3, and 4. In the main they vary with the duration of the instar. This is in contrast with the findings of Obreshkove and Fraser (1940) that the duration of the brooding periods was practically constant at 46 hours at 25° C. Whether or not the young were in the same state of development at the time of release in our experiments was not determined. It should be pointed out that the brooding period is not necessarily synonymous with embryonic period. The embryonic period is usually completed by the time the young are released. However, occasionally they may be held over in the brood chamber for some time after the embryonic period is over.

Discussion

In these experiments we have undertaken to determine the times at which various events in the life span of female *Daphnia magna* occur

when they are raised under relatively constant and reproducible conditions. The culture medium (Banta, 1921a and 1939) was completely changed at definite and frequent intervals. While this did not insure absolute constancy of environment we had what can be termed a cyclic constancy. The medium was not as satisfactory as one might wish but was no more variable than others that have been used. Algal media may have greater possibilities but even they are quite variable if Banta's (1939, p. 197) interpretation of Edlén's (1937) results are correct. Cultures made up with pure strains of bacteria are difficult

TABLE IV

Duration of the brooding period in hours for animals primiparous in

Instar	Fifth Instar		Sixth Instar		Seventh Instar	
	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations	$\bar{x} \pm \sigma_{\bar{x}}$	Number of Observations
5	48.4±0.2	19				
6	50.1±0.3	8	48.3±0.3	36		
7	54.2±0.3	17	51.3±0.3	45	46.8±0.2	23
8	53.5±0.3	15	53.9±0.3	20	50.6±0.5	20
9	55.5±0.7	21	53.9±0.4	24	51.3±1.1	6
10	55.1±1.2	10	54.7±0.4	24	52.0±0.4	7
11	57.1±0.4	16	55.9±1.0	17	53.5±0.2	21
12	61.6±1.8	7	60.6±0.9	15	48.0±	1
13	56.4±1.3	8	60.0±0.6	15	56.1±1.1	8
14	58.3±0.5	9	59.0±0.9	8	64.5±3.9	2
15	57.8±0.9	6	57.3±1.2	14	58.2±1.1	5
16	58.9±0.5	10	58.2±0.6	17	57.2±0.7	6
17	61.1±0.9	7	56.5±0.4	8	57.7±0.3	3
18	61.2±1.7	5	59.4±0.6	14	58.7±1.0	3
19	57.0±	1	56.1±0.6	7	56.7±0.7	3
20	58.0±	1	58.4±0.6	12	56.0±	1
21			60.7±2.6	3	52.0±5.0	3
22			64.3±2.6	3	58.5±1.1	2

to manipulate and not entirely satisfactory in other respects (Banta, personal communication). We have tried various culture media with yeast as a basis but they have been found wanting in many ways. Of the various culture media that have been used successfully for mass culturing (Galtsoff, Lutz, Welch, and Needham, 1937) few lend themselves for the individual culturing of Daphnia. A medium is needed that can be controlled and reproduced not only by an individual investigator but by all with a minimum of effort. Until such a medium is developed the variations in the responses of Daphnia under many types of experimental conditions will be great.

Summary

Hourly observations were made on 159 *Daphnia magna* individually reared in manure-soil medium at 25° C. throughout their whole life span.

The number of pre-adult instars varied from four to six, the most frequent number being five.

The average longevity was approximately 960 hours or 17 instars.

In general the duration of the instars increases with age. The pre-adult instars with the exception of the last require about a day, the final taking about 30 to 32 hours. Beginning with the first adult the instars last approximately two days and increase slightly with each instar.

The number of young produced increases with each adult instar up to the fifth, followed by a decrease. The use of the number of young produced as an index of the general metabolic condition of a daphnid is analysed and believed justified.

The brooding periods, i.e. the time between the deposition of eggs and their subsequent release as young, vary with the duration of the instars.

The need for a more readily controllable and reproducible culture medium is pointed out.

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DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER

V. Factors Influencing the Length of the Cycle; Observations upon the Gaseous and Dissolved Organic Nitrogen [1]

THEODOR VON BRAND, NORRIS W. RAKESTRAW AND J. WILLIAM ZABOR

(From the Woods Hole Oceanographic Institution, Woods Hole)

The experiments to be described in this paper are in part a continuation and amplification of studies previously reported (1937–1941), on the influence of temperature, the nature of the organic matter and the source of the water upon the speed of the nitrogen cycle, as well as the interrelation of the various steps involved. Our previous studies dealt almost exclusively with the fate of the particulate, ammonia, nitrite and nitrate nitrogen; in the present investigation we have also studied the behavior of two other important nitrogen fractions commonly found in sea water, dissolved gaseous nitrogen and dissolved organic nitrogen. Because of technical difficulties our study of these two nitrogen fractions is incomplete and merely preliminary. Nevertheless, the results are included in the present report, since it appears doubtful that the investigation can be continued in the near future.

The general method of the present experiments was the same as that previously described. Particulate organic matter, usually in the form of diatoms (*Nitzschia Closterium*), was added to samples of sea water in large carboys, which were kept in the dark while decomposition proceeded. Samples were removed from time to time for analysis of the various forms of nitrogen, generally particulate nitrogen, ammonia, nitrite and nitrate.

Influence of Low Temperature

In September, 1938, a culture (No. 28) of harbor water and Nitzschia was stored in the dark at 1° to 2° C. As reported previously (von Brand and Rakestraw, 1940), ammonia was the only decomposition product which had appeared up to June, 1940. A continued observation of this culture to June 19, 1941, failed to reveal any further change. It was transferred on this day to room temperature, but still kept dark. On June 27 nitrite had begun to appear, reaching its maximum ten days

[1] Contribution No. 321 from the Woods Hole Oceanographic Institution.

later and declining throughout August. The cycle was ended at the beginning of September, the nitrite having been quantitatively transformed into nitrate. A part of this culture was separated on June 27 and kept in a refrigerator at 5° C. The nitrite and ammonia in this sample remained constant to the end of September, when its observation was discontinued. Apparently the organisms responsible for nitrite formation did not develop at low temperatures. The rapid appearance of nitrite upon restoration to higher temperature, in the main portion of No. 28, proves that they were not killed by the low temperature, but only kept inactive.

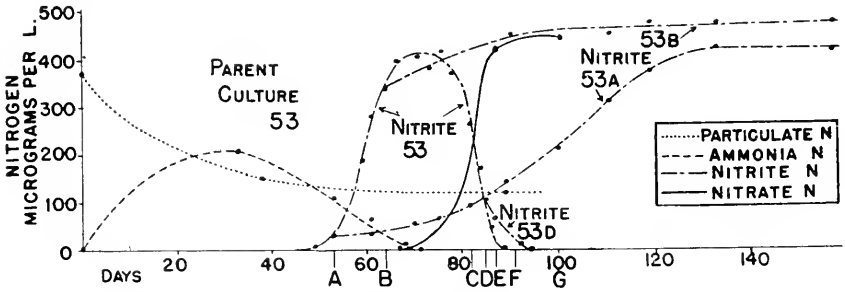


FIG. 1. Effect of low temperature. Time in days. Different forms of nitrogen in micrograms per liter. The parent culture, No. 53, consisted of Woods Hole harbor water with washed diatoms (*Nitzschia Closterium*) added. Decomposition in the dark at 20° C. Sub-cultures were removed at points on the time-scale marked *A*, *B* and *D* and preserved at 5° C. for observation of nitrite and nitrate formation, which proceeded more slowly in 53*A*, 53*B* and 53*D* than in 53. Points *C*, *E*, *F* and *G* indicate times at which other sub-cultures were removed, which are shown in Figure 5.

Series 28 is not typical of all cultures. Figure 1 shows the results in another series, Number 53. This culture also contained Nitzschia in sea water and was kept in the dark at room temperature. Sub-cultures were separated from the parent culture at various times during the decomposition, marked *A*, *B* and *D* in the figure. These were placed in a refrigerator at 5° C. and analyzed periodically, along with the parent culture, which was kept at room temperature.

The first of these sub-cultures, No. 53*A*, was separated when nitrite had just begun to appear in No. 53. Nitrite formation continued in 53*A*, but very much more slowly. At room temperature (about 20° C.) the nitrite maximum was reached 13 days after separation of the two cultures; at 5° C. only after 67 days. The average daily nitrite production was in the former case 28 micrograms per liter, the latter 5.8. The overall Q_{10} was around 3, a normal coefficient for a biological process.

Sub-culture 53*B* was separated from 53 when the nitrite was near its maximum. Nitrite continued to increase slowly in the cold, even

to a slightly higher level than in the original culture, but up to the end of the observation no nitrate had been formed.

The striking difference in temperature coefficients of nitrite formation in Series 28 and 53 might indicate that different organisms were involved. It should be kept in mind that in nature nitrite is formed at all latitudes, in waters of different temperatures. An investigation of the temperature coefficients of nitrite formation in waters of different localities might yield interesting results.

Sub-culture 53*D* was separated from No. 53 when nitrate formation in the latter was well under way. All the remaining nitrite was oxidized to nitrate in Series 53 in four days, whereas the process required nine days at the low temperature. During these periods the average daily nitrate formation was 26 micrograms per liter at the higher temperature and 11.6 at the lower. The overall Q_{10} for nitrate formation was therefore about 1.5, distinctly less than the coefficient for nitrite formation in the same series.

Nature of Particulate Matter and Source of Water

Previous studies have shown that the speed with which the nitrogen cycle proceeds is largely determined by the nature of the particulate

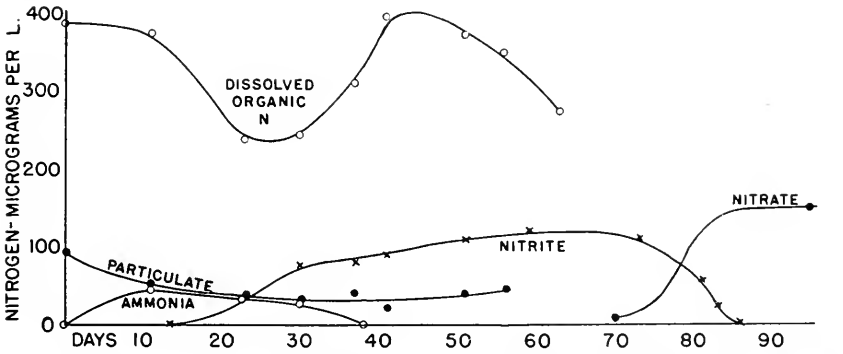


Fig. 2. Series 54. Woods Hole harbor water, with washed diatoms (*Nitzschia Closterium*). Decomposition in the dark at room temperature. Time in days. Different forms of nitrogen in micrograms per liter.

matter. The most rapid rate of decomposition was found with mixed plankton, a slower rate with diatoms, and a still slower one with plant protoplasm foreign to sea water (yeast cells). To study this relation further we undertook to compare the decomposition of diatoms (Nitzschia) with that of a protozoan (*Tetrahymena gelei*) grown in bacteria-free culture. For this purpose, two decomposition series of Nitzschia were started, No. 54 (Figure 2) in harbor water, and No. 55 (Figure 3) in deep-sea water collected from below 1000 meters depth.

In Series 57, containing harbor water, and in Series 58, containing deep-sea water, quantities of the ciliate, Tetrahymena, were introduced, which had been separated by centrifugation from the proteose-peptone culture medium and repeatedly washed with nitrate-free sea water. During this process the ciliates died but did not seem to disintegrate into too small particles to be recovered by the usual precipitation procedure after suspension.

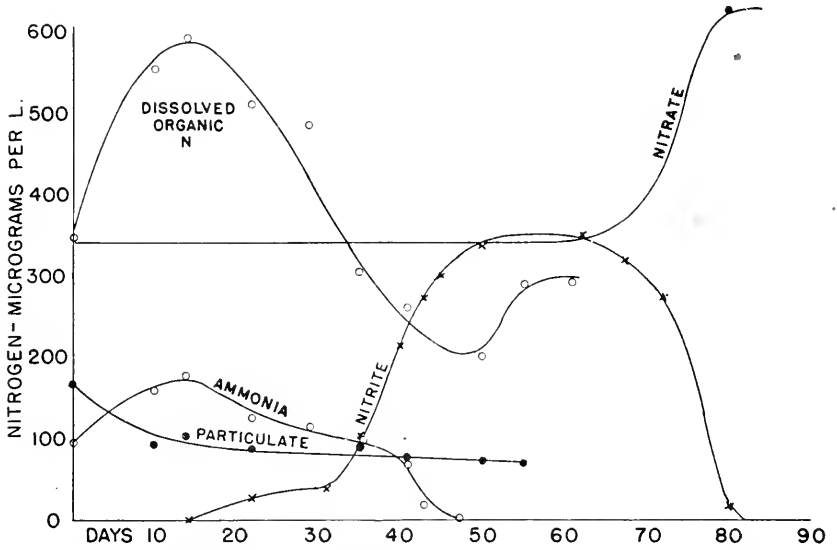


FIG. 3. Series 55. Deep-sea water (from below 1000 meters depth), with washed diatoms (*Nitzschia Closterium*). Decomposition in the dark at room temperature. Time in days. Different forms of nitrogen in micrograms per liter.

The results of Series 57 and 58 are shown in Figure 4. In both these the concentration of particulate matter was considerably higher than in Nos. 54 and 55, but it has been shown previously that this does not alter the speed of the cycle to any considerable extent.

Contrary to our expectations, the decomposition of the diatoms proceeded equally well in the harbor water and the deep-sea water. The rate of decomposition of the ciliates in harbor water was about the same as that of the diatoms. In deep-sea water, however, the decomposition of ciliates formed ammonia rapidly, but the production of nitrite was considerably retarded, and no nitrate had appeared by the time the experiment was discontinued for lack of material. The reason for this behavior is not clear.

Interrelationship of Various Steps

In our last report (1941) we pointed out that if new organic matter is added to a decomposing culture the normal sequence of "step by step" decomposition is disturbed and that under certain conditions ammonia, nitrite and nitrate formation seem to proceed at the same time. Since this situation is probably very common in nature it seemed desirable to extend the experimental study of it. Consequently, several sub-cultures were separated from Series 53, at various stages of the cycle, and new particulate matter in the form of Nitzschia was added to each. The original parent culture and the sub-cultures

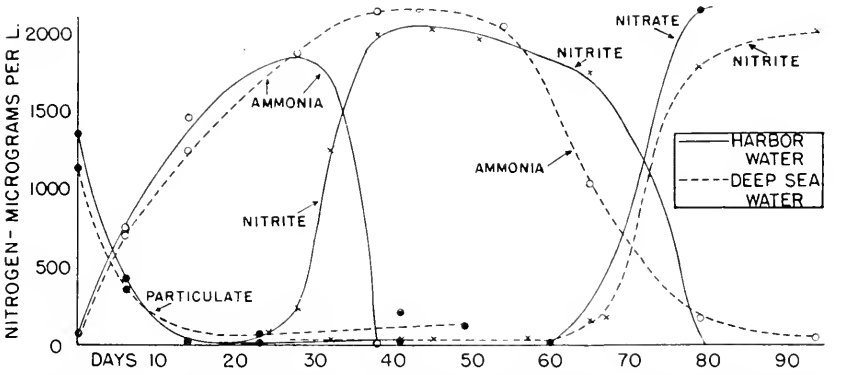


Fig. 4. Series 57 and 58. Washed cultures of *Tetrahymena gelei* suspended in Woods Hole harbor water (No. 57) and in deep-sea water (No. 58, below 1000 meters depth). Time in days. Different forms of nitrogen in micrograms per liter.

were kept in the dark at room temperature. Figure 1 shows the points at which separations were made (*C*, *E*, *F*, *G*) and Figure 5 shows the subsequent course in these sub-cultures. Series 53*C* was separated as the nitrite began to decline, 53*E* when nearly all the nitrite in Series 53 had been oxidized to nitrate, 53*F* about a week after the cycle in 53 had been completed, and 53*G* another week later.

In 53*C* practically no ammonia appeared, and the nitrite disappeared at about the same rate as in the parent culture. But due to the addition of new particulate matter the nitrate level reached a considerably higher maximum than in the parent culture. The only possible explanation is that all three processes went on at the same time, and since nitrate formation is the most rapid step, neither ammonia nor nitrite could accumulate in large amounts. This explanation is further supported by the results of sub-cultures 53*E*, *F* and *G*, separated at later stages in the cycle of the parent culture, after all the nitrogen had been completely oxidized to nitrate. In each of these ammonia

and nitrite appeared, but only in small amounts in the presence of rapid nitrate formation. This would seem to dispose of one possibility that has been suggested: that a vigorous nitrate-forming flora is capable of acting on a substratum of nitrogen compounds of high molecular weight without the formation of ammonia or nitrite as intermediates.

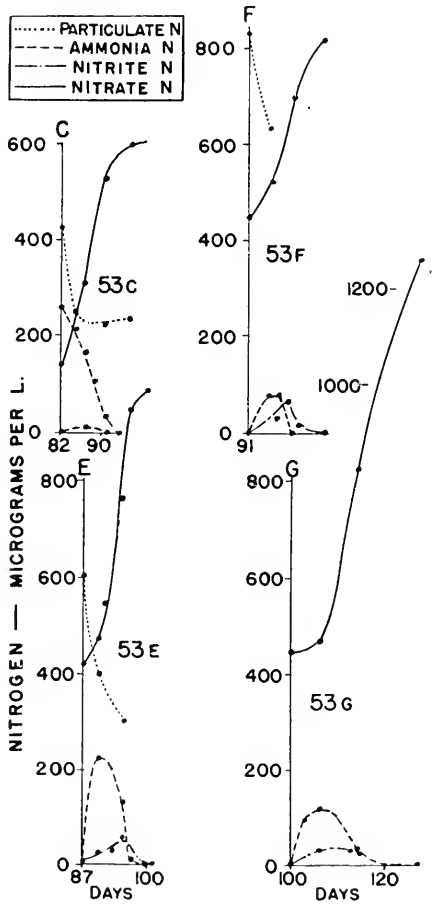


FIG. 5. Interrelationship of various steps. From parent culture No. 53, shown in Figure 1, sub-cultures were removed at times indicated by points *C*, *E*, *F* and *G*. Sub-culture 53*C* was separated when nitrate formation in 53 was proceeding rapidly; 53*E*, when nitrate formation was nearly complete; 53*F* and 53*G*, after the cycle had been entirely completed in 53. To these, new quantities of particulate organic mattre (*Nitzschia Closterium*) were added, the initial amounts being shown by the beginning of the dotted lines (except in 53*G*, in which no particulate-N analysis was made). These were preserved in the dark at 20° C. with the parent culture, No. 53. Time, in days, is measured from the beginning of culture No. 53. Different forms of nitrogen in micrograms per liter.

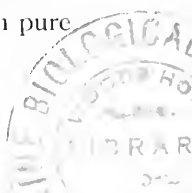
Another point that is again brought out in this series of sub-cultures is the acceleration of the cycle by a recurrent supply of particulate matter. The parent culture required 89 days for the completion of the cycle, the sub-cultures on the average only 18 days. The difference between the parent culture and the sub-cultures in this case is greater than in that described in our last report (1941). This is probably due to the fact that in the present case the particulate matter was added at a later stage in the decomposition cycle, when the most effective flora—the nitrate-forming one—was very vigorous. A continuation of these observations may lead to an understanding of the rapid succession of different organisms so frequently observed in the sea.

One further point should be noted. We had found previously that it was possible to repeat the "step by step" decomposition cycle in the same water at least three times, if the nitrate was regenerated into particulate matter between cycles by inoculating the culture with a few diatoms and exposing it to light for a period of about ten days. Why then did Series 53*G*, which was supplied with new organic matter two weeks after the original cycle had ended, not yield a "step by step" decomposition but rather a "simultaneous" one? We have no definite answer to this question, but it would appear that when the new organic matter is generated during a period of exposure to light the action of nitrite and nitrate forming organisms is somehow retarded, but when organic matter is introduced from an outside source there is no such inhibiting effect.

Gaseous Nitrogen

We have already pointed out that the quantitative nitrogen balance was satisfactory in about half of our experiments, while in the other half a more or less steady increase in total nitrogen occurred, for which no entirely satisfactory explanation has been found. One of the possibilities discussed was that of the fixation of elementary nitrogen by micro-organisms. Series 56 and 60 were designed to test this possibility. The technical difficulty involved was considerable and it was not possible to secure data for the whole decomposition cycle. It was necessary to use sealed samples in order to eliminate any exchange of gaseous nitrogen between the sample and the atmosphere. During the course of the decomposition, however, a large amount of oxygen is used, much more, as a matter of fact, than is found dissolved in a given sample of sea water. It was therefore necessary to introduce extra oxygen into the sample before sealing.

A procedure was devised by which a measured sample of water, containing particulate organic matter, could be introduced into a flask of about 500 ml. capacity, the small remaining air-space filled with pure



oxygen and the flask sealed off without any further contact of the sample with the atmosphere.

Nitrogen of course distributes itself immediately between the liquid and gas phases, but when analysis is carried out later the gas bubble is first removed, its volume measured and its nitrogen content determined. Since the total volume of the water sample is known an appropriate allowance can be made for the nitrogen in the gas phase after analysis of duplicate water samples by the method of Rakestraw and Emmel (1937).

The water not required for these analyses was later used for the determination of the other nitrogen fractions, as indicated in Tables I and II. The variations in gaseous nitrogen observed were small and

TABLE I

Series 56. Woods Hole harbor water, with fresh culture Nitzschia Closterium. Samples sealed in 500 ml. flasks with excess oxygen. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate	Dissolved organic	Gaseous
6–30–41	333	80	0	10	301	11770
"	316				333	
7–14	83	152	0		452	
7–28	78	225	0	15	443	
8– 4	70	330	0	15	270	11880
8–15	59	0	285*	65	430	11980
8–21	129	420	0	25	272	11990

* Since each sample analyzed is in a separate flask and decomposition may go more rapidly in some than in others, such irregularity is not surprising.

TABLE II

Series 60. Woods Hole harbor water, with fresh culture Nitzschia Closterium. Samples sealed in 500 ml. flasks with excess oxygen. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate	Dissolved organic	Gaseous
8– 7–41	141	25	0	15	336	11800
8–14	39	205	0	10	223	11760
8–20	35	175	0	20	319	11910
8–25	38	152	0	15	320	12000

probably within the limits of error of the method. But, while nitrogen fixation has not been demonstrated by these experiments, it should be pointed out that they cover only the first part of the nitrogen cycle and that at least in Series 60 no increase in total nitrogen was observed. Experiments are now under way to determine whether longer periods of decomposition are accompanied by changes in gaseous nitrogen.

Dissolved Organic Nitrogen

Another source of nitrogen that might conceivably account for the increase in total nitrogen is the dissolved organic fraction. Its behavior was studied in Series 54, 55, 56 and 60. The method used was that of Krogh and Keys (1934) with the slight modifications introduced by von Brand and Rakestraw (1941).

The results were not entirely conclusive. Easiest to understand are Series 55 (Figure 3) and 56 (Table I), where concurrently with the drop in particulate nitrogen an increase in dissolved organic nitrogen could be observed. This can be explained on the assumption that soluble nitrogen compounds were formed, intermediate between particulate nitrogen and ammonia. That a similar rise was not observed in Series 54 (Figure 2) and 60 (Table II) may be due to the more rapid formation of ammonia. In Series 54, however, a rather considerable drop in dissolved organic nitrogen occurred after the ammonia had reached its maximum. It seems to lie outside the limits of error of the method, but we cannot account for it. It should be remembered that the method was worked out for natural sea water, and it is quite possible that during the course of decomposition of a relatively large amount of particulate matter substances may be formed which interfere, a possibility which is unfortunately difficult to test.

The general impression gained from these preliminary experiments is that the dissolved organic nitrogen may be involved to some extent in the nitrogen cycle. The changes which have thus far been observed, however, are not sufficient to explain the nitrogen increase in some of our experiments. The most likely explanation for this is still contamination from outside sources, which is difficult to avoid in view of the very small quantities of nitrogen involved.

Summary

1. Low temperature may inhibit nitrite formation completely or may only retard it, probably depending upon the particular organisms present.

2. Oxidation of nitrite to nitrate was less inhibited by low temperature than the formation of nitrite from ammonia.

3. No clear-cut difference was observed between the decomposition of diatoms and that of ciliates.

4. Clear evidence was found that under certain conditions ammonia, nitrite and nitrate formation can go on simultaneously.

5. Preliminary experiments gave no evidence for a fixation of nitrogen, but indicated that dissolved organic nitrogen may possibly be involved in the nitrogen cycle.

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THE EFFECTS OF POPULATION DENSITY UPON GROWTH AND SIZE IN LYMNAEA PALUSTRIS

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Introduction

There is a voluminous literature on the effects of increasing or of decreasing population density. Much of the work has been done on animal aggregations confined in a small space rather than on groups of animals held together by social instinct. In some cases beneficial effects have been reported as the result of a moderate degree of crowding, and it has been suggested that such primitive advantages of association may have played a considerable part in the development of social organization. Among the lower forms such as protozoa, worms, crustacea, fish and amphibia, animals in an aggregation may be better able to withstand the effects of toxic substances in the water medium, or of extremes of temperature, than when they are living in isolation. It has been found that under certain circumstances associated animals condition the water more favorably, perhaps by adding some important substance to it, with the result that fertility or the rate of growth may be increased. In Drosophila longevity is increased to a certain degree by an increase in the density of the population, and in Tribolium the reproductive rate shows a similar relationship to population density. Summaries and critical reviews of this subject are given by Allee (1930) and by Hammond (1938, 1939).

The workers on aquatic snails have given conflicting interpretations of their tests of the effects of population density upon rate of growth, although most of them agree that even moderate crowding brings about a reduction in size. Hoffman (1927) has given a summary of the earlier literature bearing on this subject, and Crabb (1929) showed that marked retardation of growth in the presence of adequate food in *Lymnaea stagnalis appressa* may result from crowding. However, the data of some of the researches have been too scanty to be conclusive, while in many of them the natural range of variation has not been taken into account.

We have carried on extensive laboratory experiments on *Lymnaea palustris* for several years and have reared seven consecutive generations. The original animals came from a mill-pond in Newtown,

Connecticut. Scores of families and thousands of individuals have been obtained, and abundant data for numerous biological problems have been accumulated. The present paper records some of our observations on the effects of crowding upon growth and size.

Methods

In our standardized procedure, the young snails that developed from a single mass of eggs were kept together in a large nursery jar, in about two liters of water, until they were a specified age, when they were measured and isolated in quart jars containing from 500 cc. to 600 cc. of fresh spring water. Each jar contained a spray of Cabomba and a little finely-sifted earth. Green loose-leaf lettuce was used as food, and a surplus was always kept in the jars. The snails were housed in a greenhouse during the winter months until the temperature became too warm, toward the end of May, when they were brought down to the cooler laboratories.

Efforts were made to maintain a temperature as close to 70° F. as possible. The parent snails were kept in isolation, hence all the eggs deposited were self-fertilized (Crabb, 1927).

The jars were kept clear and free from scum and obvious bacterial contamination, although by no means sterile. The water level was kept constant by adding fresh water whenever needed, at least once a week. In the tripartite experiments fresh water was used for all the groups, and in no case was old medium returned to the jars.

The length of the shell was taken as the index of size. The F test was employed to determine the significance of a difference between two comparable groups. In our tables, N is the number of items in the sample or group, $\bar{X}$ is the arithmetical mean, while r is the usual coefficient of correlation. F is a critical value which, taken in conjunction with the proper degrees of freedom, may be denoted as significant or not significant by reference to an F table such as that available in "Applied General Statistics" by Croxton and Cowden (pp. 878–879). The degrees of freedom involved are: between the means, $n_1 = 1$; and within the samples, $n_2 = N_1 - 1 + N_2 - 1$, where N_1 is the number of individuals in one sample and N_2 is the number of individuals in the other sample. The level of significance adopted was .05.

Experimental Results

The fundamental problem is whether there is a correlation between the number of snails in a family aggregation and the average size of the

members of such a group. For this problem we have available a series of 54 families with varying numbers, comprising 1056 individuals in all. The animals of each family were reared together until the age of 80 days, when they were measured. The results of the statistical

TABLE I

Data of size at successive age periods

Family	80 days			160 days		
	N	$\bar{X}$	S	N	$\bar{X}$	S
		mm.			*mm.*	
A	30	4.4630	1.5101	28	6.5714	1.7686
B	37	5.3486	2.1224	22	7.5000	2.1012
C	41	2.8415	0.8628	30	5.1300	1.1847
D	49	4.6143	2.0363	46	7.7152	2.8495
E	41	6.0927	1.3378	34	6.3706	1.2440
F	44	4.7591	0.9641	43	6.4302	1.7660
G	33	2.6636	0.8605	23	8.1739	1.8811

Family	240 days			320 days		
	N	$\bar{X}$	S	N	$\bar{X}$	S
		mm.			*mm.*	
A upper	6	17.7333	1.7489	3	20.5333	0.4619
middle	16	8.1500	0.9388	12	11.0500	1.4526
lower	6	19.3500	1.2486	2	21.9000	0.8485
B upper	6	15.1667	2.6905	2	17.6500	4.3134
middle	4	11.4000	2.3036	0	——	——
lower	4	10.6750	4.0401	1	11.2000	——
C upper	4	16.6750	1.2230	2	22.4000	0.1414
middle	11	7.9091	0.7674	7	11.1000	0.8145
lower	5	15.4200	1.8566	4	18.8750	1.4773
D upper	6	18.4333	0.9331	4	19.1250	0.2986
middle	30	8.8567	2.2773	0	——	——
lower	5	16.7400	1.2798	4	17.1250	1.1701
E upper	5	16.4600	2.2052	3	18.1000	1.9000
middle	14	8.5714	1.6452	2	10.6500	2.0506
lower	3	14.6333	2.0257	3	16.1666	1.5308
F upper	6	20.2333	2.2757	2	21.3500	1.4849
middle	2	9.9500	0.0707	2	16.5500	2.4749
lower	4	20.6250	1.8392	1	19.0000	——
G upper	6	21.2667	1.6170	1	21.5000	——
middle	1	14.8000	——	—	——	——
lower	6	20.0333	0.6088	6	21.2500	0.7868

treatment were as follows:

N	SX	SX^2	SY	SY^2	SXY	F	Significance
54	1056	28884	374.26	2947.60	6484.10	6.987	positive

The figures definitely prove the reality of a highly *significant negative correlation* between the number in the nursery and the average size of the individuals composing the family group. Hence the effect of an increase in the density of the population is to reduce the size of the snails.

Two families composed of approximately the same numbers of individuals were procured from the same parent. The members of the first were isolated at hatching when they were 18 days old, while the members of the second were kept together until the age of 80 days, when they also were isolated. All were measured again at the age of 160 days. The results of their statistical comparison are as follows:

Difference in size at 80 days

	N	$\bar{X}$ mm.	SX^2	F	Significance
Family I	16	14.0625			
Family II	14	8.7071			
			4277.47	115.2926	positive

Difference in size at 160 days

Family I	16	15.7562			
Family II	14	14.3742	6909.71	8.711	none

The figures show that the effect of moderate crowding between hatching and the age of 80 days is real. In the case of Family II, they also show that escape from the effects of crowding occurred after the animals were isolated, and that this escape was practically complete at 160 days.

By random sampling, two additional groups were assembled from the available general population. As in the foregoing case, the first group comprised individuals which had been isolated at hatching while the members of the second group had grown together in their respective family aggregations until they were 80 days old. All were measured at 80 days of age. The comparison of the two groups gave the following results:

	N	$\bar{X}$ mm.	SX^2	F	Significance
Group I	26	13.6153			
Group II	35	5.8885			
			4858.28	235.694	positive

The lesser average size ($\bar{X}$) of the members of Group II shows that here

too the effect of population density is to reduce the amount of growth of the animals in an aggregation. The greater value of F in this instance, as compared with that in the preceding analysis, may be due to greater inequalities in the numbers of the comparable groups, or to genetic divergence and distinction.

We now present additional data that not only confirm the conclusions based on the previous material but also show to what extent recovery from the effects of crowding may ensue upon isolation. The subjoined data relating to three different families give the average sizes, in millimeters, of the members of these families at successive times in their lives, as follows:

	80 days		160 days		240 days		320 days		400 days	
	N	Size	N	Size	N	Size	N	Size	N	Size
		mm.		*mm.*		*mm.*		*mm.*		*mm.*
Family I	39	4.24	39	14.34	39	17.22	19	19.98	—	—
Family II	17	6.56	16	7.37	13	14.88	7	18.08	5	17.90
Family III	48	3.56	48	6.39	11	11.31	10	14.00	6	16.17

In the experiments in question, the individuals of Family I were isolated at 80 days, when the average size was only 4.24 mm. Their subsequent growth to more than 14 mm. at 160 days was about as usual under the circumstances. Family II was allowed to grow as an aggregation until 160 days, when its members were isolated. The average size very nearly equalled that of the first group at the 320-day period. The snails of Family III were not separated until they were 320 days of age at which time the ten surviving individuals had gained the average size of only 14 mm., a figure considerably below the average sizes of the other two groups at the same period. The three families had the same genetic origin as they were all grown from eggs laid by the same parent.

The data show that the longer the snails were kept in an aggregation, the less able they were to recover from the inhibiting effects of crowding. Nevertheless, crowding during only the first 80-day period had little, if any, effect upon final size. The normal rate of growth is evidently susceptible to modification during the earlier stages without permanent result, but if the effects of crowding are prolonged, full recovery does not follow.

Still another procedure was followed in the present series of studies. A single family was reared in a nursery jar; the animals were measured at 80 days and were then returned to the same jar where they remained

until they were 160 days old. At this time they were measured again, and the six largest and six smallest individuals were isolated, while the intermediate members of the graduated series were kept together in the original nursery. Not a single individual died before the age of 240 days when all were again measured, but some members of each of the three subordinate groups failed to reach the age of 320 days. The average sizes of the members of the groups are given for the several age-intervals as follows:

	80 days	160 days	240 days	320 days
	mm.	*mm.*	*mm.*	*mm.*
Upper 6	7.00	9.50	17.83	20.50
Middle 16	4.28	6.37	8.26	11.25
Lower 6	2.83	5.17	19.17	22.00

The six largest snails, constituting the "upper" group at 160 days had evidently grown at a more rápid rate than the others, either by virtue of an inherent predisposition to do so, or because they were relatively less susceptible to the inhibiting effects of crowding. On the assumption that all of the snails had kept their places in the scale of size between 80 and 160 days, it would seem that the absolute amounts of growth were about the same for the three series, although the relative amounts were different. Considering the figures for 240 days, it is clear that both the upper and the lower groups grew very rapidly after the time of isolation, while the components of the middle group were markedly retarded as the result of their continued aggregation. At 320 days the 12 surviving snails of the intermediate group averaged only 11.25 mm. in length, a figure that is only a little more than half of the average for the lower group. The two living members of the last-named were actually longer than the survivors of the upper series at this last age interval; but their relation was exceptional.

Seven additional families of the third pedigreed generation belonging to a single clone were treated in the same way to ascertain whether the above relations and phenomena were consistent. The results are given in Table I. The several families differed somewhat in the quality of average size at 80 days, but the figures are uniformly low. In all of them the amount of growth from 80 days to 160 days was relatively small because the animals were living in association. In every case the components of the upper and lower groups enlarged at a notably rapid rate after their isolation. Also in every case the animals of the intermediate groups which were not isolated lagged far behind the solitary individuals.

Conclusions

In general the larger the number of snails grown in a confined aggregation the smaller will be the average size of the members of the association.

Isolated individuals of *Lymnaea palustris* grow faster and attain larger sizes than do individuals grown in an aggregation.

When growth is retarded by crowding during only the earlier period of the life cycle, recovery is usually complete after isolation. Continued crowding to a later time in the growth cycle produces a lasting effect from which full recovery does not follow. The result is a diminished final size of the snails.

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ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1942

JULY 21

The effect of some vitamins of the B-complex on respiration of mutants of Neurospora. A. C. Giese and E. L. Tatum.

Using the standard Barcroft-Warburg technique the effect of the addition of various vitamins to cultures of Neurospora appropriately starved for the vitamin was determined. Addition of B_1 to a culture of a "thiaminless" mutant of Neurospora resulted in a small but significant rise in the rate of respiration.

Experiments were next performed with three vitamins of the B-complex-pantothenic acid, p-aminobenzoic acid and B_6 (pyridoxin) whose respiratory function, if any, has not yet been demonstrated. Addition of pantothenic acid to the "pantothenicless" mutant, of p-aminobenzoic acid to the "p-aminobenzoicless" mutant and of B_6 to the "pyridoxinless" mutant resulted in each case in a significant rise in the rate of respiration. This indicates that each of these vitamins has a respiratory function in Neurospora.

The increase in the rate of respiration is almost immediate in the case of B_1, somewhat slower in the other three cases suggesting that in the latter three the effect of the vitamins may be indirect but the experiments do not permit of a decision at present.

AUGUST 4

The effect of temperature on vertebral variations in Fundulus heteroclitus. M. L. Gabriel.

In spite of much study of temperature-controlled variation of vertebral numbers in wild populations of fishes, evidence from laboratory experiments is still needed to assure adequate control of temperature as well as genetic control otherwise impossible. In the present study, *Fundulus heteroclitus* eggs were fertilized *in vitro,* each stripping was divided into three parts, and the eggs were raised in constant temperature baths at 24.5° C., 18.7° C., and 13.5° C., the sibs being kept separate. The adult number of vertebrae is present at hatching and vertebral counts of alizarin preparations were made.

Significant differences in the mean vertebral number were obtained: the mean is $32.73 \pm .03$ (S.E.) at 24.5°, $33.10 \pm .04$ at 18.7°, and $33.44 \pm .11$ at 13.5° ($t = 3.12$, $P < .01$; $t = 6.44$, $P < .01$). At a single temperature (24.5°), the vertebral number was found to be related to rate of development. In fishes grouped according to time at hatching in two-day intervals, the mean rises linearly from $32.45 \pm .06$ at 9–10 days to $33.10 \pm .13$ at 19–20 days.

The hypothesis is advanced that the rates of somite separation and mesodermal histodifferentiation may be so related that under accelerating conditions the latter becomes precocious relative to somite formation so that vertebral rudiments may fail wholly or partly to separate. Fewer vertebrae would thus be formed, the modification taking place in the caudal region where the rate discrepancy would be greatest. It has been shown (Ford, 1933, *Jour. du Conseil,* **8**) that the caudal

region is the site of modifications in vertebral number. It is thought possible that "fused" or "complex" vertebrae, most common in the caudal region and at high temperatures, may be produced by such rate discrepancies between somite separation and histodifferentiation.

Some effects of temperature in the regeneration of Tubularia. Florence Moog.

In furtherance of recent work (Spiegelman, Goldin, and Moog) indicating that the determination of size of the hydranth in regenerating Tubularia is independent of the rate of formation to a limited degree, studies were made of the effects of temperature on the two factors. Temperatures of 7°, 10.8°, 13.5°, 18.7°, and 20.8° C. were used.

The relation between the velocity (the inverse of the time to the appearance of the constriction between the primordium and the rest of the stem) and temperature is linear, the steepness of the line varying directly with the rate at the highest temperature. The average Q_{10} was 1.8. The fact that the more sluggish material was least affected by temperature suggests that the velocity was being limited by low concentration of substrate.

The size of the primordium averaged 35 per cent longer at 7° than at 21°, the relation between size and temperature taking the form of a sigmoid curve. The average Q_{10} for the size effect was 1.29, and it did not vary much with size or speed. Apparently the determination of size is limited principally by a physical factor, probably diffusion. The change in size was too great to be accounted for on the basis of the increase in oxygen tension with decreasing temperature; more likely the slowing of the velocity constants of the processes of formation of the hydranth has the effect of raising the supply of substrate and oxygen available for conversion.

The effect of temperature change on the tentacle number is two-fold, the average number in the oral circlet increasing from 11.7 at 21° to 13.7 at 7°, while the basal circlet average decreased to 13.3 to 11.2. The ratio of the number in the oral to the number in the basal group shifted from 0.88 to 1.22, and the relationship between the numbers in the two circlets showed a significant correlation at each temperature, indicating that the processes determining the numbers in the two groups are interdependent. The data offer no basis for deciding whether the relative increase of activity of the oral portion of the primordium is an intensification of the normal dominance of the stem, or merely a local situation.

Mass and time relationships in the regeneration of Tubularia. S. Spiegelman.

Definitions of ratio of regeneration are necessarily arbitrary. Most early investigators were content to use 1/t as a measure of regeneration rate, where t was the time from section to a specified morphological stage in the process. This particular definition necessarily ignores the masses of tissue transformed, an important aspect of the rate problem. Accordingly Barth (*Physiol. Zool.*, 11: 179, 1938) formulated a rate in terms of the ratio of the length of the primordium formed and the time required, i.e., L/t.

The mass of the primordium formed is proportional to $\pi r^2 L$, where r is its radius and L its length. Therefore the L in the definition is proportional to the mass of the regenerating tissue. It is thus seen that two distinct factors are combined in the ratio, one being the mass of tissue transformed into hydranth and the other, the time necessary to arrive at a particular morphological event in the process. It is clear that the rate of regeneration may be varied either by affecting separately the mass of tissue involved, or the time to constriction or both simul-

taneously. There is, therefore, no *a priori* reason for believing that regeneration rates modified by different reagents or even various concentrations of the same reagent are directly comparable since the same resultant rate could be obtained in several different ways involving entirely different mechanism. If this is true then similar effects on rates by two agents does not necessarily imply that they are acting through the same mechanism.

To test whether a separation of the mass and time factors is experimentally obtained experiments were carried out with various respiratory inhibitors. Two representative experiments are cited.

In the case of ethyl methane over a concentration range which yielded a 67 per cent decrease in the time factor, the mass factor was increased by 11 per cent. The resulting effect of course being a decrease in the L/t curve. In the case of cyanide within a concentration range which yielded over a 200 per cent decrease in the l/t factor, no significant change occurred in the mass.

It is possible to interpret these results in terms of a synthetic reaction requiring an energy source in a system approaching the steady state. However, whatever be the final interpretation of the data, it is obvious that under the circumstances the l/t definition of rate so commonly used is inadequate, and if the whole story is to be obtained both the mass and the time must be considered.

August 18

The contractile mechanism in unicellular chromatophores (melanophores of Fundulus). Douglas A. Marsland.

Contractility in the melanophore, whereby the pigment granules are withdrawn from the numerous branches and concentrated in the center of the cell, appears to depend upon a gelation of the protoplasm (plasmagel). As in the case of amoeboid movement, the streaming movements of plant cells and cleavage movements of egg cells, contractile properties develop in proportion to the degree of protoplasmic gelation.

Hydrostatic pressure in the range up to 8,000 lbs. per in.^{2} limits the gelation capacity in a fashion that parallels the inhibition of contraction. This is true not only in the steady contraction induced by KCl and adrenalin solutions, but also for the alternating contractions which appear when pulsation of the melanophores are induced by the Spaeth method. The pressure effect is the same regardless of whether or not there is a survival of the nerve fibres which normally initiate the expansion and contraction of these melanophores.

The contracting effects of KCl and adrenalin can be counteracted by reducing the temperature to 6° C., and the expanding effects of NaCl, acetylcholine and physostigmine solutions are cancelled at 32° C. To appreciate the significance of these results it must be remembered that all of the protoplasmic gels thus far studied display a behavior opposite to that of gelatin. All the protoplasmic gels have their counterpart in such gels as myosin and methylcellulose in which gelation is fostered by increasing temperature, and by decreasing pressure.

The stiffness of the protoplasmic gel in melanophores is considerably greater than in most other cells previously studied. However, with a centrifugal force of 80,000 × greater one can always demonstrate a displacement of the pigment granules in expanded specimens, but never in contracted ones.

Comparing the melanophore to an amoeboid cell,—the clear region which can be seen in the center of expanded and half-expanded specimens, probably represents the plasmasol, which is surrounded by the pigment-laden plasmagel. During the contraction of the plasmagel it appears as though the hyaline plasmasol is squeezed forth into the outlying branches of the cell, replacing the pigmented plasmagel as it retreats from these branches.

Aggregation of separate cells of Dictyostelium to form a multicellular body. Ernest H. Runyon.

The fruiting body of Dictyostelium is fungus-like, consisting of a multicellular stalk anchored to the substratum, and a terminal mass of spores; but each cell of this structure was previously an independent protozoan-like amoeba (myxamoeba). Thousands of amoebae, after a period of feeding and multiplication come together in characteristic patterns forming a pseudoplasmodium, in which there are no cell fusions, but nevertheless as has been well demonstrated by Raper, definite co-ordination and dominance. The pseudoplasmodium is the immediate precursor of the fruiting body.

Study of the aggregation phase was found to be facilitated by the use of non-nutrient agar over which are thinly spread the amoebae, previously washed, concentrated, and roughly separated from the associated mass of bacteria by centrifuging. Size, pattern, and rate of development of aggregates are much affected by the thickness of the film or layer of water in which the amoebae are dispersed. If the culture is relatively dry (the aqueous film very thin) collecting points (centers) are many and aggregation progresses rapidly. In thicker films aggregation is slower, centers fewer, and the amoebal strands leading to the centers longer and less compact. Under these conditions, also, spiral aggregation patterns are frequent. Under water 0.2 to 0.5 mm. deep the aggregation pattern is a network of amoebal clumps connected by much elongated but loosely placed amoebae; there are no well delimited centers nor compact strands. The aggregation influence seems to be diffuse. Under water deeper than 1 mm. aggregation has not been observed, but the relatively small proportion of the amoebae that comes into the surface film does aggregate there. Potts in 1902 recorded fruition of Dictyostelium under oil. The pattern and rate of aggregation under a layer of paraffin oil is much the same as without the oil: the collecting points are many and aggregation progresses rapidly. Aggregation with center determination occurs under a glass cover slip or dialyzing membrane (Visking) placed over the amoebae on agar. Amoebae which are on top of such a dialyzing membrane become oriented corresponding to patterns of aggregation below the membrane: the amoebae on top accumulate along strands and centers below the membrane. Thus it seems likely that the determination of centers of aggregation depends upon the diffusion of a substance that is water- but not oil-soluble, active in thin films, and of a molecular character such that it can pass through a dialyzing membrane.

The structure of biologically active membranes. Dorothy Wrinch.

The membranes under consideration are plasma membranes, flexible and highly specific systems containing a protein component. Plainly we must assume that the proteins are in their native state, since only then is high specificity evinced. Further the membranes must be continuous with the cytoskeletons within the cell, so that theories attributing to the membranes structures of radically different types from those of protoplasm are necessarily excluded. Actually, extending to the membranes the type of structure already proposed for the cytoskeletons, on the basis of the newer knowledge of native protein structure (Wrinch, *Cold Spring Harbor Symp.,* 1941), the required properties result.

Thus native proteins are characterized by their ability to form associations. On this view the membranes consist of native proteins linked to form a surface skin, continuous with the interior cytoskeletons. Interlinks are of two types, less polar links where the hydrocarbon groups cushion together and more polar hydrogen bridges or ion rivets which are known to impose a rather open structure showing that our membrane should have pores. The passage of molecules will be distinctively different in the two cases. Growth, proceeding by the incorporation of new molecules, must exhibit the highest specificity and this "activity" the most

characteristic feature of all living systems may be studied as an expression of the high and specific associability of native proteins.

This picture of membranes as surface networks of native proteins suggests a new line of experimental attack, in which *the native protein* is the central theme. This theme cannot be set aside lightly, for the proteins are ubiquitous and their structure-determining powers in native form are approached by few other molecular species and rivalled by none. Given a fuller extension of Langmuir's powerful techniques, there is no apparent reason why these experiments should not be extended to yield information as to the formation of mixed films of native proteins and other biologically significant ions and molecules.

August 25

Experimental modification of molts and coat-color-changes by controlled lighting of the short-tailed weasel. Thomas Hume Bissonnette and Earl Elmore Bailey.[1]

For over three years, two short-tailed weasels (*Mustela cicognanii cicognanii* Bonaparte), similarly fed and cared for, under temperatures 40–70° F., were subjected alternately to controlled daily periods of available light. Both maintained normal molts and color cycles on normal light-cycles. Normal pelt-cycles were resumed following return to normal days after experimental light-manipulations. Molting, followed by growth of more or less white pelt, followed shortening daily light periods, even in March–April, when control, in the same room, on normal days turned brown. While experimentally lighted, one passed a summer in piebald coat, after incomplete color-change in both directions, and in almost complete white the next summer on normal days, followed by normal autumn molt to complete white and spring molt to nearly complete brown coat. One whitened in October, on short days, and returned to brown in December, on long days. The other responded likewise under similar light-manipulation. Gradually or suddenly shortened days induced whitening; lengthening days, brown, regardless of previous color. Molting was stopped short of completion by appropriate lengthening or shortening of daily periods of available light. Temperature-changes did not condition these differences. Changes in daily lighting constituted the major factor inducing and controlling these molts and color-changes in these Pennsylvania-bred weasels, studied in Connecticut.

Thiamin (vitamin B_1) deficiency in the cat. Sixteen millimeter film. Guy M. Everett and Dietrich C. Smith.

A sixteen millimeter motion picture film was made showing the behavior of two cats during the development of thiamin deficiency. After three weeks on the vitamin B_1 deficient diet, during which time the two cats lost approximately 20 per cent of their body weight, the animals showed muscular weakness and marked disturbances in postural tone. Walking was awkward and unsteady. The righting reactions were much impaired and pupillary constriction in bright light was lessened. Interest in food had ceased. At this time any slight stimulus may have produced a convulsive seizure lasting several minutes. Both clonic and spastic spasms occurred.

Upon the injection of one milligram (333 units) of thiamin chloride the animals showed within an hour improved muscular coordination and a return of

[1] Aided by grants from the Penrose Fund of the American Philosophical Society, 1939, and the American Academy of Arts and Sciences, 1942.

appetite. Convulsive seizures could no longer be evoked. In twenty-four hours recovery was practically complete.

Papers by Title

Permeability of Pelomyxa carolinensis to water. W. H. Belda.

Pelomyxa carolinensis (Wilson) is a multinucleate amoeboid organism of relatively large size. The changes in volume which occur when specimens are kept in solutions of different osmotic concentration can readily be measured.

A culture of Pelomyxa was obtained from the General Biological Supply House, Inc., which designates this organism *Chaos chaos*, Schaeffer, Strain A. The specimens were grown in Hahnert solution in glass finger bowls and were fed on *Stentor coeruleus*. At intervals, large specimens of Pelomyxa were put into fresh Hahnert solution for 48 hours, until all food vacuoles had disappeared, and the volume of each was measured by means of a volumescope. This apparatus consists of a glass tube having a bore about 0.25 mm. in diameter. When a specimen of Pelomyxa is drawn by suction into the tube the organism becomes cylindrical in shape. Its volume is then directly proportional to its length.

Specimens kept in Hahnert solution without food showed an average decrease in volume of 8% every 24 hours.

Specimens transferred to 0.1 M., 0.2 M., and 0.3 M. solutions of non-electrolytes (mannitol, erythritol, and lactose) in Hahnert solution, and to distilled water underwent various changes in volume, as shown by the following table. Each quantity represents the average volume of 50 or more specimens in terms of per cent of the original volume in Hahnert solution.

Medium	Time in hours after transfer					
	1	2	4	6	12	24
0.1 M. non-electrolytes	92.7	91.1	84.6	80.3	74.5	69.3
0.2 M. non-electrolytes	82.6	73.2	59.4	51.9	29.9	33.2
0.3 M. non-electrolytes	78.1	63.8	disintegrated			
Distilled water	108.2	102.5	103.9	97.5	99.6	

After having been kept in 0.1 M. solutions of non-electrolytes for periods of from one to 12 hours, the specimens usually recovered their original volume if they were returned to Hahnert solution for an equal period of time. Specimens which had undergone shrinking in higher concentrations did not recover their original volume completely.

The rate of output of fluid from the contractile vacuoles of Pelomyxa was measured. Specimens kept in Hahnert solution eliminated 3.8 per cent of their total volume per hour. In distilled water the rate of output was 6.4 per cent of the total volume of the organism per hour. After transfer from Hahnert solution to 0.1 M. solutions of non-electrolytes the output of fluid from the contractile vacuoles decreased rapidly to nearly zero within one hour.

The value of k, the permeability constant for water in *Pelomyxa carolinensis*, was calculated. Under various conditions of swelling and shrinking k was usually between 0.02 and 0.03 cubic micra of water per square micron of surface per minute for each atmosphere of pressure.

The effect of thiamin chloride on the oxygen consumption and the development of Arbacia punctulata at different stages. Matilda Moldenhauer Brooks.

Experiments on the rate of O_2 consumption of different stages in the development of Arbacia as affected by different concentrations of thiamin chloride were done using the Warburg-Barcroft method. Fourteen concentrations were used from 2×10^{-3} to 2×10^{-11} and from 4×10^{-5} to 4×10^{-9}, diminishing in value by the factor of 10. The stages used were unfertilized eggs, fertilized eggs, early cleavages, morula, blastula, gastrula and pluteus at a temperature of 15.9° C. There was no change in the rate of O_2 consumption in any of these experiments during three to four hours in which the experiments were conducted.

The rate of development appears to have been affected. Thiamin chloride, in concentrations of 1×10^{-7} and 10^{-8} was added as follows to eggs in sea water: (A), immediately before fertilization; (B), immediately after; (C), at the 2 to 4 cell stage; (D), the morula; (E), the blastula; (F), the gastrula; (G), the pluteus. All experiments were done under similar conditions as to egg suspension, volume of solution, temperature (running sea water.) At the end of 24 hours (A) contained 50% gastrula and 50% pluteus, while the controls contained 95% gastrula and 5% pluteus; in (B) there were 100% pluteus; in (C), 100% pluteus. The size of the pluteus from the tip of the spine to the end of the larva was measured, in (A) to (F). These showed a length of .35 mm. in 3 days as compared with .27 mm. in the controls. They remained larger for a longer time than the controls and lasted 14 days in (G) as compared with 6 days (controls).

These experiments show that there is no direct relation between the utilization of thiamin or the rate of development, and the O_2 consumption; there appears to be a direct relation between the intake of thiamin and the rate of development and length of life especially marked in the early stages and the pluteus.

It would appear that the oxidation end of the redox scale in metabolism is not directly affected by thiamin chloride.

Intake and loss of phosphate ion by eggs and larvae of Arbacia and Asterias. S. C. Brooks.

This material was immersed in and sampled from a beaker, or each sample taken from a separate dish, or flooded in a Buchner funnel, using radioactive sodium phosphate in low concentrations (0.175–0.81 mM. L^{-1}). The samples were separated by washing with isotonic erythritol (which removed much of the radioactivity), or simply by hard centrifugation, and the phosphate content of eggs determined by counts. The radioactivity as measured with a counter was translated into concentration of the phosphate ion.

It was found that phosphate had often been absorbed in two distinct periods separated by a period of loss of this ion. Assuming arbitrarily that the concentration was the driving force during intake, it is found that the early rate indicates a permeability for Arbacia of $36–70 \times 10^{-10}$ and for Asterias 2280×10^{-10} GM. $cm.^2$ $hr.^{-1}$ $(GM.L^{-1})^{-1}$. For the late intake, values of $2.2–70 \times 10^{-10}$ were found. The maximum concentrations found in the eggs or larvae of both vary between 0.0050 and 8.3 mM. L^{-1}. Inverse correlation was intimated by the data between concentration or radioactivity and permeability. Tests of intake of radioactive sodium were done. Full publication will discuss the effects of the β-radiation (the sole radiation of P*), the part played by the stage of the egg or larvae, the effects of the methods used, the dimension of different ions and theories of absorption.

Oxygen consumption in caffeinized Arbacia eggs. Ralph H. Cheney.

As a fundamental step in a general investigation regarding the effects of caffeine upon the reproduction and growth of animals, experiments were conducted

to determine the action of caffeine directly upon the micro-respiration (in terms of oxygen consumption) of the fertilized eggs of *Arbacia punctulata*. Mature eggs were fertilized and prepared in the form of a 35 per cent suspension in sea water. The following percentages of caffeine-in-sea-water were employed: 2, 0.5, 0.2, 0.1, 0.02, 0.004 and 0.002. Five-tenths of one cubic centimeter of the 35 per cent suspension of fertilized eggs was used in all experiments. Sea water or the proper percentage of caffeine-in-sea-water was added to Warburg flasks so that a total volume of 2 cc. of fluid was maintained in all vessels. Experiments were conducted at 25° C. The determination of the oxygen consumption per hour for a total of three hours was made by means of the Warburg manometers and method.

Total oxygen consumption of controls and the experimental series was plotted as a time relationship curve in cu. mm. of oxygen consumed over a three hour period. Variations from the untreated controls were from 0.5 to 61 per cent. Changes of less than 10 per cent, whether inhibitory or stimulatory, were considered not significant in biological material of this sort. The oxygen consumption of fertilized eggs of Arbacia was inhibited by concentrations equivalent to 0.1 per cent or above. In the series employed, lower percentages caused a variation of 10 per cent or less and were interpreted as not significant.

A typical experiment (number 20 of the series) inhibited the oxygen consumption over a three hour period statistically as follows: 2 per cent caffeine-in-sea-water reduced oxygen consumption 60.6 per cent in comparison with controls, 0.5—58.6 per cent, 0.2—41.4 per cent, 0.1—22.9 per cent reduction. Eggs were examined with 100 × magnification after each experiment to check the cleavage stage in each vessel against the controls and against the corresponding percentages in other parallel experiments of the series.

Caffeine effect on fertilization and development of Arbacia eggs. Ralph H. Cheney.

To determine the extent of the effect of caffeine upon the ability of the egg to become fertilized and of the sperm to accomplish fertilization, eggs and sperm were shed separately into caffeine-in-sea-water and into normal sea water. The germ cells could then be mixed as desired in the various combinations available. The production of the fertilization membrane and subsequent cleavage stages developing in time periods comparable to the normal controls were studied. Normal time periods for the different developmental stages were accepted as presented by E. B. Harvey (*Biol. Bull.*, **79** (1): Plate II, photographs 16–32 inclusive, August, 1940). The following percentages of caffeine-in-sea-water were employed: 0.00, 0.002, 0.004, 0.02, 0.04, 0.1, 0.2, 0.5, 1.0 and 2.0. Eggs and sperm were shed into the normal sea water or into each of the above concentrations separately. They remained in the solution in which they were shed for fifteen minutes prior to mixing in any combination.

For each percentage used in the series, the combinations of normal and caffeinized eggs and sperm were mixed as indicated below:

1. Normal non-treated egg × normal non-treated sperm and development allowed to occur in normal sea water. These served as controls for each day's run.
2. Normal egg × caffeine-treated sperm. Development in sea water.
3. Caffeinized egg × normal sperm. Development in sea water.
4. Caffeinized egg × normal sperm. Development in sea water—caffeine.
5. Caffeinized egg × caffeinized sperm. Development in sea-water.
6. Caffeinized egg × caffeinized sperm. Development in sea-water—caffeine.

Experiments were conducted at 25° C. An examination of the eggs was made at intervals of 10, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180 minutes and at longer periods up to two to three days or until either pluteus larvae developed or cytolysis occurred.

Plutei developed in normal *time* and *form* in all combinations of 0.002 per cent and 0.004 per cent caffeine-in-sea-water. All combinations except the caffeinized egg × caffeinized sperm developed in caffeine-in-sea-water in 0.02 per cent and 0.04 per cent, developed plutei of normal form but in delayed time. In 0.1 per cent and 0.2 per cent cleavage was arrested at various stages and the echinochrome pigment became localized. Higher percentages affected the egg so that the cleavages became progressively more abnormal and the arrest of division occurred at an increasingly earlier stage. Two percentum prevented the formation of a fertilization membrane in combinations numbers four and six. There were many evidences that the egg was more sensitive than the sperm to the action of caffeine.

Effects of irradiated water on Arbacia sperm. Titus C. Evans.

In previous investigations on the influence of the medium on the radiosensitivity of Arbacia sperm, check experiments using irradiated water showed no effect on unirradiated sperm. Recent attempts have been made to determine whether slight amounts of a toxic agent produced in sea water irradiated with very large doses of x-rays could be detected. Positive results have been obtained with doses of the order of 100,000 roentgens; using very dilute concentrations of sperm (1 : 4000); and allowing the sperm to stay in the irradiated water for a sufficient length of time. The irradiated sea water under these conditions caused the sperm placed therein to die sooner than the control sperm in sea water. In addition, it was found that sperm kept in irradiated sea water until approximately 50 per cent had died, caused a delay of about 10 minutes (at 22° C.) in the time of cell division of eggs fertilized therewith.

The deleterious agent produced by the radiation in the sea water is apparently hydrogen peroxide. Tests with titanic chloride reveal a very faint color change in heavily irradiated sea water which roughly corresponds to a concentration of hydrogen peroxide between 1 : 1,000,000 and 1 : 2,000,000. Hydrogen peroxide in a concentration of 1 : 1,000,000 affected the sperm in much the same way as did the irradiated water.

Protein metabolism and embryonic growth rates. Otto Glaser.

Nitrogen in chicks (Glaser) supports Schoenheimer's conclusion that protein metabolism in growing organisms and adults differs mainly in the ratio synthesis/destruction. Temporary storage of blood proteins or the capacity to retain more than usual quantities of particular amino-acids (van Slyke: Riker) fail to demonstrate storage of amino-acids unless as substituents in existing proteins or as building blocks specifically located in new molecules.

With certain native proteins immediately or indirectly responsible for all other accumulations and syntheses, the only amino-acids that control our measurements are the ones that settle in appropriate locations. Granted adequate income, the number of amino-acid units that settle during a given time equals the number of available locations. During development, progressive cell differentiation reduces the rate at which new locations are produced. Hence the rate of amino-acid settlement should decline by steps proportional to differences between the squares of consecutive whole numbers (Glaser).

This fact, usually obscured by heterogonic diversifications, leaves its imprint on properly timed growth rates. In the linear equation

$$\log w = k \log(2t + 1) + C,$$

C represents a weight at zero time, w a weight $2t + 1$ conventional time units later and k, the rate. Here the time-scale has intervals systematically proportional to differences between the squares of consecutive whole numbers. By this scale k for the entire chick equals k for its genetic proteins. For isolable organs and native

chemical fractions, the k's have ratios identical with the corresponding allometric constants. The only processes to which this numerical system can plausibly apply, are protein maintenance and synthesis. Moreover, a metrical system so specifically implicating surfaces would be exceedingly unlikely unless the native protein molecule is organized as a compact three-dimensional structure, carrying its many specificities on a well-defined surface.

Rate of breaking and size of the "halves" of the Arbacia punctulata egg when centrifuged in hypo- and hypertonic sea water. Ethel Browne Harvey.

Arbacia eggs kept in hypotonic (60 per cent, 80 per cent) sea water break less rapidly and those kept in hypertonic (125 per cent) sea water break more rapidly than those in normal sea water when centrifuged in sugar solutions of corresponding tonicities at 10,000 × g. The break occurs so that the red (granular) "half" is practically the same size in all the solutions, and the increase (in hypotonic) and decrease (in hypertonic) in size is in the white (clear) "half."

Some observations with a simplified quartz microscope. George I. Lavin and Arthur W. Pollister.

A quartz microscope has been assembled for work with the mercury resonance line 2537 Å. The light source is a quartz mercury whose output is about 90% 2537 Å. The visible light is removed by means of a liquid filter (mixture of $CoSO_4$ and $NiSO_4$) described by Bäckström, 1940 (*Arkiv. for Kemi. Mineralogi Och Geologi,* **13**: 1). In operation an image of the material to be examined is focussed on a synthetic willemite screen which is fastened, face down, to the usual ground glass holder. It has been found that when the image is in focus on this screen it is in focus when a plate is taken with the 2537 Å line as the light source. This line is useful in biological work because it is close to the region of maximum absorption of the purine and pyrimidine components of nucleic acid. Photographs of fibers of nucleohistone show intense absorption. Photographs of liver cells that have been treated by a method known to remove thymus nucleohistone shows that the nuclei have lost practically all of their nucleic acid, since they absorb very slightly. Living spermatids of insects have been photographed. The mitochondrial body or nebenkern (a sphere 20 micra in diameter in *Notonecta undulata*) absorbs no more intensely than does the surrounding cytoplasm; so the indications are that the mitochondria are poor in nucleic acid. The Golgi bodies (acroblasts) show appreciably more absorption than the general cytoplasm.

Pituitary function in the chromatic physiology of Opsanus tau. Richard E. Lee.

The melanophore and xanthophore pigments of the toad-fish, *Opsanus tau*, become dispersed over a black background and assume a partially concentrated condition over a white background. The chromatophores of denervated bands behave similarly under these conditions, but their pigment migration is much slower and less extensive than that found in the innervated cells.

Hypophysectomy has no apparent effect on this ability of toad-fish to change color. Likewise, denervated chromatophores of hypophysectomized specimens will repeatedly disperse their pigments after having assumed a concentrated state in response to white backgrounds or to electrical stimulation of the denervated region. The ventral integument of hypophysectomized animals, however, contains approximately 50 per cent of the usual number of melanophores; and 20 to 30 per cent of these remaining cells generally show signs of degranulation and melanin loss.

Biological assay of toad-fish pituitaries reveal that the average gland contains 525 frog units of melanophore dispersing principle. At least 1300 frog units of this medium (obtained from toad-fish pituitary glands or commercial pituitrin) are necessary to produce a slight transitory dispersion of all toad-fish chromatophores within a radius of 0.5 cm. about the point of a subcutaneous injection.

It is concluded that the toad-fish pituitary gland functions to sustain coloration by pigment maintenance, but it has no apparent role in the more overt "physiological" color changes of this animal.

The interfibrillar material in the central nervous system of mosquito larvae (Culex pipiens). A. Glenn Richards, Jr.

Sudan dyes used as indicators of the distribution of oil solvents (e.g., xylol) via the tracheal system give bright coloration to only the central nervous system. The concentration of dye agrees with the known concentration of intercellular and interfibrillar material. Ganglia just beginning to be stained by the above method show that the dye diffuses from the small tracheae and then along the fibers. These data suggest an interneuronal lipoid but these nerves are not medullated since they do not reduce osmic acid except after heat fixation. After formalin or acetic acid fixation this lipoid is stained as a non-resolvable diffuse color along fiber tracts and between cells, especially with Black Sudan B. It can be similarly stained by brief immersion of living ganglia in the alcoholic stain but alcohol soon releases Sudan-stainable particles (mostly $< 10\,\mu$) which are readily removed by strong alcohol and slowly by weak alcohol or water. These particles have irregular shapes, a high melting point ($> 100°$ C.) and negative birefringence. Clearly they are different from the lipoids of adipose tissue. This substance seems to be a bound phospholipid.

Data published in a previous paper show histological degeneration of the fiber tracts of the central nervous system due to toxic petroleum oils. One of the effects of such oils seems to be the destruction of this interfibrillar material. It is suggested that this material permits the rapid penetration of oils and oil-borne toxins into the central nervous system. If this substance is a phospholipid its breakdown may under some circumstances produce degeneration products which are neurotoxins.

A fourteen-day rhythm in the left-right spiraling ratio of the common marine ameba Flabellula citata. A. A. Schaeffer.

In common with other species of amebas and human leucocytes (polymorphs), the common marine ameba moves around a capillary glass tube in a helical spiral path. A part of the path is twisted in a right spiral and a part in a left. The amount of the path to the left as compared with that to the right is the left-right ratio.

This summer daily records of the left-right ratios of Flabellula from a pure line culture (27.7° C.) were obtained. When these ratios are plotted vertically on cross section paper with the days as a horizontal time scale, pronounced 14-day rhythms are observed in the ratios. These rhythms sweep diagonally across the graph from the left to the right. Each rhythm takes about 14 or more days for completion. For the greater part of the time two rhythms are present at the same time. The ratio of the amebas in any one rhythm become more right as time goes on, at the rate of .1 each day. That is, if the ratio is 1.7 left to 1 right on July 24, then on July 25 the ratio will be 1.6 : 1. When this rhythm reaches a ratio of about 1 : 2 right, it stops and a new rhythm appears with a predominant left ratio. Thus, while the rhythm from left preponderance to right preponderance is steady and continuous, there is visible no return rhythm from right to the new left rhythm. That is, the rhythms are discontinuous as represented on the graph.

The presence of a 14-day rhythm in this ameba is of great interest, for two years ago a 14-day rhythm was found in human leucocytes at normal temperature (37.6° C.). This and similar earlier researches are guided by the hypothesis that the chief controlling mechanism consists of two stereoisomers of which the left one is more labile and breaks down at a constant percentage rate.

The early embryonic development of Amaroecium constellatum. Sister Florence Marie Scott.

Amaroecium constellatum produces heavily yolk-laden eggs that develop to the free-swimming stage within brood spaces in the adult. The first cleavage divides the egg into a smaller left blastomere and a larger right blastomere. This disparity in size of cells from left to right continues through subsequent divisions. The second cleavage separates two larger anterior cells from two smaller posterior cells. The third cleavage produces four micromeres at the animal pole and two heavy macromeres at the vegetative pole.

In the sixteen cell stage the embryonic areas may be distinguished. They correspond to the areas of the less heavily yolked Protochordates, Styela and Amphioxus, consisting of an anterior chorda-neural and a posterior mesodermal crescent enclosing an endodermal area. The areas are delineated at the animal pole, the vegetative hemisphere being composed of the yolk macromeres.

Gastrulation is initiated at the 22-cell stage. Divisions on the right side lag behind those on the left. The smaller size of cells and the accelerated rate of division of the mesodermal cells on the left concur to facilitate invagination on that side whereas the mass of yolk and slowed rate of cleavage interfere with ready invagination of mesoderm at the ventral and right lateral lips. The chorda-neural cells divide into their derivatives, the chordal cells turning in between the endoderm and the neural plate. The endodermal cells change in shape and they become slightly depressed but there is no invagination. By the approximation of neural cells and the ectoblastic derivatives of the ventral lip the blastopore closes.

The neural cells reacting, possibly, to the organizing influence of the leftwardly directed mesoderm grow down on the left side of the embryo. The neural plate thus curves through an angle of 90°. The dorsal region differentiates into the brain vesicle and ganglion, the lateral part into the neural tube. The neural tube lies to the left of the notochord which grows directly backward from the ventral lip differentiating partly from chordal cells and partly from cells enclosed by the process of overgrowth. The first mesoderm to invaginate at the lateral lips becomes mesenchyme. The remaining mesoderm is converted into muscle cells of the tail.

Endoderm, lacking an archenteron, is established definitely by the process of overgrowth, mesoderm by the process of invagination. The asymmetry of the nervous system obtains from interference with invagination at the ventral lip. The pattern of differentiation in Amaroecium in the same as that of the less heavily yolked Protochordates but the yolk introduces modifications of the plan that alter gastrulation by interfering with the convergence of morphogenetic substances toward the median axial plane.

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THE CULTURE OF EUDIPLODINIUM NEGLECTUM, WITH EXPERIMENTS ON THE DIGESTION OF CELLULOSE

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In the fermentation which occurs in the rumen of cattle and related forms there is a significant decomposition of cellulose (Tappeiner, 1884). Microscopic examination of the rumen contents discloses a microcosm teeming with protozoa and bacteria. Certain among these are the agents responsible for the cellulose digestion. However, identification and isolation of the cellulose-digesting organisms have proved extremely difficult.

Becker, Schulz, and Emmerson (1929) and Winogradow, Winogradowa-Fedorowa, and Wereninow (1930) defaunated ruminants and found no decrease in the amount of cellulose digested in their alimentary tracts. Margolin (1930) and Westphal (1934) succeeded in growing the rumen protozoa in mass cultures for several weeks and concluded that they did not digest cellulose. On the other hand, Schuberg (1888), Braune (1914), Schulze (1924), and Trier (1926) concluded from microscopic observations that cellulose was digested, and Weineck (1934) has described microchemical tests which indicate that sugar is present around cellulose particles in the digestive sack of certain rumen protozoa.

The present investigation was undertaken in order to develop methods for growing the rumen protozoa and to obtain further evidence on their capacity to digest cellulose. Because of the similarities between the mode of life of the termite protozoa and those in cattle it seemed desirable to test on the cattle ciliates the effect of factors known to be important in culturing the termite flagellates. These factors are: the presence of cellulose, proper concentrations of the inorganic salts in the culture medium (especially sodium chloride), anaerobic conditions, a suitable pH, and the concentration of protozoa (Trager, 1934; Hungate, 1939, 1942).

Culture Experiments

1. *The Effect of Sodium Chloride Concentration*

Powdered cellulose was obtained by treating absorbent cotton for several days with a strong solution of hydrochloric acid until the cotton broke up into small particles. The cellulose particles were filtered off, washed free of chlorides, air dried, and ground in a mortar. A solution of inorganic salts in tap water was prepared as follows: 0.1 per cent $NaHCO_3$, 0.1 per cent KH_2PO_4, 0.01 per cent anhydrous $MgSO_4$, and 0.01 per cent anhydrous $CaCl_2$. Thirty milliliters of the solution were placed in each of ten 50-milliliter Erlenmeyer flasks containing a few milligrams of the powdered cellulose. Sodium chloride was added to give a series of concentrations ranging from 0.1 to 1.0 per cent in increments of 0.1 per cent. The flasks were placed in a water bath at 38° C. and an oxygen-free gas mixture containing 95 per cent nitrogen and 5 per cent carbon dioxide was bubbled through each vessel. One-half milliliter of fresh rumen contents containing numerous protozoa of many species was added to each flask during the latter part of the bubbling. The flasks were then stoppered tightly with a rubber stopper and incubated in a water bath at 38° C. They were inspected daily for protozoa by holding them up toward a ceiling light and examining the bottom with a hand lens. After three days the protozoa were dead in all flasks except the ones with 0.5, 0.6, and 0.7 per cent sodium chloride. In these a few large protozoa could be seen actively swimming about.

Microscopic examination of a sample of the culture showed that the protozoa resembled *Diplodinium* (*Eudiplodinium*) *neglectum* of which several forms were described by Dogiel (1927). Considerable variations in the size and shape of the protozoa in the laboratory cultures have been observed and individuals similar to the forms *dilobum*, *monolobum*, and *bovis* have been noted. Kofoid and MacLennan (1932) considered these forms to be distinct species of a new genus, *Eremoplastron*. However, Poljansky and Strelkow (1934) have shown that the forms, *bovis* and *monolobum*, occur in clones obtained from a single individual of the form *bilobum*. For this reason it seems preferable to include them all in the species *neglectum*, subgenus *Eudiplodinium*, as arranged by Dogiel. The cultures used for experiments on cellulose digestion contained all three forms.

The freezing point depressions of the fluid portions of the rumen contents of two cows were determined, the values obtained being 0.54 and 0.59° C., respectively. The freezing point depression of the inorganic solution containing 0.6 per cent sodium chloride was found to be

0.47° C. The similarity in osmotic pressure between the optimum inorganic solution and the rumen contents suggests that the sodium chloride concentration is important because of its osmotic effect. In all the following experiments a concentration of 0.6 per cent sodium chloride was used.

2. *The Reaction of the Medium*

Six flasks containing the inorganic medium plus cellulose were adjusted with sodium hydroxide or hydrochloric acid to pH's of 7.6, 7.1, 6.6, 6.1, 5.6, and 5.1, respectively, using the glass electrode. The experimental gas (oxygen-free nitrogen and carbon dioxide) was bubbled through the cultures while the pH was adjusted. Each flask was brought to a temperature of 38° C. and then inoculated from a sample of rumen contents. Examinations for survival were made at frequent intervals. The protozoa in cultures with an initial pH ranging from 6.1 to 7.6 survived for about nine days. In the cultures with a pH of 5.6 and 5.1 the survival time was reduced to five days and one day, respectively. Later experiments have shown that the protozoa die in cultures having an initial pH of 8.0. It has also been observed that in old cultures the protozoa become inactive when the pH drops to 5.5.

In this experiment again the medium appeared to be selective for *E. neglectum* as this was the protozoan which survived longest.

3. *Size of the Inoculum*

The influence of the size of the inoculum on the longevity of the protozoa was investigated by adding varying amounts of rumen fluid to fixed quantities of inorganic medium plus cellulose. With an inoculum equal to one-third to one-half the volume of the inorganic medium the time of survival of the protozoa was much less than with an inoculum of one-half milliliter in 30 milliliters of medium. The effect was not due to exhaustion of the cellulose substrate which was present in excess. It may be accounted for by the accumulation of metabolic products which reach a toxic concentration sooner with a large than with a small inoculum. Toxic metabolic products are of particular importance where fermentative organisms such as the cattle protozoa are studied, for under anaerobic conditions acids such as formic, acetic, and lactic are formed in relatively large quantities. Slight concentrations of these acids are often toxic.[1]

[1] Levine and Fellers (1940) have reported upon the inhibiting effect of acetic acid on *Aspergillus niger*. The rapid death of Colpidium, noted by Hall (1941) when exposed to an acetate buffer can be explained as due to the toxicity of the acetic acid present. Elliot (1935) noted that acetic and butyric acids were toxic to the proto-

When very small inocula are used the protozoa often die within a few hours. The factors causing this have not been fully analyzed but death is probably due to toxic materials in the medium, among which oxygen may be mentioned. With larger inocula the combined protoplasts of the protozoa can absorb without permanent injury quantities of toxic materials that are fatal to one or a few organisms.

4. *Composition of the Organic Portion of the Medium*

Cellulose was used as a source of carbohydrate in the present experiments, since it was desired to study primarily any protozoa in the rumen which might be able to utilize this material. When *Eudiplodinium neglectum* was transferred from an initial culture of inorganic medium plus cellulose to a subculture of the same medium, no growth occurred. Evidently growth in the original culture was supported by materials in the inoculum, but in subcultures these materials were no longer present in sufficient quantity. In particular, it seemed probable that nitrogenous substances needed to be included in the medium.

Addition of 0.1 per cent ammonium sulfate did not improve conditions. Beef extract (0.003 per cent) and peptone (0.005 per cent) were added but subcultures still failed to show growth. Filtered rumen fluid was then included with the inorganic solution plus cellulose, and with this medium successful subcultures were obtained. Apparently the rumen fluid supplied requirements of the protozoa that were not met by beef extract and peptone. This is in agreement with the results of Westphal (1934) who concluded that factors present in the rumen were required for growth by the protozoa.

Since green plant parts are an almost universal component of the food of ruminants it seemed possible that the necessary requirements might be supplied by the grass or other green food consumed by these herbivores. This was tested by adding a little grass to the inorganic medium containing cellulose, beef extract, and peptone. The grass was added in the form of a dried powder prepared by drying and grinding fresh, green, winter grass (*Bromus catharticus*). Some cultures using this medium showed growth of Eudiplodinium but not all were successful and great variations in the numbers of individuals in surviving cultures were observed. It was then found that omission of the beef-extract and peptone improved the uniformity of the growth without sacrificing numbers of protozoa. Thus, a medium containing grass

zoan, Colpidium, whereas the salts of these acids exerted no influence or were slightly beneficial. Burnett (1940) observed that the pH range for growth of *Trichomonas termopsidis* is affected by citric acid. In its presence the optimum pH is shifted in an alkaline direction.

and cellulose as the only organic constituents proved entirely satisfactory.

Since grass contains some cellulose it seemed possible that grass alone might support growth of Eudiplodinium. However, omission of the cellulose resulted in death of the protozoa after a few transfers. Later it was found that grass alone would support the growth of the protozoa if they were transferred daily, but they did not become as numerous as when cellulose was added.

Experiments were performed to determine the optimum concentration of organic materials. It was found that dependable results were obtained with concentrations of 0.04 per cent grass and 0.04 per cent cellulose. Higher concentrations supported excellent growth for a few transfers, but it was difficult to maintain an optimum acidity and considerable variations in numbers resulted.

The following routine culture method was adopted. The inorganic medium was placed in a graduated cylinder in the water bath, and the oxygen-free gas mixture was bubbled through it for 15 minutes. Twenty milliliters of this solution were added to a 50-ml. Erlenmeyer flask containing 16 milligrams of cellulose and 16 milligrams of grass. Then 20 ml. of a vigorous *in vitro* culture of Eudiplodinium were added. Oxygen was displaced by bubbling the nitrogen-carbon dioxide mixture through the cultures. The flasks were then tightly stoppered and incubated at 38° C. Although the large inoculum used in these transfers proved unsatisfactory in the case of rumen contents, it was found that the large inoculum from an *in vitro* culture gave more uniform results, and it was not necessary to observe as great precautions in removing oxygen. The rumen contents contain a much greater percentage of organic material than the *in vitro* cultures and the bad effects of large inocula of the former may be ascribed to this factor.

Each culture undergoes a series of changes. The acidity increases from an initial pH of about 7.0 to one of 6.0 at the time of transfer. At first the cellulose and much of the grass rest on the bottom of the vessel. The protozoa swim actively about; many of them remain near the grass and cellulose but they also are found in the upper layers of the culture. As fermentation of the grass and cellulose progresses these materials tend to be carried up in the liquid by the bubbles of fermentation gases and in later stages of the culture are largely collected near the surface. The protozoa become less active and accumulate on the bottom of the flask. Subcultures should be prepared at this point or earlier because the protozoa begin to die after this time. In transferring the culture it was vigorously rotated to distribute the protozoa and undecomposed

substrate evenly throughout the medium. Twenty milliliters were then transferred to a fresh flask.

Using this procedure *Eudiplodinium neglectum* was cultured from March until June, 1940, when a failure in the temperature regulating apparatus caused the death of the cultures. In October, 1940, another culture was obtained from rumen contents and at the present time it has been maintained in the laboratory for 22 months.

Two species of grasses and one legume were tested to see if they were equally suitable for culturing *E. neglectum*. Italian winter rye (*Lolium italicum*) as well as *Bromus catharticus* supported excellent growth when the dried ground grass was used but bur clover (*Medicago arabica*) prepared in a similar fashion was unsatisfactory. With the latter the protozoa were dead after two transfers. An extract of the suitable grasses obtained either by boiling one minute in the medium or by soaking over-night could not replace the particles of dried grass.

Westphal (1934) reported success in maintaining suitable acidities by using urea in the cultures. Decomposition of the urea by bacteria liberated quantities of ammonia which neutralized the metabolic acids. In the present experiments a concentration of 0.1 per cent urea produced a reaction which was too alkaline. With a concentration of 0.02 per cent the protozoa survived well, but no better growth or regulation of acidity was obtained than when the urea was omitted. It is perhaps significant that in Westphal's experiments the species of Eudiplodinium did not survive throughout the culture period, only Entodinium showing marked growth.

5. *Numbers and Division Rate of Protozoa*

The protozoa in the routine cultures transferred at two-day intervals were counted just before transferring. The flask contents were thoroughly mixed and a 0.1 milliliter sample was withdrawn and examined under a dissecting microscope. The protozoa were counted as they were drawn up into a capillary mouth pipette. This gave an accurate count in the sample. The error due to sampling was of the order of ten per cent. The counts over a 14-day period are shown in the solid lines in Figure 1.

The curve shows the concentration of protozoa in each subculture and indicates which subculture was used for further transfer. The continuous line represents the continuous culture series.

The concentration of organisms at the time of transfer was maintained throughout the period in which counts were made and therefore it may be concluded that the protozoa divided on an average of once in 48 hours. The way in which the protozoa settled during the later

stages of the culture and the fact that they died if not transferred on schedule, suggested that the cultures were less favorable for growth in the later stages. Consequently it was anticipated that a more rapid division rate would be observed if the transfers were made more frequently. In order to test this, a second series of cultures with daily transfers was run in parallel to the first.

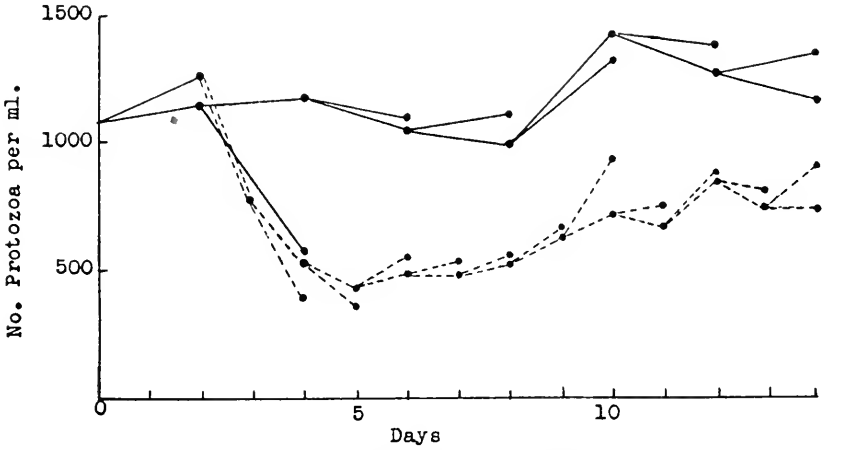

FIGURE 1. Concentration of protozoa.

The results of the second series are shown as broken lines in Figure 1. It may be seen that after an initial drop in concentration as compared with the 48-hour series the division rate increased sufficiently to maintain and even to increase slightly the concentration of protozoa at each 24-hour transfer. It follows that on the average a division occurred at least once each day.

6. *Purification of the Protozoa*

A preliminary attempt was made to grow the protozoa free of bacteria but it was unsuccessful. The wash methods which have been used to obtain pure cultures of other protozoa present special difficulties in the case of Eudiplodinium. It is necessary to maintain a suitable temperature of the wash medium and to prevent exposure to oxygen. Special anaerobic wash dishes were constructed and single protozoa were washed through as many as five transfers. They ceased moving at or before the fifth transfer and settled on the bottom. Such individuals failed to divide even though sterile grass and cellulose were present. On the other hand, in three different instances a single indi-

vidual transferred directly from a routine culture into one of the wash vessels containing sterilized cellulose and grass survived and gave rise to a thriving clone.

Physiological Characteristics

The Effect of Oxygen

The gas passed through the cultures described thus far consisted of 95 per cent nitrogen and 5 per cent carbon dioxide from which traces of oxygen were absorbed by the chromous oxygen absorbent described by Stone and Beeson (1936). The bacteria and protozoa present in the culture rapidly absorbed any traces of oxygen present initially or which might gain entrance. Thus, the protozoa grow in laboratory cultures under strictly anaerobic conditions. In their natural environment, the rumen, there is also an almost complete absence of oxygen.

Occasionally in the routine cultures the pressure in a flask due to gaseous fermentation products became so great that the stopper would be blown out, allowing free access of air to the surface of the culture. If left exposed for any length of time, the protozoa in such flasks were killed. Although presence of oxygen is the most probable cause, the death of the protozoa might also be explained as due to the growth of unfavorable bacteria, or to a change in acidity through loss of carbon dioxide.

A more convincing demonstration of the toxicity of oxygen was accomplished as follows: a gas mixture containing air and 5 per cent carbon dioxide was bubbled through samples of a thriving culture of Eudiplodinium and the effect compared with that of 95 per cent nitrogen-5 per cent carbon dioxide. Changes in acidity were the same since both gas mixtures contained 5 per cent carbon dioxide. Possible unfavorable influences exerted by aerobic bacterial growth were decreased by an experimental arrangement which permitted detection of the toxic effects of oxygen before appreciable bacterial growth could occur. The gas to be tested was brought into rapid equilibrium with the culture liquid by introducing it through a sintered glass plate of fine porosity. The plate extended across the middle of a 12-millimeter Pyrex glass tube which was immersed in the water bath to within a few centimeters of the top. The experimental gas supply was connected at the bottom of the tube, and by applying suction at the top the gas was drawn through the tube until it completely filled the part below the sintered glass. The suction was discontinued and 5 milliliters of a protozoan culture were added to the upper portion of the tube. Slight suction was then carefully applied so that a copious supply of fine bubbles of the gas passed up through the culture. At periodic intervals

the suction connection was removed and a sample of the culture withdrawn for microscopic examination.

When oxygen-free 95 per cent nitrogen-5 per cent carbon dioxide was passed through the suspension no decrease in motility of the protozoa could be detected after two hours. Using the same technique but with 95 per cent air-5 per cent carbon dioxide some of the protozoa retracted their membranelles and ceased moving after bubbling for five minutes. After ten minutes most of the protozoa were motionless and after 15 minutes no motile individuals could be observed. Similar rapid immobilization of the protozoa was obtained with alveolar gas which also contains oxygen and has a carbon dioxide content of about 5 per cent. This indicates that the protozoa are sensitive to oxygen and may be considered obligate anaerobes. They are catalase negative. When carefully washed samples of active protozoa from two cultures were treated with hydrogen peroxide no gas was evolved.

Cellulose Digestion

The fact that cellulose is a necessary ingredient of the culture medium for *E. neglectum* suggests that these protozoa use it as food. Microscopic examination shows that they ingest large quantities. However, cellulose-decomposing bacteria are present in the cultures and the beneficial effect of the cellulose as well as its ingestion might be due to its use by bacteria which in turn are fed upon by the protozoa.

Since it was not possible to grow the protozoa free of bacteria, tests for cellulose digestion by extracts of the protozoa from the crude cultures were performed. The two chief problems involved in obtaining an extract of the protozoa were (1) to raise them in sufficient numbers for extraction and (2) to free them of the cellulose-decomposing bacteria which were also present in the culture. The number of routine flask cultures of protozoa was increased to 32. Sixteen of these were used to supply protozoa for the experiments, while each of the remaining cultures was transferred to two subcultures.

Separation of the protozoa from the single bacterial cells offered little difficulty due to the large size differences. But many particles of grass and cellulose were about the same size as the protozoa and of almost the same density. Filtration and centrifugation did not accomplish a separation. After several trials the following techniques were developed and yielded protozoa almost entirely free of both large and small particles. Some of the powdered cellulose was ground with water in a pebble mill to give a very fine suspension. The upper 20 milliliters of culture medium in each of the 16 flasks used to supply protozoa was pipetted off, leaving the lower half containing the undis-

turbed protozoa. Then one-half milliliter of the fine cellulose suspension, 16 milligrams grass, and 20 milliliters of fresh inorganic medium were added, the cultures made anaerobic, and incubated. Practically all of the finely divided cellulose in these flasks was carried to the top of the medium by the fermentation gases and appeared as a cap just beneath the surface. In its rise it caught and carried upward most of the grass and debris, but the protozoa were left free and could be found swimming about near the bottom of the flask. When the debris appeared to be well separated from the protozoa, the culture fluid near the bottom of the vessel was pipetted off with as little disturbance of the surface cap as possible. It was strained through bolting silk (144 meshes to the inch) into a shallow culture dish with vertical sides. The bolting silk retained any large particles of debris but permitted the protozoa to pass through. By adding ice the filtered suspension was cooled to the point where the protozoa became immotile and settled to the bottom. The fluid above, containing bacteria and many fine particles still in suspension, was pipetted off. Fresh inorganic medium was added, the dish was agitated to wash the protozoa, and after they settled the wash solution was removed. This was repeated until the wash water showed no cloudiness and the protozoa were left relatively free of bacteria and other very small particles. The particles of debris left with the protozoa in the bottom of the dish were removed as follows. The dish was gently rotated in such a manner that the protozoa and debris were carried to the center of the dish. By further agitation of the liquid the heavier protozoa were caused to collect at the bottom of the central heap. The other material collected in clumps at the top and sides and was removed with a capillary pipette under the dissecting microscope. The remaining protozoa were almost completely free of external particles of grass and cellulose. They presented a green appearance due to the ingested grass.

Following the washing the protozoa were transferred to a centrifuge tube narrowed at the base to a small-bore tube of known diameter. They were collected in the small tube by centrifuging and their total volume was measured. Twenty to 70 microliters of washed protozoa were obtained from 640 milliliters of the original cultures.

The centrifuged protozoa were resuspended in several times their volume of inorganic medium and ground in a small mortar with a little sand. After rubbing to a fine paste additional liquid medium was added and the extract was filtered through an asbestos filter. In the first trials the final volume of the extract was about one milliliter and no evidence of cellulose digestion was observed. Karrer, Schubert, and Wehrli (1925) found that the cellulose-digesting capacity of snail

digestive juice was decreased by dilution. In later experiments the protozoan extract was kept at a volume of 0.2 ml. or less and in these tests definite evidence of cellulose digestion was obtained.

The digestion experiments were performed as follows. The fine aqueous suspension of cellulose obtained with the pebble mill was added to the enzyme extract. Small amounts of toluene were added to retard bacterial growth and the tubes were incubated at 38° C. After 24 hours the test suspensions were freed of cellulose by filtering. To the clear filtrate was added an equal volume of Benedict's solution and the resulting mixture was heated three minutes in a boiling water bath. Amounts of reducing materials were estimated by visual comparisons of the tubes. The results of a number of experiments and appropriate controls are summarized in Table II. The amount of reducing material is shown by plus signs. Several types of controls were run but due to the small volume of extract not all of these could be run at the same time.

TABLE I

Results of experiments and controls on cellulose digestion by extracts of Eudiplodinium

	Unboiled extract, cellulose	Boiled extract, cellulose	Unboiled extract, no cellulose	Boiled extract, no cellulose	Unboiled extract of debris, cellulose	Boiled extract of debris, cellulose
Expt. 1	+	−				
Expt. 2	++		±			
Expt. 3	+				−	
Expt. 4	+	−			−	−
Expt. 5	++		±	−		

Inspection of Table I shows that significant quantities of reducing materials were always formed in the tubes containing the unboiled protozoan extract plus cellulose. When no cellulose is added to the unboiled extract there is a slight reduction but it is not comparable in amount to the reduction when cellulose is present. The slight reduction in the absence of cellulose is probably due to digestion of non-reducing soluble carbohydrates in the extract. In order to determine if glycogen was present, the extract was mixed with three volumes of alcohol and the resulting precipitate was tested with iodine-potassium iodide solution. No reddish-brown color indicative of glycogen was observed whereas the precipitate did give a positive protein test with Millon's reagent. Formation of reducing materials is completely inhibited by boiling of the extract.

The debris control was performed in the same manner as the experiment except that the extract was prepared from the partially decom-

posed grass and cellulose in the cultures. This control was included because it seemed possible that the cellulose digestion observed in the experiment might be due to enzymes of bacteria ingested with the grass and cellulose. Since extracts of the debris gave no reduction it is evident that the demonstrated cellulase is not due to ingested bacteria. It may be concluded that it is elaborated by *E. neglectum*.

Some experiments were run to test the influence of pH on the activity of the cellulase. Small amounts of indicator were added directly to the extracts and the acidity adjusted to the desired point with sodium hydroxide or hydrochloric acid. A control showed that the indicator did not diminish the enzyme activity. The dilution of the extract resulting from addition of the acid or base was taken into account and proper amounts of water added to the other tubes to give the same final concentration of enzyme in the extracts at the different acidities. In these experiments the cellulase was found to be active in the pH range between 4.0 and 6.6. The greatest reduction was observed with a pH of about 5.0. Weineck (1934) noted that the endoplasm of the rumen protozoa was distinctly acid. Thus the optimum acidity for cellulose digestion by the extract is similar to the acidity at the site of cellulose digestion in the protozoa.

The nature of the reducing substances formed by the action of the Eudiplodinium enzymes on cellulose was tested as follows. An extract of the protozoa was prepared and adjusted to a pH of 5.0. Cellulose and toluene were added and the suspension was incubated 24 hours at 38.0° C. The cellulose was filtered off and to the filtrate were added 4 milligrams of anhydrous sodium acetate and 4 milligrams of phenylhydrazine hydrochloride. The tube containing the solution was stoppered and placed in the boiling water bath for 45 minutes. Yellow needle-like crystals formed while the tube was still hot and on gradual cooling of the bath these increased in number. Microscopic examination showed the rosettes, sheaves, and needles characteristic of glucosazone. They were insoluble in hot water. The crystals were transferred to a slide on which were also some crystals of pure glucosazone. The slide was heated on a hot stage on the microscope and the melting point determined. The temperature was followed by means of a thermocouple. Both groups of crystals melted at 207° C., uncorrected.

Weineck (1934) postulated that cellobiose occurred as an intermediate product in the breakdown of cellulose to glucose by the rumen protozoa. If this is true the protozoan extract should be expected to show a cellobiase action. Tests for cellobiase were run in the same manner as those for cellulase except that cellobiose was substituted for the cellulose and the products were tested with phenylhydrazine in-

stead of by reduction. The extracts were adjusted to a pH of 5.0 and the control was heated to boiling. After 24 hours of incubation sodium acetate and phenylhydrazine hydrochloride were added to experimental and control tubes and they were heated in the water bath. Copious crystals resembling glucosazone formed in the experimental tube before cooling but none were present in the control. When transferred to hot distilled water these crystals did not dissolve. Typical crystals of cellobiosazone appeared in the control tube on cooling and to a lesser extent in the experimental tube. These crystals readily dissolved when transferred to hot distilled water. These results indicate that *E. neglectum* contains a cellobiase enzyme and support the hypothesis that cellobiose is an intermediate step in the digestion of cellulose by this protozoan.

In the course of the enzyme extract experiments an interesting confirmation of the attachment of the nucleus to the ectoplasm was observed (Bretschneider, 1934). In the residue left after filtering off the protozoan extract the empty bodies of the protozoa could be found. The process of grinding had forced out all of the endoplasm, including the grass and cellulose particles, but the outer case of the animals was left intact. By staining with methyl green and acetic acid the nucleus could be seen still present in all of the ectoplasmic shells.

Discussion

The interior of the rumen is characterized by conditions which are not commonly met in nature and which seem to be necessary for the growth of the rumen protozoa. The success of *in vitro* culture methods is determined in large part by the extent to which they duplicate the natural conditions in the rumen. The present method for growing *E. neglectum* provides an environment which in many respects is similar to that of the rumen although there are certain differences.

The culture solution and the rumen contents are similar in being anaerobic, and in having the same temperature, pH, and osmotic pressure. The grass and cellulose supplied in the flasks represent the plant materials of the rumen but they are present in much smaller concentrations. Whereas the cultures furnish a total of 0.8 milligram of cellulose and grass per milliliter, the concentration of foodstuffs in the rumen is many times as great. This is one of the chief differences between the flask cultures and the natural environment of the protozoa. The abundant substrates in the rumen are actively fermented by the organisms present, both bacteria and protozoa, and large quantities of products are formed. These are absorbed or are neutralized by the alkaline saliva and thus are prevented from reaching a concentration

that would injure the protozoa. Under these conditions the number of protozoa normally reaches one million protozoa per milliliter (Ferber, 1928).

In the flask cultures, buffering by the medium and dilution through subculturing prevent toxic concentrations of products, but the capacity for accommodating the fermentation products is small in comparison with the rumen. Favorable conditions are maintained only if the amount of substrate is small. The limited substrate supports relatively few protozoa.

Other species of Diplodinium ingest plant parts in a fashion similar to *E. neglectum* and they, too, probably digest cellulose. Additional experiments with these forms will be necessary to determine with certainty their capacity for cellulose digestion but on the basis of the present results it appears probable that many of them can utilize this material.

Some of the protozoa in the rumen are passed on into the succeeding divisions of the stomach where they are digested and absorbed. Their importance in the diet of the host can be estimated from the growth rate of the protozoa in the rumen. The division rate of *E. neglectum* is much greater than the average rate of seven per cent of fissions each day assumed by Ferber and Winogradowa-Fedorowa (1929). Westphal's experiments (1934) also indicate more rapid division, namely, once every 12 hours for Entodinium. It is probable that the average division rate of the rumen protozoa is about that of *E. neglectum* since the latter is one of the medium-sized species. According to this assumption, the rumen protozoa, if none were removed, would each day double their weight. However, some of the protozoa are passed more or less continuously into the succeeding portions of the stomach and do not divide. Consequently, during one day, fewer protozoa are produced than if all were retained in the rumen.

In order to estimate the number of protozoa supplied to the ruminant let it be assumed that the excess protozoa are continuously removed from the rumen so that the number left is the same at all times. The rate of fission of these latter would determine the number of excess protozoa formed.

The formula expressing the number of organisms, N, produced in time t through fission by an initial number, N_0, with a division rate of r is

$$N = N_0 2^{rt}.$$

Differentiating, we obtain

$$\frac{dN}{dt} = Nr \log_e 2 = 0.69Nr.$$

When r equals unity, as with a division rate of once each day, the number of organisms produced in one day is $0.69N$. Thus, the protozoa in the rumen produce daily about 69 per cent of their weight of rumen protozoa. Ferber and Winogradowa-Fedorowa (1929) calculated that 2 per cent of the protein requirements of the sheep were supplied in the form of protozoa, assuming that 7 per cent of the protozoa were used each day. With the value of 69 per cent, as calculated from the division rate of *E. neglectum*, it is evident that the protein supplied to the ruminant in the form of protozoa constitutes more nearly 20 per cent of its nitrogen requirement. Schwarz and Bienert (1926) concluded from their investigations that the protozoa in the caecum of the horse supplied their host with one-fourth to one-third of its required protein. These estimates show that the protozoa compose an appreciable fraction of the nitrogenous diet.

The older work of Wilsing (1885) and Henneberg and Stohmann (1885) indicated that the products of the cellulose fermentation in the rumen are absorbed by the host. Thus, the protozoa are concerned with the carbohydrate as well as the protein nutrition of the ruminant. Whether their total role is helpful or harmful cannot be determined as yet. However, the data at hand do indicate that some of them are helpful.

The experiments of Becker, Schulz, and Emmerson (1929) and Winogradow, Winogradowa-Fedorowa, and Wereninow (1930), showing that defaunated animals digested about as much cellulose as those with protozoa, do not disprove a cellulolytic action by the protozoa. The experiments show that the protozoa are not essential for cellulose digestion, but the probable explanation is that some of the bacteria in the rumen also digest cellulose and in the absence of the protozoa they become sufficiently numerous to carry the entire burden. When the protozoa are present, some of them also participate in cellulose digestion. Since the ruminant is unable to digest cellulose it is evident that any cellulose digestion by the protozoa is helpful and in this respect the cellulose-digesting forms are definitely symbionts.

Summary

A successful method for culturing a cattle ciliate, *Eudiplodinium neglectum*, has been developed. A medium containing grass and cellulose in addition to inorganic salts has supported continuous culture for a period of 22 months. The osmotic pressure of the medium must be suitable and oxygen must be excluded. The protozoa are obligate anaerobes.

The protozoa have been grown in sufficient numbers to permit preparation of an enzyme extract which is shown to be active in cellulose digestion. The optimum acidity for action of the cellulase is about pH 5.0, a reaction similar to that in the endoplasmic sack of the protozoa. Glucose occurs as an end product of cellulose digestion and cellobiose is probably an intermediate product since cellobiase is present. Because of its cellulose-digesting capacity, *E. neglectum* is helpful to the host and the relationship between the two is one of symbiosis.

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DIGESTION OF FAT IN THE RHIZOPOD, *PELOMYXA CAROLINENSIS*[1]

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Introduction

It is well known that there is fat in the form of discrete globules in the cytoplasm of protozoa. It is, therefore, assumed that they use fat as food, but the evidence in favor of this assumption is meager. Greenwood (1886–87) maintains that digestion of fat in rhizopods is doubtful, and Nirenstein (1905) says it has not been observed in any protozoa. Dawson and Belkin (1928) injected various oils into *Amoeba dubia* and measured the diameter of the globules each day for several days. Globules of cod liver, olive, cotton seed, sperm, and peanut oil decreased in size but those of paraffin and oxfoot oil and oleic acid did not. They concluded that *Amoeba dubia* digests the former but not the latter. They (1929) repeated the experiments with *Amoeba proteus* and concluded that *Amoeba proteus* digests all the oils tested except paraffin. However, as Mast (1938) points out: "It is obvious . . . that the decrease in the size of the globules may have been due to oxidation rather than to digestion." Mast and Hahnert (1935) and Mast (1938) demonstrated, however, that fat in *Colpidium striatum* ingested by *Amoeba proteus* is broken down into fatty acid and glycerine, and that these substances pass from the food vacuoles into the cytoplasm, and there unite to form neutral fat.

The following experiments were made to ascertain whether *Pelomyxa carolinensis* digests fat in ingested organisms and fat injected into the cytoplasm.

Fat in Ingested Organisms

About one hundred pelomyxae were passed through five different 5 cc. portions of sterile culture fluid and left in 50 cc. of this fluid without food for three weeks. Then several were taken at random and tested for fat with Sudan black. No food vacuoles were found in any of them. A few had a small number of fat globules and all had

[1] The writer wishes to express his sincere thanks to Professor S. O. Mast, who suggested the problem and under whose immediate direction the work was done, for constant encouragement and invaluable aid in preparing the manuscript.

several minute granules which stained black with Sudan black and were probably fat.

The remaining pelomyxae were put into culture fluid with numerous specimens of *Colpidium striatum* and left until each pelomyxa had ingested several. The pelomyxae were then taken out, passed through five separate portions of culture fluid, and put into 50 cc. of the fluid. Some of these were immediately stained with Sudan black and Nile blue sulfate respectively,[2] and others at intervals of two hours. The results obtained follow:

Immediately after ingestion there were numerous globules in the colpidia, which stained black with Sudan black and blue with Nile blue sulfate and only a few in the cytoplasm of the pelomyxae and these stained pink with Nile blue sulfate. The former consisted, therefore, of fatty acid and the latter of neutral fat (Parat, 1927). Four hours after feeding there were still numerous globules of fatty acid in the colpidia and only a few globules of neutral fat in the pelomyxae. Six hours after feeding, the food vacuoles had divided into two or more smaller ones and all contained globules of fatty acid, but there were now numerous small globules containing a mixture of neutral fat and fatty acid in the cytoplasm of nearly all the pelomyxae. Eight hours after feeding most of the food vacuoles still contained fatty acid, but there were many small globules and several large ones containing neutral fat and fatty acid in the cytoplasm of all the pelomyxae. Ten hours after feeding only a few of the food vacuoles contained fatty acid and there were now numerous small globules and several large ones containing neutral fat and fatty acid in the cytoplasm of all the pelomyxae examined. Twenty-four hours after feeding there were no fatty acid globules in any of the vacuoles and the cytoplasm of all the pelomyxae contained a great many globules consisting of a mixture of neutral fat and fatty acid.

The results obtained indicate, therefore, that the fatty acid in the colpidia passed out of the food vacuoles into the cytoplasm of the pelomyxae and that some of it there united with glycerine to form neutral fat.

Twelve pelomyxae, taken from a thriving culture, were centrifuged[3] and cut so as to remove nearly all the globules of fatty material,

[2] Nile blue sulfate stains neutral fat pink, fatty acid blue, and a mixture of both purple (Parat, 1927).

[3] If specimens of *Pelomyxa* are suspended in gum arabic solution and centrifuged, the protoplasmic constituents stratify as follows, in order of decrease in weight: refractive bodies, vacuole refractive bodies in food vacuoles, nuclei, crystals, beta granules, hyaloplasm, contractile vacuoles, and fat. It is, therefore, easy to cut out of centrifuged specimens nearly all the fat, refractive bodies, and food vacuoles.

refractive bodies, and food vacuoles. They were then left for several hours in culture fluid to recover from the "shock" of the operation. A few were now stained with Sudan black and the rest put into culture fluid with numerous colpidia. After each pelomyxa had ingested several colpidia, they were put into sterile culture fluid and left twenty-four hours; then some were stained with Sudan black and others with Nile blue sulfate.

The results obtained with Sudan black show that before feeding there was very little fat (Figure 1*a*) in the cytoplasm of the pelomyxae but that twenty-four hours after feeding there was much (Figure 1*b*) and the results obtained with Nile blue sulfate show that the globules in the cytoplasm after feeding consisted of a mixture of neutral fat and fatty acid, for they became purple (Parat, 1927). These results, therefore, support the conclusion reached in the preceding experiment, that the fatty acid in the ingested colpidia passes out of the food vacuoles into the cytoplasm and that some of it there unites with glycerine to form neutral fat.

This conclusion is not in full accord with the results obtained by Mast (1938) in observations on *Amoeba proteus*, for he found only neutral fat in the cytoplasm. It would seem, therefore, that the metabolism of fat is not the same in *Pelomyxa* as it is in *Amoeba*. The colpidia which he used were, however, grown in a bacteria-free medium and contained only neutral fat, whereas those used in the experiments described above were not, and they contained only fatty acid. It might, therefore, be argued that the difference observed in the metabolism of fat was due to the difference in the fat used. The following experiments concern this proposition.

Injected Olive Oil

One hundred pelomyxae were starved and put into sterile culture fluid as in the preceding experiments and an olive oil droplet [4] injected into the cytoplasm of each. The pelomyxae were then separated into two equal groups, after which the diameters of the oil droplets in one group were measured daily, and specimens taken daily from the other group and stained respectively with Sudan black and Nile blue sulfate.

The results obtained in measurements of seven of the 50 pelomyxae (taken at random) are presented in Table I. This table shows that

[4] Olive oil droplets of this kind were mounted in culture fluid on a slide, covered with a cover-slip, stained with Nile blue sulfate, and examined under high magnification. They became intensely pink and, consequently, consisted largely if not entirely of neutral fat.

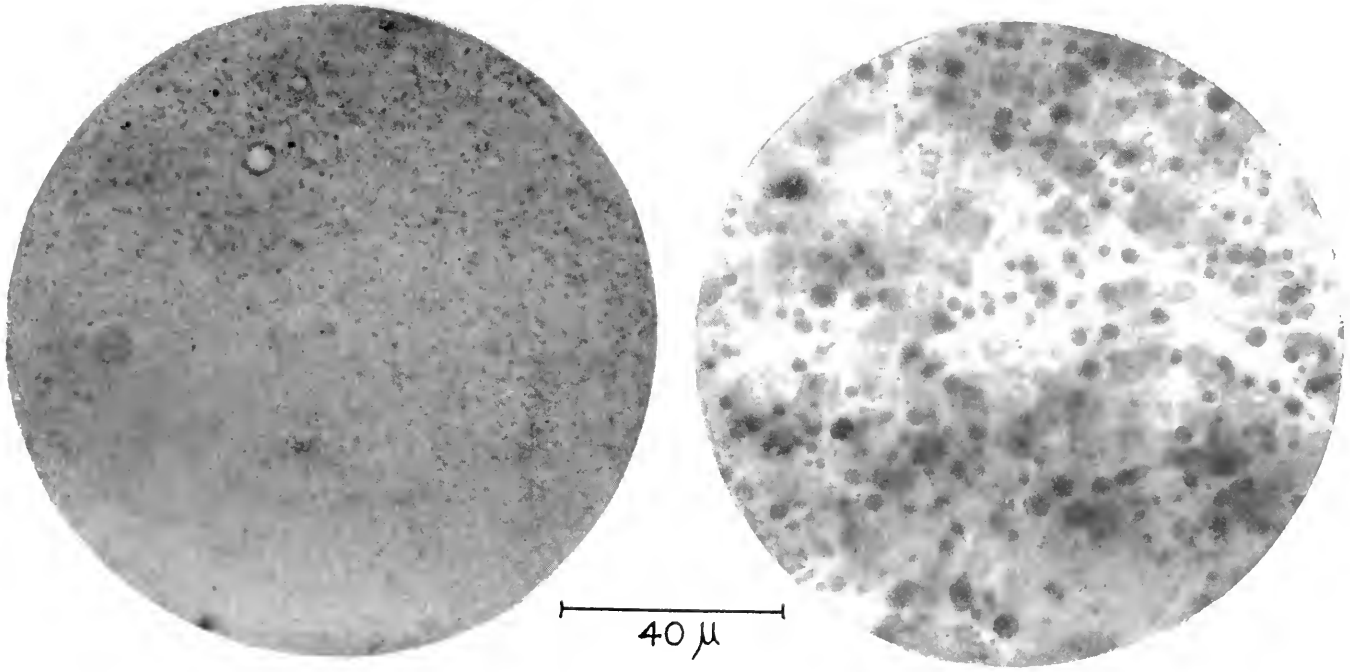

FIGURE 1. Photomicrographs of portions of specimens of *Pelomyxa carolinensis* stained with Sudan black, showing the increase in the amount of fat in the cytoplasm due to feeding on *Colpidium striatum* containing much fatty acid. A, a pelomyxa in which the fat had been removed by centrifugation; B, a centrifuged pelomyxa after having fed on *Colpidium striatum* for 24 hours; black and gray dots, fat globules. Note that there were only a few globules in the cytoplasm of A but very many in that of B.

the droplet of injected olive oil decreased in volume from day to day in all the specimens but that the rate of decrease varied with the individuals.

The observations on the stained pelomyxae show that as the injected oil droplets decreased in size, the number of fat globules in the cytoplasm increased and that these globules invariably stained purple with Nile blue sulfate, indicating that they consisted of a mixture of fatty acid and neutral fat (Parat, 1927). They show also that the injected droplets at first stained pink with Nile blue sulfate but that after they had decreased considerably in size they stained blue and that as they decreased in size they became irregular in form and a non-staining hyaline fluid formed around them.

TABLE I

Changes in the size of globules of olive oil injected into the cytoplasm of Pelomyxa carolinensis

Designation of pelomyxae	Diameters of globules in micra on successive days after injection						
	0	1st	2nd	3rd	4th	5th	6th
1	83.3	73.5	60	0			
2	34.3	19.6	14.7	0			
3	78.4	68.6	58.9	53.9	44.1	39.2	0
4	60	45	30	19	0		
5	100	40	20	0			
6	25	23	oil ejected				
7	120	60	30	0			

The fact that the droplets became irregular in form indicates that their viscosity increased greatly, and the fact that they stained blue with Nile blue sulfate indicates that they contained much fatty acid. This seems to show that at least a part of the olive oil (neutral fat) was hydrolyzed before it passed into the cytoplasm. The facts that the fat globules in the cytoplasm increased in number as the injected droplet of oil decreased in size and that these globules contained neutral fat as well as fatty acid, show that if all the injected oil was hydrolyzed a portion of it united with glycerine in the cytoplasm. They also show that the suggestion, that the decrease in the volume of injected oil observed in *Amoeba* may be due to oxidation, does not apply to *Pelomyxa*.

The color of the fat stained with Nile blue sulfate in the cytoplasm of the pelomyxae which had digested injected olive oil was repeatedly compared with that in the cytoplasm of those which had digested

fatty acid in the food vacuoles, but no difference in shade was detected. It is therefore apparent that the form in which fat is stored in the cytoplasm of *Pelomyxa*, as indicated by the staining reaction, is the same whether neutral fat or fatty acid is taken into the organism.

The results of the above two series of experiments indicate that fat metabolism in *Pelomyxa* is not the same as in *Amoeba*, for neutral fat passes from the food vacuole into the cytoplasm and is stored there in globules which in the one consist of a mixture of neutral fat and fatty acid and in the other of neutral fat. Probably all rhizopods digest fats and store them in the cytoplasm as food reserve, but the form in which they are stored is not the same.

Summary

1. If *Pelomyxa carolinensis*, which contains no fat ingests *Colpidium striatum*, containing fatty acid, the fatty acid in the food vacuoles disappears and simultaneously globules of a mixture of neutral fat and fatty acid appear in the cytoplasm of *Pelomyxa*. This indicates that fatty acid passes out of the food vacuoles into the cytoplasm and that some of it there combines with glycerine to form neutral fat.

2. If droplets of olive oil (neutral fat) are injected into the cytoplasm of pelomyxae, which contain no fat, the injected droplets gradually decrease in volume and finally disappear and numerous globules of a mixture of neutral fat and fatty acid appear in the cytoplasm. This indicates that the neutral fat in the injected droplets hydrolyzes and passes out into the cytoplasm.

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THE PERMEABILITY OF YEAST CELLS TO RADIOPHOSPHATE

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Data have been presented by Hevesy, Linderstrøm-Lang and Nielsen (1937) giving the analytical and radioactive phosphorus contents of yeast after twenty-four hours immersion in radioactive phosphorus with an external concentration of 8.7 mg. per cent phosphorus. Their data showed that inactive and active phosphorus were taken up in the same ratio as that found in the external solution, and that phosphorus uptake was negligible without sugar at 20° or with sugar at 0°. Hevesy et al. suggest either that the phosphorus content of the cell is not exchangeable, or that the cell is only permeable to phosphorus when growing. Lawrence, Erf, and Tuttle (1941) also found sugar to be essential to radioactive phosphorus uptake and demonstrated an inhibition of radioactive phosphorus uptake by fluoride ion in the presence of glucose. Further, 20 per cent of the radioactive phosphorus taken up was in nucleoprotein fractions and 80 per cent in the acid soluble fraction of yeast. They concluded that the radioactive phosphorus uptake curve of yeast could be superimposed on a curve for the metabolic rate but not for the growth curve. O'Kane and Umbreit (1942) have shown for *Streptococcus fæcalis* that when glucose is not present, organic phosphorus is lost from the cell to the medium, and that the medium suffers an increase in the concentration of both organic and inorganic phosphorus, indicating that the organic phosphorus lost from the cell is broken down extracellularly. When glucose is added, there is a marked decrease in the inorganic phosphorus both intra- and extracellularly. Later inorganic phosphorus is released. These results are in accord with theories of phosphorylating glycolysis. Ketchum (1939) using the marine diatom *Nitzschia closterium*, finds that it is possible to produce a "phosphorus debt" in this organism, when it is grown in light without phosphorus. Normally the diatom will not take up phosphorus in the dark, but when a phosphorus debt has been incurred, it can be repaid by uptake in the dark. This phosphorus uptake is independent of the external concentration of phosphorus and this suggests that the reaction is a definite protoplasmic combination with the element. This view is further substantiated by the observation that in light, phosphorus

uptake is proportional only to the number of new cells formed and not to concentration.

In order to clarify the effects of metabolic factors on phosphate transfers, the cells of *Saccharomyces cerevisiæ* were studied under conditions where the following were varied: oxygen tension, temperature, and carbohydrate supply. Measurements were then made of radioactive phosphorus (P*) and analytical phosphorus (P) taken up by the cell.

Experimental

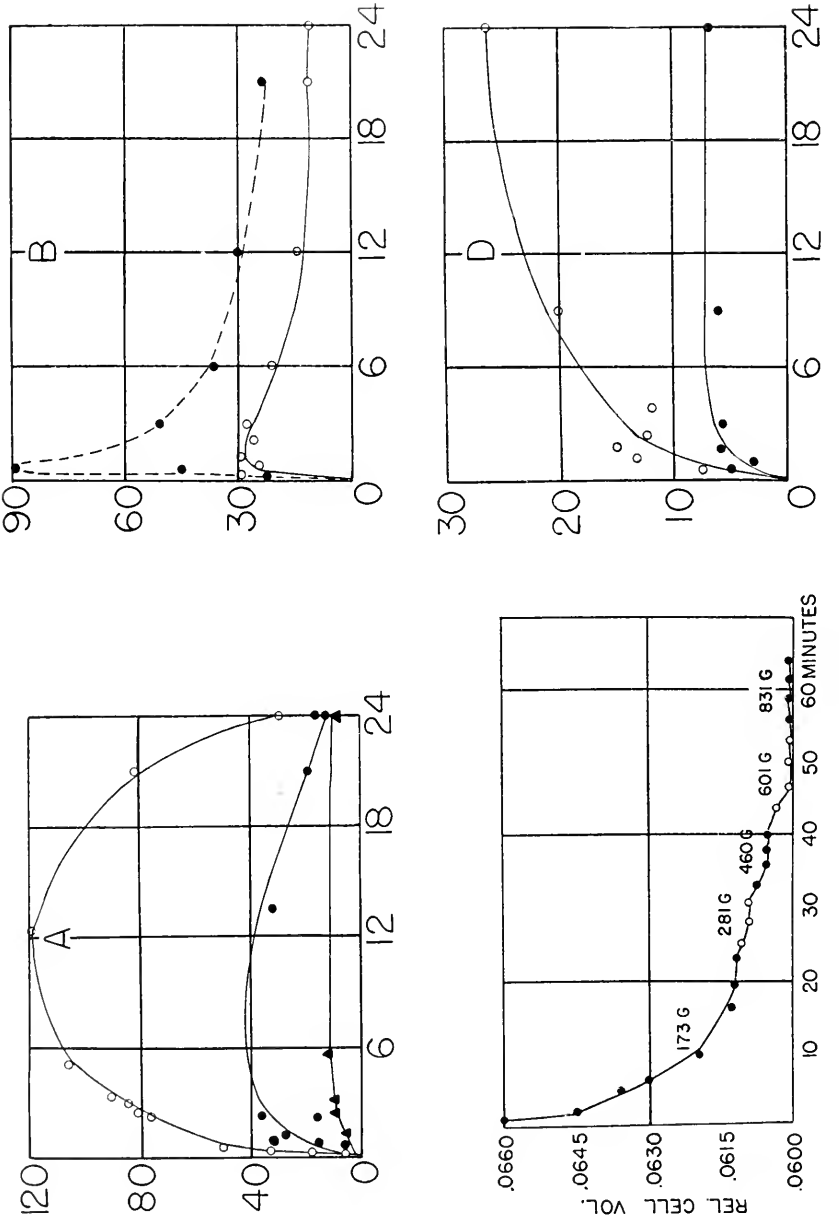
Yeast cells were obtained in cakes and freed of extraneous material by centrifugation and washing with distilled water or saline. The cells were then suspended in approximately 100 times their own volume of standard medium [1] (sugar, nitrogen, and phosphate = SNP) and the suspension was placed in a water bath at 37° C. where it was continuously aerated and stirred by a stream of bubbles of (CO–CO_2 free) O_2 gas saturated with water vapor. After 12 hours growth, the cells were again washed with fresh medium and finally suspended in the particular experimental solution desired to which had been added 5 mg. per liter $Na_2HP^*O_4$, 20 microcuries (β) per liter (μC/L).[2] In all cases pH was adjusted to 4.5 as measured by a quinhydrone electrode. This pH varied from 4.5 to 4.2 during the experiments. After the desired times of immersion in thermostats at various temperatures and at various gas tensions, a uniform sample of the suspension was obtained and centrifuged to constant volume at 830 × G, washed in either non-radioactive medium or distilled water, dried to constant weight, digested with nitric acid, evaporated to dryness, and counted under a Geiger-Müller counter.[3] Average moisture content was determined

[1] Standard Medium = 0.02M $(NH_4)_2SO_4$
(SNP) 0.03M KH_2PO_4
5.0% Brown Sugar
(NP) = 0.02M $(NH_4)_2SO_4$
0.03M KH_2PO_4
(P) = 0.02M Na_2SO_4
0.03M KH_2PO_4.

[2] The radioactive isotope P* is $_{15}P^{32}$; half-life, λ = 14.30 days. Na* and Br* designate the respective isotopes $_{11}Na^{24}$ and $_{35}Br^{82}$. The absolute radioactivity is given in all cases. For P*, since no γ-ray activity has been observed, the activity is compared with the β activity of Ra. For Na* and Br* their γ activity is compared with γ-radiation of Ra in equilibrium with its decay products.

[3] The various units used in radioactive work are as follows:

Term	Symbol	Quantity
Radioactivity	R	counts/min. (c/m) [As measured by a Geiger-Müller Counter]
Standard Radioactivity	R_s	c/m/gm. of solution or tissue
Specific Radioactivity	ψ'	c/m/mole of element
Relative Specific Radioactivity	ψ'_R	ψ' (tissue)/ψ' (solution bathing tissue)


A
B
D
0
6
12
18
24
120
80
40
90
60
30
30
20
10
.0660
.0645
.0630
.0615
.0600
REL. CELL VOL.
10
20
30
40
50
60 MINUTES
173 G
281 G
460 G
601 G
831 G

FIGURES 1a–d.

to be 86 ± 4 per cent, and no significant difference in this figure could be produced by varying the experimental conditions of growth. In experiments where hydrogen gas was used to obtain anaerobic conditions, tank hydrogen was passed through a heated combustion tube filled with copper and was distributed to the experimental solution through block tin tubing. Other connections in the system were either of glass or copper tubing. The experimental flask was so arranged that samples could be obtained without disturbing anaerobiosis.

After counting, the dried nitric acid digest was ashed in platinum with sodium carbonate, and the ash was analyzed for phosphorus by the method of Kuttner and Lichtenstein (1930). Colors were determined with a balanced cell photoelectric colorimeter and determinations in duplicate were accurate to ± 0.5 per cent.

In the use of cell volumes, as read from graduated centrifuge tubes, for the calculation of wet weight, due cognizance must be taken of the "packing fraction" or that fraction of the indicated volume of the sediment which is actually occupied by cells. In order to determine this value accurately, a 5 per cent (by volume) suspension of yeast was made up in standard medium (SNP) saturated with oxygen and held at 37° C. for one hour. Samples of this suspension were then taken (1) for measurement of cell volume by centrifugal sedimentation (mycocrit), (2) for a determination of the number of cells, using a haemocytometer chamber, and (3) for a microscopic measurement of cell dimensions. The mycocrit measurements were carried out in short graduated capillary tubes so arranged that the entire tube projected above the metal centrifuge tube holder. By the use of a stroboscope mounted above the centrifuge, the progress of the sedimentation could be followed visually.

The results of such studies are shown in Figure 1c where the mycocrit is plotted against the time of application of a given centrifugal acceleration. The curve shows that in 50 minutes the cell volume had reached a constant value equal to 0.060 of the total volume of the

FIGURE 1a. Ordinates, R_s or the standard radioactivity of yeast; abscissae, time of treatment in hours. All curves are for yeast grown in sugar, nitrogen (as NH_4+) and phosphate (SNP). Open circles, O_2 at 37° C.; solid circles, O_2 at 10° C.; solid triangles, H_2 at 10° C. All yeast suspensions were initially 5%.

FIGURE 1b. Ordinates and abscissae as above; curves are for yeast in phosphate alone, no (S) or (N). Solid circles, in O_2 at 37° C.; open circles in O_2 at 10° C.

FIGURE 1c. Ordinates, observed cell volume relative to total volume of suspension; abscissae, time of sedimentation in minutes. Numbers on the curve are the relative centrifugal acceleration.

FIGURE 1d. Ordinates, mM/L, Na^* or Br^*; abscissae, time of treatment in hours. Medium is SNP with $Na+$ concentration = 50 mM/L and Br^- = 30 mM/L, O_2 was supplied and temp. = 37° C. Open circles are Na^* and solid circles, Br^*.

suspension. The measurement of major and minor axes of 100 cells gave, as averages, the figures 6.5μ and 5.0μ respectively. Assuming a prolate spheroid, the average volume then was 88 $\pm$ 4μ^3 or 88 $\times$ 10^{-12} cm.^{3}. By actual count of the suspension, the number of cells was 6.3 $\times$ 10^8/cm.^{3}. Therefore, the actual volume occupied should be 88.0 $\times$ 10^{-12} $\times$ 6.3 $\times$ 10^8 = 0.055 cm.^{3}. Now the mycocrit gave 0.060 cm.^{3} as the volume sedimented so .055/.060 = the "packing fraction" 0.92 $\pm$ .05. This is the percentage of the volume of the mycocrit which was really cells. It is obvious that this figure is only slightly different from 1.0; however with some cells this correction can be appreciable.

Results

In Figures 1a and 1b are shown the result of some experiments in which the standard radioactivity of the yeast cells is plotted against time of immersion in the solution. The two upper curves in Figure 1a show that the uptake of radioactive phosphorus is much accelerated

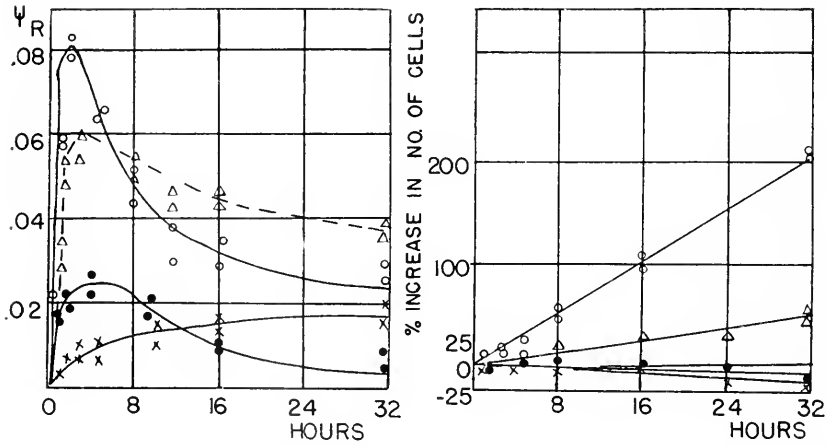


Figure 2a. Ordinates, relative specific activity; abscissae, time of immersion of cells in P* solution (SNP medium). Open circles, cells at 37° C. in O_2; open triangles, cells at 37° C. in H_2; solid circles, cells at 10° C. in O_2; crosses, cells at 10° C. in H_2.

Figure 2b. Ordinates, per cent increase in number of cells growing in SNP medium. Abscissae, time in hours. Open circles, cells growing in O_2 at 37°C. Open triangles, cells at 37° C. in H_2; solid circles, cells at 10° C. in O_2; crosses, cells at 10° C. in H_2.

by a rise of temperature from 10° C. to 37° C. while the two lower curves show that it is much inhibited by the absence of oxygen. In oxygen (upper curves) the count passes through a maximum at 6 to 12 hours and then declines. This decrease in the count indicates

perhaps a breakdown of the phosphorylated compounds due to exhaustion of the sugar in the medium.

Similar curves in the absence of sugar and nitrogen supply are shown in Figure 1b. In this case the maximum occurs sooner and disappears more quickly and must be attributed to the presence in the yeast of some residual carbohydrate capable of phosphorylation. The same marked decrease in radioactivity due to the lowered temperature is evident here and the high temperature coefficient indicates that the penetration is not due to mere physical exchange of radioactive phosphorus for phosphorus but to some chemical reaction. (Ketchum, loc. cit.)

The metabolic nature of the penetration of phosphorus is made more evident when it is compared with the penetration of radioactive sodium and bromine as illustrated in Figure 1d. In both cases the radioactivity gradually approaches a maximum value without the subsequent decline which characterizes the penetration of phosphorus.

Interpretation of Figures 1a and 1b is facilitated by Figure 2a which shows the relative specific radioactivity of the yeast phosphorus. The two curves for oxygen at 37° C. and 10° C. show a similar rapid rise to a maximum and subsequent decline. These data from Figure 2a would indicate that the decline in Figure 1a is not due to loss of both phosphorus and radioactive phosphorus but to loss of radioactive phosphorus relative to phosphorus. It could not be due to a gain in phosphorus relative to radioactive phosphorus because all the phosphorus outside the cells has a high P*/P ratio. The difference in the time relation between Figures 2a and 1a must be due to differences in the yeast used for the two experiments. A loss of radioactive phosphorus without much change in phosphorus is possible if it is the newly phosphorylated compounds which are broken down. The remainder of the cell phosphorus is evidently more permanently bound and it does not readily exchange. Figure 2b shows the relative rates of growth of yeast, as measured by cell counts, during the various experimental conditions described.

Conclusions

From the experimental evidence presented, it would seem as though the various factors responsible for the transfer of phosphorus from the external solution to the yeast cell could be enumerated as follows: a. phosphorus is used by the cell in the synthesis of relatively permanent constituents of the protoplasm; b. phosphorus is transferred in both directions across the cell membrane in connection with the phosphorylation and oxidation of carbohydrate; c. phosphorus enters the

cell by diffusion or exchange for outgoing phosphorus and is not synthesized into any organic compounds in the cell. (This is in reality the situation found in (a.) but is treated separately since the eventual fate of the phosphorus is different.) Experimentally it has been shown that radioactive phosphorus enters the cell to a relatively slight extent at 37° C. in the absence of carbohydrate. This would indicate that the amount of phosphorus required for synthesis into proteins is relatively small, since one would expect that phosphorus would be taken up for such synthetic purposes, even in the absence of sugar, if there were a need of such phosphorus. In the case of (b.) it may be stated that ample carbohydrate supply in either oxygen or hydrogen at 37° C. allows large phosphorus uptakes; the fact that this uptake is sensitive to temperature but not to oxygen would indicate that an enzymatic process of some kind was going on, and the close interdependence between phosphorus and sugar would suggest phosphorylation. As for the purely physical diffusion or exchange processes (c.), by which ions reach a concentration inside the cell which bears a direct relationship to their initial concentration outside the cell, and thus permit the computation of a "permeability constant," the data would suggest that such a permeability must at least be very low. These diffusion processes, if purely physical, should not be either temperature or oxygen sensitive to any marked degree, and hence that data for yeast in hydrogen at 10° C. should show the extent to which the phosphate ion would penetrate the cell. As can be seen from the curves, the phosphorus uptake under these conditions is very small, as compared with uptake in carbohydrate. The average phosphorus content of yeast is about 84 mM/kg., and the initial slope of the uptake curve for phosphorus in hydrogen at 10° C. is 0.25%/hr. Therefore the rate of uptake is 2.1×10^{-2} M/kg./hr.

The author wishes to acknowledge the kindness of Professor S. C. Brooks for arranging for laboratory facilities for this work. Thanks are due to Dr. Daniel Mazia for assistance with some of the experiments, and acknowledgment is also due Professor E. O. Lawrence for the supply of radioactive phosphorus, radioactive sodium, and radioactive bromine used in these experiments.

Summary

The data presented indicate that the transfer of phosphate across the yeast cell membrane is dependent almost entirely on the carbohydrate metabolism of the cell. A maximum quantity of phosphorus is transferred into the cell when high external carbohydrate concentration is maintained and when the temperature is held at 37° C. The

presence or absence of oxygen is not very important in promoting phosphorus transfer. Very little phosphorus will diffuse into yeast at 10° C. in the absence of sugar.

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THE INFLUENCE OF CERTAIN DRUGS ON THE CRUSTACEAN NERVE–MUSCLE SYSTEM

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Introduction

The physiological problems involved in the peripheral innervation of the decapod crustacea differ in many ways from those encountered in vertebrate nerve-muscle preparations. There are many structural and functional differences; the five which are most important from the standpoint of the effects of drugs will be briefly considered.

A. The number of efferent fibers innervating a crustacean muscle is very small, the largest number yet found being five, the smallest, two. In contrast to the vertebrate motor-unit concept, all of the muscle fibers in a given muscle receive a branch from each of these nerve fibers (van Harreveld, 1939b). The number of fibers innervating a particular muscle of a certain species of the decapod crustacea is constant (Wiersma, 1941a). The two muscles used in the present investigation, namely the opener (abductor of the dactylopodite) and the closer (adductor of the dactylopodite) of the cheliped of *Cambarus clarkii*, receive two and three nerve fibers respectively.

B. In contrast to the single nerve ending usually found for each muscle fiber of the vertebrates, the crustacean muscle fiber receives many branches of each of the nerve fibers innervating that muscle, and the nerve endings are well distributed over the entire length of the muscle fiber (van Harreveld, 1939a). From this and other evidence it has been concluded that the spread of excitation over the crustacean muscle fiber is due not to muscular but to nervous conduction. The contraction, then, is the sum of the many local contractions; the muscle action potential, the sum of the local action potentials set up at the individual nerve endings. (For a more complete discussion see Wiersma, 1941b.) It is doubtful, in fact, that direct muscle stimulation is possible in the crustacea to any extent greater than a local contraction at the electrodes (van Harreveld, 1939c).

C. Unlike the skeletal muscles of vertebrates, most of the leg muscles in the crustacea can contract in several ways. This was first

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observed by Richet (1879) and was subsequently confirmed by a number of authors (Lucas, 1917; ten Cate, 1927; Blaschko *et al*, 1931; Wiersma, 1933; Wiersma and van Harreveld, 1934; Knowlton, 1942). In 1936, however, van Harreveld and Wiersma showed that for the closer of the claw of the crayfish, *Cambarus clarkii*, stimulation of one (the thicker) of its two motor neurons (prepared as single fibers) invariably produces a "twitch" contraction, whereas stimulation of the other (the thinner) produces always a contraction of the "slow" type. The muscle action potentials resulting from stimulation of the prepared fibers of such a doubly motor innervated muscle are also of two distinct types. That the contractions of the two types occur, indeed, in the same muscle fiber, as would be expected from the anatomical innervation, has been established. By direct microscopic observation of small groups of muscle fibers in the closer of Cambarus, van Harreveld (1939c) found that stimulation of either of the two motor axons separately produces contraction in the same muscle fibers.

D. Whereas in vertebrate striated muscle a single nerve impulse causes a maximal twitch contraction, most crustacean muscles do not show any mechanical response unless a number of impulses reach the muscle within a limited time interval. This need for facilitation is characteristic of all crustacean slow systems, and appears, although to a lesser extent, in most fast ones (see Wiersma and van Harreveld, 1938). The fast system of the closer of Cambarus is, in this respect, an outstanding exception, since here a single nerve impulse causes both a maximal muscle action potential and a twitch contraction. Repetitive stimulation of the single motor axon to the opener produces a contraction of the slow type (van Harreveld and Wiersma, 1936).

E. The presence in the crustacea of centrifugal nerve fibers whose function is specifically inhibitory has been demonstrated by their isolation as functional single axons (van Harreveld and Wiersma, 1937). The mechanisms involved in this peripheral inhibition have been subjected to considerable investigation (Marmont and Wiersma, 1938; Wiersma and Helfer, 1941; Wiersma, 1941a, b; Wiersma and Ellis, 1942). With respect to the opener muscle of the crayfish it was found that, whereas inhibition of the contraction is usually not accompanied by depression of the muscle action potentials (simple inhibition), under certain circumstances such reduction can be observed (supplementary inhibition). It was also found that inhibition is of different efficiency [2] with regard to the contractions of different

[2] By efficiency is meant the number of excitatory impulses that can be suppressed by one inhibitory impulse. This number was found to vary from three to one-fifth in different muscles (Wiersma and Ellis, 1942).

muscles. The opener of the crayfish shows a very efficient inhibitory system, whereas the inhibitor of the closer is for practical purposes without influence on either the fast or the slow contractions.

In the present investigation the effects of drugs on the fast and slow types of contraction and on inhibition have been studied. The closer and opener systems of *Cambarus clarkii* were selected because of the great dissimilarity between the fast and the slow contractions of the closer, the negligible influence of inhibition on the closer, the ease with which the opener can be inhibited, and the presence of supplementary inhibition in the opener system.

Methods

Two types of experiments were carried out involving the use of drugs; in one group the substance was injected into the animal, in the other, the drug was applied directly to the isolated claw preparation by injecting it hypodermically through the hole obtained by removing

Table I

Classification of drugs as to their generally accepted pharmacological action

Group	Sympathetic	Parasympathetic	Curare-like
Drug	Epinephrine Amphetamine Hydrastinine Yohimbine 933F	Choline Acetylcholine Mecholyl Doryl Muscarine Pilocarpine Physostigmine Atropine	Curare Trimethylammonium salts Tetramethylammonium salts Brucine Strychnine
Group	Insecticides	Local anaesthetics	Muscle drugs
Drug	Nicotine Pyrethrum Rotenone	Procaine Diothane Nupercaine	Caffeine Digitalin Veratrine

the pollex of the propodite. In additional experiments, a limited portion of the prepared nerve was treated with a solution of the drug in a narcotic chamber.

The drugs were selected to represent a rather wide variety of known pharmacological actions, and can be grouped as 1) sympathetic drugs, 2) parasympathetic drugs, 3) curare-like drugs, 4) insecticides, 5) local anaesthetics, and 6) muscle drugs. The ones used as representatives of these groups are shown in Table I. A wide range of dosage was used in each case. In all instances, dilution of the drug

was made in the balanced physiological solution for crayfish reported by van Harreveld (1936).

The effects of these drugs on the two types of contractions of the closer muscle necessitated isolation of the single axons by the method of van Harreveld and Wiersma (1936) to permit selective stimulation of either fiber. As the inhibitor to the opener muscle does not lie in the small nerve bundle in which the motor axon is to be found, the opener preparation did not necessitate exposing the single fibers for mechanogram studies. It was simpler just to separate the small nerve bundle from the two larger ones, and avoid interaction of the closer muscle on the opener contractions by cutting the tendon of the closer. For electrogram studies, however, the single inhibitor neuron was prepared as an isolated fiber in order to avoid the muscle action potentials of the closer.

Results

Drugs lengthening the refractory period of the nerve. Bayliss *et al* (1935), working with the nerves of Maia, and Marmont (1941) with the isolated nerve fibers of the rock-lobster, Panulirus, have reported that yohimbinized nerve fibers show a most significant lengthening of the refractory period. In Cambarus a similar result is obtained; thus after injecting a saturated solution of yohimbine (1 : 20,000 according to Bayliss *et al*) into the claw, single fast closer twitches of normal height can be obtained for an indefinite period after the injection, yet if the preparation is stimulated repeatedly with shocks following each other at intervals of about one second, a twitch can be seen to drop out occasionally (Figure 1, A). Faradic stimulation (45/sec.) causes, instead of a tetanus, a series of twitches of normal magnitude which occur at a frequency of about 1/sec. (Figure 1, A). Stimulation of the slow fiber under these conditions no longer results in a contraction, nor does stimulation of the opener. The muscle action potentials of the slow closer contraction and of the opener are correspondingly absent. The muscle action potentials of the fast twitch show little effect of the drug. During faradic stimulation a series of such action potentials is usually obtained. These effects are generally found either after injection of the drug into the claw or after treating the nerve in a narcotic chamber. Sometimes, however, when the drug is injected into the claw, faradic stimulation results in a row of action potential spikes of variable frequency and magnitude, only the larger of which are accompanied by any visible contraction (Figure 1, C). This is probably due to the unequal distribution of the drug with respect to the finer branches of the nerve fibers in the muscle; part of

the nerve fiber branches have a different refractory period than those of the rest of the arborization.

It is interesting to note that stimulation through electrodes placed on the nerve or in the muscle shows the same effect on the mechanogram. In both cases a series of twitches is obtained on faradic stimulation. Only with very strong stimulation through electrodes placed on the muscle can a continuous contraction be obtained. This indicates that unless excessively strong stimuli are used, stimulation remains indirect. The contraction obtained by strong direct stimulation is usually irreversible.

Local anaesthetics and veratrine also produce a lengthening of the refractory period of the nerve, but with veratrine this is usually more or less masked by other effects which will be described later.

Local anaesthetics. Injection of drugs of this type into the claw caused a gradual diminishing of the fast contractions, and ultimately resulted in a complete excitatory failure. When the nerve was treated in a narcotic chamber, however, the twitch was either present, full size, or was entirely absent (Figure 2). This shows that in the single fast nerve fiber the impulse is either conducted or not conducted in the region beyond the chamber. The intermediate size of the contractions on injecting the muscle can be considered to result from blocking the smaller branches of the nerve fiber at different times as introduction of the drug by injection is not instantaneous and all parts in the muscle are not acted on simultaneously by an equal concentration of the drug. At first only part of the branches are blocked, but with further spreading of the drug more and more of the nerve endings are affected, and only after some time does the entire muscle show the true drugged response.[3]

[3] Since direct stimulation of the muscle is not possible in the crustacea one cannot be sure whether the drug acts on the muscle as well as on the nerve or not. In view of the completeness of the nerve block in the chamber experiments it seems likely that the action is only on the nerve. The fact that a markedly lower concentration will produce the effects when injected into the muscle than is necessary to block the nerve is due, in part, to the more readily accessible position of the nerve fiber branches, in part, to the thinner nature of the fiber.

FIGURE 1. *Cambarus clarkii*, closer muscle. The effects of a saturated solution of yohimbine hydrochloride injected into the claw. A.—Contractions of the closer muscle in response to stimulation of the fast axon. It should be noted that the fourth, seventh, eighth, tenth, and eleventh single induction shocks were without mechanical effect. B.—Stimulation of the fast fiber with single induction shocks and the slow fiber faradically. Note the complete absence of contraction during stimulation of the slow fiber. C.—Electrogram and mechanogram during stimulation (faradic) of the fast fiber. Note the higher spikes and evidence of mechanical contraction at 2 and 3. M = mechanogram, E = electrogram, S = signal of stimulus, and T = time. In A and B time is in seconds, in C, in 0.1 second.

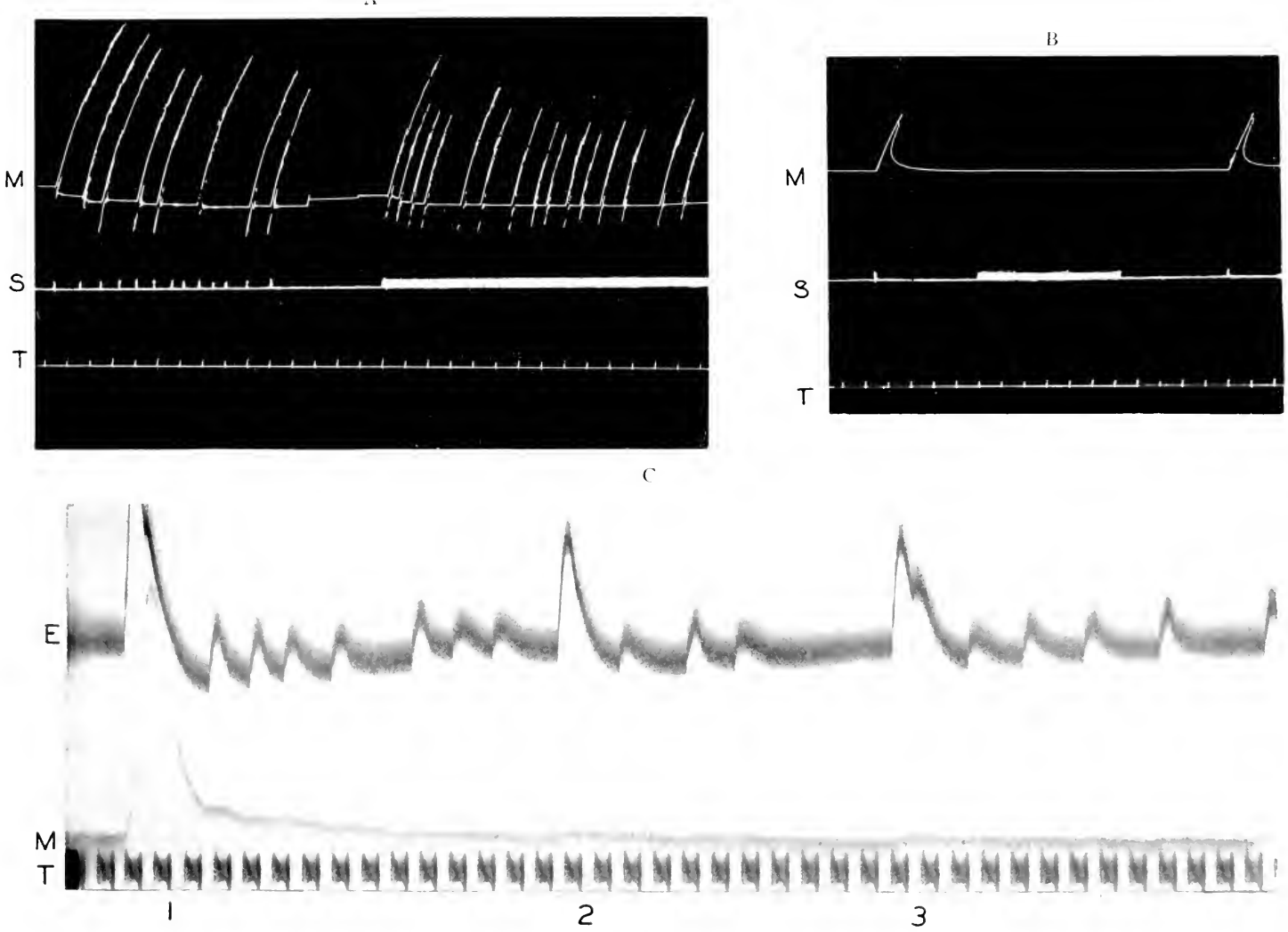

FIGURE 1.

When the fast and the slow systems were stimulated separately the twitch contraction of the closer could be elicited at a time when faradic stimulation of the slow did not result in any visible effect. This might be the result of a selective blocking of the smaller slow fiber before the larger fast one, as was shown by Escobar (1937) to be characteristic for the narcotic block in vertebrate preparations. The difference in the diameters of the "thick" fast closer fiber and the "thin" slow closer axon (58μ and 38μ respectively, see van Harreveld and Wiersma, 1936), however, is hardly great enough to warrant explanation of the early failure of the slow system on this basis. It is a well established fact that in the early stages of narcosis nerve

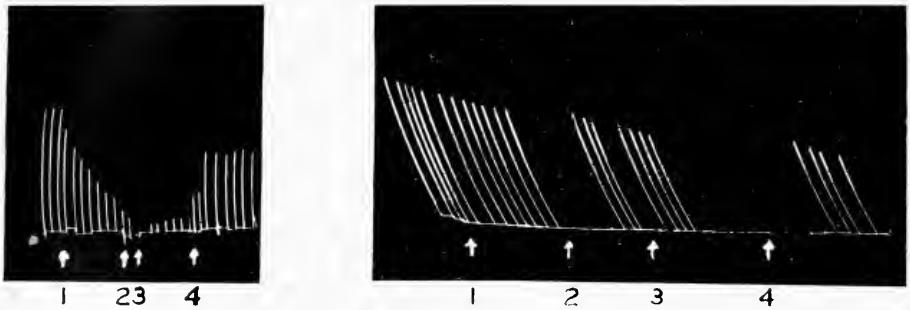

FIGURE 2. Mechanogram of the twitch closer contraction of *Cambarus clarkii* after treatment with procaine. A.—At 1, ½ ml. 0.1 per cent solution of procaine was injected into the claw. At 2, 3, and 4 the claw was washed with van Harreveld solution. B.—The nerve was treated with a 1 per cent procaine solution in a narcotic chamber at 1 and 3. The chamber was emptied and refilled with van Harreveld solution at 2 and 4. Single induction break shocks were given every 30 seconds in A; every minute in B. Note that in A the contraction disappears and reappears gradually, in B, all at once.

fibers exhibit a lengthening of the refractory period (Wedensky, 1904). Because of the dependence of the slow contractions upon facilitation (repetitive stimulation), lengthening of the refractory period would make it impossible for the impulses to follow each other at a high enough frequency to produce a contraction. This seems the more likely explanation for the failure of the slow system before the fast system, especially since in this state faradic stimulation of the fast shows a smaller contraction than the normal one. Drugs producing this anaesthetizing effect when injected into the claw are nupercaine (0.03%), procaine (0.1%), diothane (0.4%), amphetamine (1%), and atropine (1%). The concentrations indicated produced failure in the slow system within five minutes.

Drugs increasing tendency to repeated discharge. Stimulation of crustacean nerves with strong stimuli commonly results in repeated discharge of the nerve fibers. This tendency can be so intensified by treating the preparation with appropriate drugs that a single impulse set up in a normal portion of the nerve fiber, upon reaching the poisoned portion, causes a series of discharges to be initiated at the drugged region. A volley of muscle action potentials is recorded upon stimulation of the nerve with single shocks of weak intensity. Drugs producing this effect are physostigmine, 933F, hydrastinine, pyrethrum, veratrine, and brucine. Veratrine and pyrethrum are effective in much lower concentrations than the others. Injection of these drugs initiates spontaneous contraction in the claw; the larger the dosage the more pronounced is this contraction, and the longer is its duration. These spontaneous contractions are especially pronounced with physostigmine and pyrethrum.

With the electrodes on an undrugged part of the nerve, the concentration of the drugs required to produce repetitive discharges in the slow fiber in response to single shock stimulation is lower than that needed to produce this effect in the fast system. The duration of the burst of repetitive impulses is longer in the slow system than in the fast. In consequence, it has been possible to obtain contractions of the slow type on stimulation of the slow fiber with single weak induction shocks (Figure 3, B 4 and B 5). Repetition of discharge in the fast system results in an increase in the height of the contraction, which is clearly the result of summation of a limited number of impulses following each other within a very short time interval. When 933F was used, for example, a direct relation was observed to exist between the number of action potential spikes in each burst and the magnitude of the contraction. Only with very strong concentrations does a more prolonged fast contraction take place. Pyrethrum [4] (0.02%), 933F (0.1%), and brucine (2.5%) produced only these effects of repetitive discharge.

Hydrastinine presents a somewhat more complex picture. In a concentration of 1 per cent it shows, in the slow systems, essentially the same picture with regard to the number of muscle action potential spikes, thus single impulses will cause contractions. These are, how-

[4] Pyrethrum was kindly supplied by the United States Department of Agriculture in the form of concentrates of Pyrethrin I and Pyrethrin II. The Pyrethrin I concentrate consisted of Pyrethrin I, 57.0 per cent; Pyrethrin II, 13.7 per cent; unknown composition, 29.3 per cent. The Pyrethrin II concentrate consisted of Pyrethrin I, 5.2 per cent; Pyrethrin II, 74.2 per cent; unknown composition, 20.6 per cent. Dilution was made with alcohol to 1 mg./100 ml. This was further diluted with van Harreveld solution to the desired concentration.

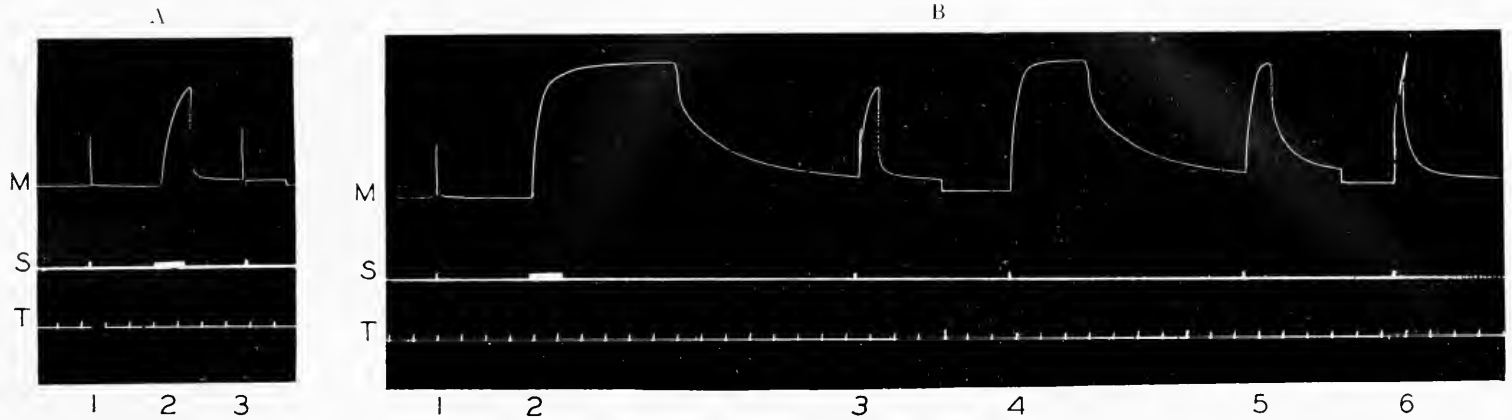

FIGURE 3. Mechanogram of the closer muscle of *Cambarus clarkii* after treating the claw with a 0.001 per cent solution of veratrine. A.—1—normal fast contraction, 2—normal slow contraction, 3—normal fast contraction. B.—1—fast contraction 6 minutes after injection, 2—slow contraction after 6 minutes, 3—fast contraction immediately following 2, 4—contraction following stimulation of slow fiber with single induction shock 7 minutes after injection, 5—slow fiber stimulated with single shock, 6—fast fiber stimulated with single shock 8 minutes after injection. Note that in B-2 the contraction continued for some time after stimulation ceased, and compare this with B-4 in which the response is to a single stimulus. T = 1 second.

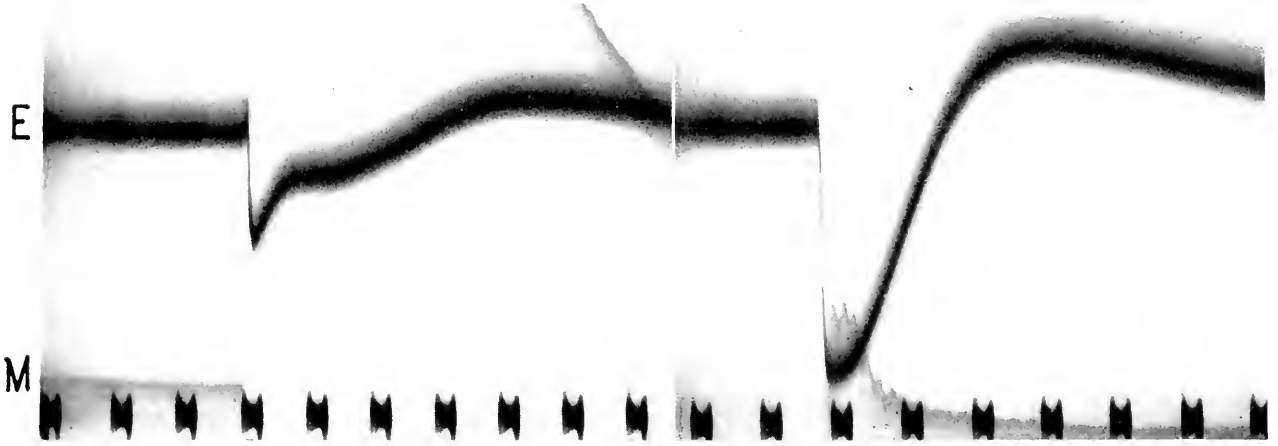

FIGURE 4. Electro- and mechanograms of the fast closer contraction of *Cambarus clarkii*. A.—before and B.—after treatment with 1 per cent hydrastinine hydrochloride. Note the increased size of the muscle action potential and at the same time the diminished size of the mechanical contraction. T = 0.1 second.

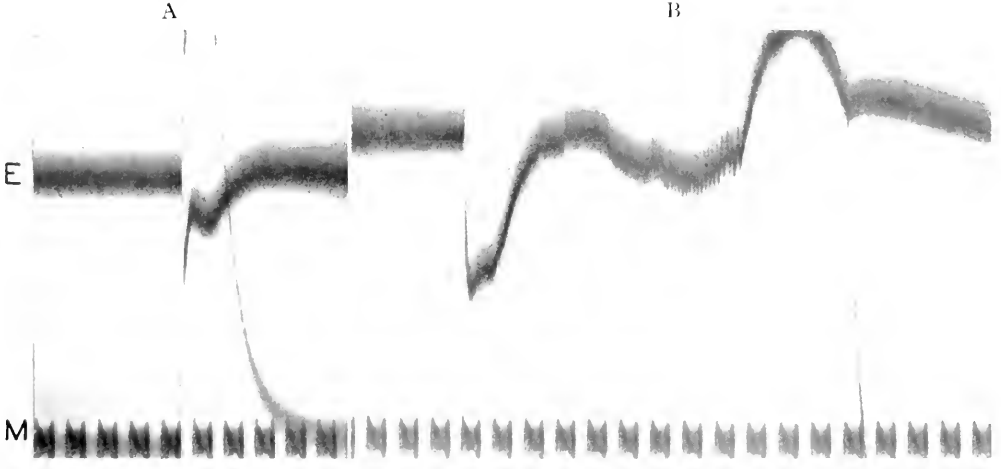

FIGURE 5. Electro- and mechanograms of closer muscle of *Cambarus clarkii* on stimulation of the fast fiber with single induction shocks. A.—normal preparation, B. —same preparation following injection of the claw with a 0.001 per cent solution of veratrine. T = 0.1 second.

ever, of a smaller magnitude than would be expected from a 933F treated preparation. In chamber experiments the fast system shows a higher contraction which is associated with repeated discharge of the nerve fiber, but when hydrastinine is injected into the claw, the mechanical contraction of the fast system diminishes in height, whereas the action potentials show a very pronounced increase in amplitude (Figure 4). Hydrastinine, therefore, appears to exert opposite effects on the muscle action potential and on the mechanical contraction; its effect on the nerve is clearly limited to setting up repeated discharges.

The action of veratrine is due, for the most part, to the initiation of repetitive discharge, but shows, in addition, considerable complication. When the whole nerve is treated in a narcotic chamber there ensues on a single stimulus a twitch of an increased height, sometimes followed by a secondary slow-rising top (Figure 3, B 3). When the slow fiber is removed, however, the contractions show only an increase in the size of the twitch, indicating that the secondary top in the first instance is due to a repetitive outburst in the slow fiber. Upon injection of the claw, single shocks to the slow fiber give rise to more or less prolonged contractions, and faradic stimulation for a short period may be followed by a markedly prolonged slow contraction (Figure 3, B 2). Stimulation of the isolated fast fiber following injection of the claw, however, not only produces contractions of a larger size, but, in addition, the secondary slow-rising top again becomes apparent. Under these circumstances the muscle action potentials show an initial high-topped spike with evidence of repetition, followed by a more or less prolonged burst of much smaller rhythmical action potentials diminishing in frequency, growing in size, and clearly accompanying the second top of the contraction (Figure 5). After the claw has been exposed to the drug for some time, the size of the fast action potentials again diminishes, as does the size of the action potentials of the slow closer contraction and of the opener. The lengthening of the refractory period by veratrine has already been mentioned.

Drugs without apparent effect. A number of substances had little or no effect on the peripheral system in the crayfish, even in high concentrations. These include choline (0.2%), the choline derivatives including mecholyl, doryl, and acetylcholine (0.1%), epinephrine (0.2%), digitalin (1%), nicotine (1%), caffeine (1%), curare (1%), muscarine (0.5%), pilocarpine (1%), and strychnine (0.2%). When injected into the whole animal, however, epinephrine (0.1 mg./g.), curare (0.3 mg./g.), strychnine (0.5 mg./g.), and the tetramethylammonium salts (0.1 mg./g.) had a marked depressive influence on

reflex excitability. Mecholyl (2.5 mg./g.), doryl (0.5 mg./g.), pilocarpine (2 mg./g.), the trimethylammonium salts (0.1 mg./g.), and acetylcholine (1 mg./g.) showed a more or less marked excitatory state as shown by reflex activity. Several of the others, e.g., nicotine (0.01 mg./g.) showed first an increased reflex excitability, but later a depressive effect became evident. These influences indicate an apparent dissimilarity in response between the central and peripheral nervous systems.

Inhibition

As has been pointed out, the experiments on inhibition have made use of the opener preparation in which only a single motor axon and an inhibitory fiber are involved. The drugs which had no effect on excitation were likewise found to have no effect on inhibition. The local anaesthetics and those drugs which lengthened the refractory period had no effect in concentrations which did not depress the contraction, and in stronger concentrations no especial effects were discernible.

The drugs which produce repeated discharges in the nerve fibers produce several interesting effects on the inhibitory mechanisms which can be attributed to this tendency. Because one or both of the fibers may fire repetitively, give after-discharges, or fire spontaneously without any stimulation, the results of stimulation become quite unpredictable, and analysis of them is often not possible. In certain cases, however, analysis can be made with some certainty. The following phenomena have been observed:

After-discharge of the inhibitor is found when, on simultaneous stimulation of the excitor and inhibitor fibers, a sudden stopping of inhibitory stimulation is accompanied by a prolongation of the inhibitory effect.

Both repetitive firing and after-discharge of the excitor have been encountered. The former condition has been observed under certain circumstances quite free from any parallel activity in the inhibitor. Stimulation of both excitor and inhibitor at a frequency of 30/sec. was accompanied, as shown by the muscle action potentials, by three excitatory impulses per stimulus. The mechanical contraction, under these circumstances, was only partially suppressed. This is not surprising since the *frequency of inhibition/frequency of excitation* ratio for complete inhibition in this system is 0.40, two inhibitory impulses being able to suppress 5 excitatory ones. Stimulation of the inhibitor at a frequency of 90/sec. resulted, as expected, in complete inhibition. It is obvious, therefore, that repetitive discharge was limited to the excitor in this case.

An interesting demonstration of the after-discharge of the excitor was seen on releasing inhibition shortly after stimulation of the excitor was discontinued. The resulting contraction could only be due to such an after-discharge.

Combined repetitive activity or after-discharge of both inhibitor and excitor would result in a situation so nearly normal that no clear-cut differences could be observed.

In several instances treatment with hydrastinine (1%) was accompanied by a state in which faradic stimulation of the inhibitor resulted in an enhancement of excitation, or even in contraction of the opener muscle in the absence of stimulation of the excitor (Figure 6). This

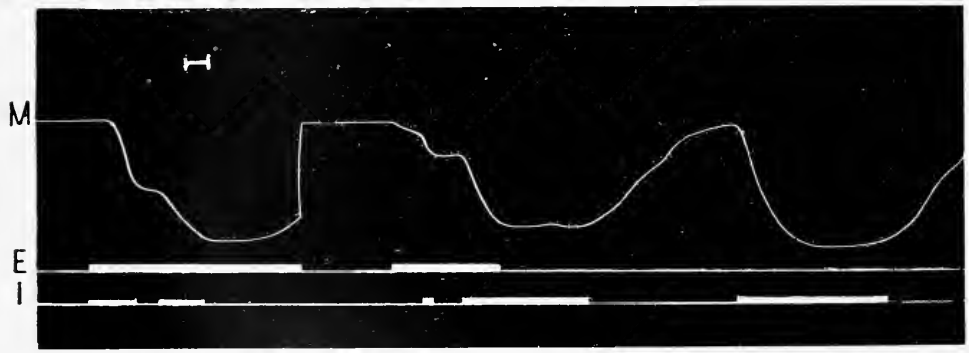

FIGURE 6. Mechanogram of the opener muscle of *Cambarus clarkii* after treatment with hydrastinine hydrochloride, showing reversal of inhibitor. The excitor and inhibitor were isolated so as to prevent stimulation by current escape. T = 1 second, E = excitatory stimulation, I = inhibitory stimulation.

effect was also obtained following injection of pyrethrum (0.02%) into the claw. It should be pointed out that due care was taken in each case to avoid stimulation of the other fibers by current escape. An explanation of this interesting phenomenon will be given in the discussion.

Supplementary inhibition. The presence of an independent secondary inhibitory process (supplementary inhibition) resulting in a depression of the muscle action potentials has made it interesting to investigate the effects of the drugs on this process. Supplementary inhibition occurs only when the time of arrival at the muscle of the inhibitory impulse just precedes that of the excitatory impulse within a very narrow time interval. When yohimbine was used it was found that with concentrations which were just high enough to ultimately produce the characteristic block of excitation, supplementary inhibi-

tion was present, apparently unchanged, up to the very moment of the excitatory block. This was also the case with the local anaesthetics. Hydrastinine, veratrine, and pyrethrum showed no selective effect on this process. These three latter substances, however, by virtue of their tendency to set up multiple discharges in the nerve upon stimulation, produce a state in which many of the excitatory impulses are firing at times which make their arrival at the muscle fibers with respect to the arrival of the inhibitory impulses fall outside the narrow time limits necessary to produce depression of the muscle action potentials. By using a cathode ray oscillograph with the sweep synchronized with the stimulation frequency, it was possible, in several instances, to perceive, instead of one, two or more action potentials in each sweep. Only the first of these showed any reduction during supplementary inhibition.

Pyrethrum (0.0001%) greatly increased the excitability of the axons, giving rise to a state in which almost any degree of activity could be obtained. In the intervals between spontaneous discharges, reduction of the muscle action potentials during inhibition was still possible. Veratrine in low concentrations (0.001%) resulted in a situation quite similar to that following injection of hydrastinine; the first action potential was reduced, the repetitive ones were unchanged. It appears, therefore, that these substances have little effect on the mechanism of supplementary inhibition.

Discussion

In view of the fact that the innervation of a muscle fiber in the decapod crustacea presents a picture so completely different from that of vertebrate skeletal muscle, it is not unexpected that responses of such a nerve-muscle preparation to drugs should be, in general, quite different. Straub (1900) and Katz (1936) have shown that curare, e.g., which is known to block the transmission mechanism at the end-organ in vertebrate preparations, has no noticeable effect on the crustacean system. Our results further illustrate the fundamental differences in the action of drugs on vertebrate and crustacean preparations.

The hope that by using a number of drugs it would be possible to study more clearly the mechanism involved in the transmission of the nerve impulse to the contractile mechanism in the crustacea has not been realized. Katz (1936) reported a "curarization" effect on crustacean muscle on treating the preparation with an excess of Mg^{++}, but it was certainly not well borne out by Waterman (1941), nor has it been possible in our laboratory to reproduce this effect. In fact

it has not been possible to observe any effects which could be limited to the transmission mechanism between nerve and muscle. A large majority of the substances used have clearly exerted their effects on the nerve itself, the response being the same or very similar whether the drug was injected into the muscle or whether the nerve was treated in a narcotic chamber. Both hydrastinine and veratrine show, besides an effect clearly limited to the nerve fiber, an effect which seems to be located somewhere between the nerve impulse and the contractile

TABLE II

Classification of drugs as to their action on Crustacean peripheral nerve-muscle systems

Group	Local anaesthetics	Lengthened refractory period	Increased tendency to repeated discharge	No effect (concentrations up to 2 per cent)
Drug	Procaine* Diothane* Nupercaine† Amphetamine Atropine	Yohimbine‡ Veratrine‡	Veratrine‡ Pyrethrum† Physostigmine† 933F* Hydrastinine Brucine	Choline Acetylcholine Mecholyl Doryl Muscarine Curare Strychnine Trimethylammonium salts Tetramethylammonium salts Pilocarpine Digitalin Epinephrine Nicotine Caffeine Rotenone

* Effective in concentrations between 1 and 0.1 per cent.
† Effective in concentrations between 0.1 and 0.01 per cent.
‡ Effective in concentrations below 0.01 per cent.

mechanism. The change brought about on the nerve impulses, however, prevents satisfactory analysis of this additional effect.

In many cases noticeable effects were obtained only by using strong concentrations of substances which, in mammals, are known to work in very low concentrations. One hesitates, therefore, to consider the action of substances like prostigmine, which gave little effect in concentrations of less than 2 per cent as specific. Certain of the results, however, can be discussed with a reasonable degree of confidence (Table II).

Aside from the narcotizing effect of the local anaesthetics, the effects of the drugs on the nerve seem to be of two types; either the

refractory period is so lengthened that the nerve cannot carry impulses at an effective frequency, or the excitability is so increased that multiple discharges are set up. Either of these effects provides interesting relationships to the problems involved in the "twitch" and "slow" contractions of crustacean muscles. The characteristic need for facilitation in the slow contractions is emphasized by the former type of response, i.e., lengthening the refractory period to such an extent that facilitation becomes impossible, whereupon no slow contraction is obtainable. On the other hand, those drugs which increase the excitability and thereby give rise to multiple discharges on single shocks, show contractions of the slow type on stimulation with single weak shocks which normally produce no response whatsoever.

It should be pointed out that the fundamental mechanisms involved in inhibition were apparently little influenced by even the most effective drugs used in this investigation. Drugs which lengthen the refractory period of the nerve cannot be easily investigated as to their effect on peripheral inhibition, since their effect on the excitatory mechanism in "slow" systems usually results in a complete excitatory failure. It is probable, however, that they exert an effect on the inhibitory axon identical with that on the motor fiber. Reduction of the muscle action potentials during "supplementary" inhibition was found to apply only to the action potential set up by the motor stimulus; subsequently occurring repetitive discharges, being asynchronous with inhibitory ones, showed no such depression. Simple inhibition, at least qualitatively, appeared to be quite normal.

The "reversal" effects in which stimulation of the inhibitor results in contraction, or stimulation of the excitor results in inhibition, need a more extended discussion, especially in the light of Segaar's (1929) claim that inhibitory stimulation at times becomes excitatory and vice versa. He attributed this to an inherent ability of the axons to produce reciprocal effects, i.e., the inhibitory axon actually becomes excitatory under certain influences, and vice versa. The present investigation has provided some pertinent information concerning this type of behavior, which makes explanation of the phenomena possible without having to assume any alteration of the inherent specificity of the nerve fibers. The reversal effects are not normally encountered, and are only produced with great difficulty. They are rarely, if ever, encountered together in the same preparation.

In the crayfish opener system, the motor axon is much more prone to repeated discharge than is the inhibitor. This is seen on cutting the leg from the intact animal, whereupon, following a short-lasting initial closing, a prolonged opener contraction is almost always present.

Stimulation for this contraction is a spontaneous volley arising at the severed end of the motor axon. Were the inhibitor equally prone to repetitive discharge, the contraction would be suppressed by the spontaneous impulses arising from the cut end of the inhibitory fiber. Observations in this laboratory have shown that of all the nerve fibers in the claw, the opener axon shows by far the most prolonged after-discharge following transection of the nerve fibers. It is not surprising, then, that certain drugs (veratrine, pyrethrum, etc.) should set up repetitive discharges in the motor axon to a greater extent than in the inhibitory fiber, nor is it difficult to conceive that under these circumstances a spontaneous motor discharge is set up at a frequency such that the nerve fiber is barely able to follow. Additional stimulation of the motor axon would then result in a Wedensky-block of the opener contraction. This concept is further supported by the complete absence of muscle action potentials, showing that the excitatory fiber is quiescent. If the absence of contraction were due to inhibition, action potentials of at least 25 per cent of the normal height should be present.

The other reversal effect in which stimulation of the inhibitor produces a contraction or an enhancement of the one set up by excitation, however, cannot be explained on this basis. The total absence of muscle action potentials shows that the excitor and inhibitor were not both firing spontaneously and thus suppressing the contraction until released by a Wedensky-block of the inhibitor. It is apparent, therefore, that some other mechanism is involved. It seems likely that the nerve fibers (probably in the smaller branches) become so sensitive under the influence of the drug that passage of an impulse along the inhibitory fiber produces sufficient polarization to excite the hypersensitive adjacent motor axon. In these motor branches each such stimulus sets up repeated discharges, raising the excitatory frequency above that which the inhibitory frequency can suppress. Such cross excitations have been reported (Jasper and Monnier, 1938) and the interaction between adjacent fibers has been demonstrated and studied by Katz and Schmitt (1940, 1942).

Summary

The effects of a wide selection of drugs on the peripheral nerve-muscle preparations of the cheliped of the crayfish, *Cambarus clarkii*, were studied.

Of all drugs used, the local anaesthetics were the only ones which, as a group, showed the customary vertebrate effects on crustacean preparations.

In general, the effects of the drugs used in the investigation were limited to changes in the excitability of the nerve fiber. These effects were of two types; either the refractory period of the nerve was markedly lengthened, or the nerve fiber became so hypersensitive that excitatory stimuli set up multiple discharges in the nerve.

The effects of the drugs on peripheral inhibition were studied, with the conclusion that the substances exert little effect on the mechanisms involved in these phenomena, especially on those responsible for supplementary inhibition.

An investigation was made into certain "reversal" effects in which stimulation of the inhibitory axon showed an excitation, and in which stimulation of the excitatory axon showed an inhibitory effect. Of these, the former is ascribed to stimulation of the adjacent hypersensitive motor fiber upon stimulation of the inhibitor; the latter, to a Wedensky-block.

The central effects appear to be quite different from the effects on the peripheral systems.

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OXYGEN CONSUMPTION OF FOX SPERM

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Measurements of oxygen consumption have been made on human spermatozoa (Shettles, 1940; MacLeod, 1941) and on those of some domestic and laboratory animals (Ivanow, 1931; Redenz, 1933; Windstosser, 1935; Comstock, 1939; Lardy and Phillips, 1941; Henle and Zittle, 1942). The determinations have been carried out on suspensions of epididymal sperm derived from excised gonads and suspensions of seminal sperm obtained by emission.

Natural and technical difficulties attending the measurement of respiration have hitherto required certain preparatory changes to be made in the normal sperm suspensions before the respiratory measurements were made. It has often been necessary to dilute densely packed samples of epididymal sperm suspensions, and most investigators have found it expedient to remove the sperm altogether from the normal medium by centrifugation and to resuspend the sperm in Ringer-glucose or salt solution (MacLeod, 1941; Henle and Zittle, 1942).

In suspensions in which the count is low, it has often been necessary to concentrate the sperm by centrifugation in order to secure sufficient respiring material to give a measurable oxygen uptake. Frequently samples have been pooled for determinations (Henle and Zittle, 1942). This procedure supplies much material for study but cancels individual variation. It was found necessary to use one ml. of suspension in the Warburg apparatus (MacLeod, 1941), but samples of that size are not easily obtained from small animals. A further important drawback in many investigations of the respiration of sperm is the considerable delay that is allowed to lapse between the time the sperm are collected and the determinations made.

By the use of the microrespirometer of Scholander (1942), it is possible to eliminate several of the difficulties encountered in previous measurements. The measurements were made on the semen from silver foxes, for in the short breeding season of these animals objective criteria of the viability of sperm are significant. Very small quantities of ejaculate, containing relatively few sperm, could be measured,

* Agent, Fish and Wildlife Service, United States Department of the Interior.

and the sperm were studied in the seminal fluid, essentially as they are under natural conditions. Furthermore, only a short period (about ten minutes) intervened between the time the sperm were collected and the respiratory measurements begun. A continuous record of oxygen consumption could be obtained over a two-hour period.

Methods and Materials

Apparatus

The volumetric microrespirometer described by Scholander (l.c. Figure 3) was used in this investigation. It was necessary to change the apparatus in several respects to adapt it to this problem (Figure 1).

A bulb-shaped respiratory chamber, *a*, of about 0.25 cc. total capacity, replaces the original straight-sided chamber. This vessel offers a larger surface on which to place the sperm suspension, and at the same time is easier to manipulate, without decreasing the sensitivity of the instrument.

The opening of the capillary tube into the compensatory bulb, *b*, is sealed with a piece of rubber held in place by a brass clamp, *c*. This seal avoids the use of grease which often and especially when wet oxidizes sufficiently to indicate a considerable oxygen consumption. The blank obtained when grease is used is too large to be disregarded.

Determinations on mammalian sperm require a water bath kept at 37°–38° C. (Figure 2). The thermobarometer does not require closer control of temperature than to within one degree. Two baths were used, one within the other, to prevent sudden temperature changes around the apparatus. The entire instrument, including the micrometer, was immersed in the inner bath.

Sensitivity

When the apparatus was set up without respiring material, the volume change was not greater than 0.01 cu. mm. an hour. This figure represents the limit of sensitivity and stability. Under these conditions there is practically no blank value. It was found that measurements of oxygen consumption could be made on suspensions containing as few as 500,000 sperm. With fewer sperm the values were open to question. When suspensions containing approximately 100,000 sperm were tested, the oxygen consumption could barely be detected. Satisfactory determinations of sperm respiration were obtained with 0.02 cc. samples in which the concentration of sperm was as low as 30,000,000/cc. In the fox the volume of the ejaculate, obtained by the electrical stimulator, varies between 0.5 and 3.0 cc., of

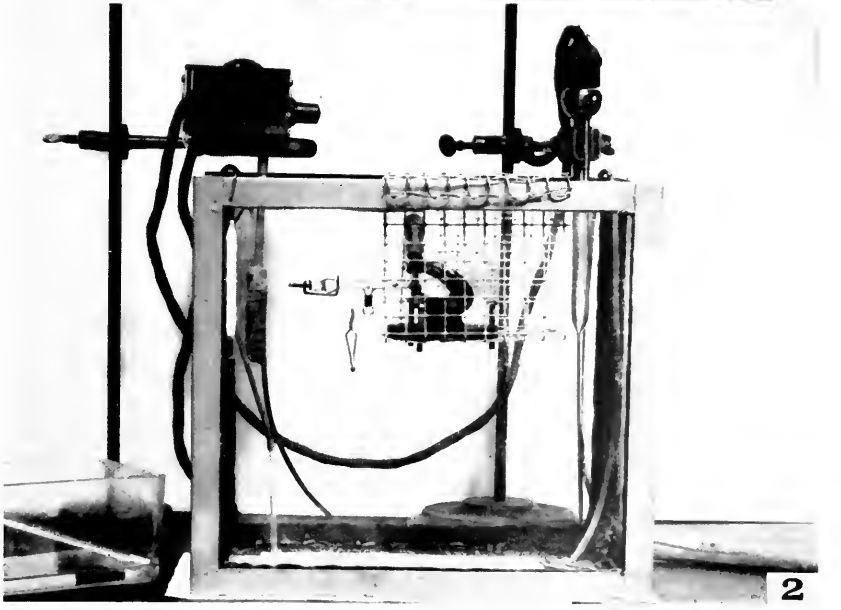

FIGURE 1. The microrespirometer showing *a*, the respiratory chamber; *b*, compensatory bulb; *c*, brass clamp which holds rubber seal in position.

FIGURE 2. The microrespirometer in position in outer water bath with heater and thermoregulator; inner bath removed.

which one-half to one-fourth is sperm fraction, the remainder being relatively sperm-free prostatic and preputial secretions. The sperm concentration normally lies between 25–240 million/cc.

Procedure

The spermatozoa of the silver fox, *Vulpes fulva*, were obtained by means of an electrical stimulator (Benham and Enders, 1941). The ejaculate was collected in a glass-tipped syringe and immediately transferred to small centrifuge tubes. Mild centrifugation by means of a hand centrifuge (60 r.p.m. for 25 seconds) was sufficient to separate the sperm from tissue debris and preputial oil globules. The concentration of the sperm in the middle of the suspension remained unaltered.

Approximately 20 cu. mm. of sperm suspension were introduced into the bottom of the respiratory chamber, which together with the rest of the respirometer, had been kept in an oven at about 37° C. Potassium hydroxide (0.9 per cent) was added to the platinum wire loop suspended from the T-tube so as to project into the respiratory chamber. The chamber, containing the sperm, was then placed firmly in position, after which a drop of 2 per cent Tergitol was introduced into the capillary tube through the compensatory vessel, and the tube closed with the rubber seal. The apparatus was immersed in the water bath, care being taken to compensate for the initial drift of the Tergitol drop owing to temperature changes. About ten minutes elapsed between seminal emission and the beginning of the respiratory determinations.

As oxygen was consumed by the respiring material, and carbon dioxide absorbed, the volume of the gas phase in the animal chamber and the capillary tubes became smaller as indicated by the drift of the meniscus in the tube. By screwing in the plunger of the micrometer this drift could be compensated and the meniscus returned to the same position in the capillary tube. Oxygen consumption was read in micrometer scale divisions and these converted into units of volume.

The oxygen consumed in one hour was on the order of one cu. mm., less than 2 per cent of the total oxygen in the system at the beginning of a determination. No oxygen was added to the reservoir during the course of the measurements.

The sperm suspension was spread in a thin film, about 0.5 mm., in thickness, on the bottom of the respiratory chamber. Diffusion was believed to be sufficiently rapid so that agitation was not necessary.

After the respiratory measurements were made, the sample was transferred to a graduated tube and diluted 500 times. With a blood-counting pipette a known volume of the sample (one part in

11,000) was placed on a hemocytometer slide, and the number of sperm determined. Duplicate counts with an average error of less than 10 per cent were obtained.

Results

Oxygen consumption of fox sperm suspensions

The initial uptake of oxygen by the sperm suspension frequently is very high, but after a stabilization period, from two to 15 minutes,

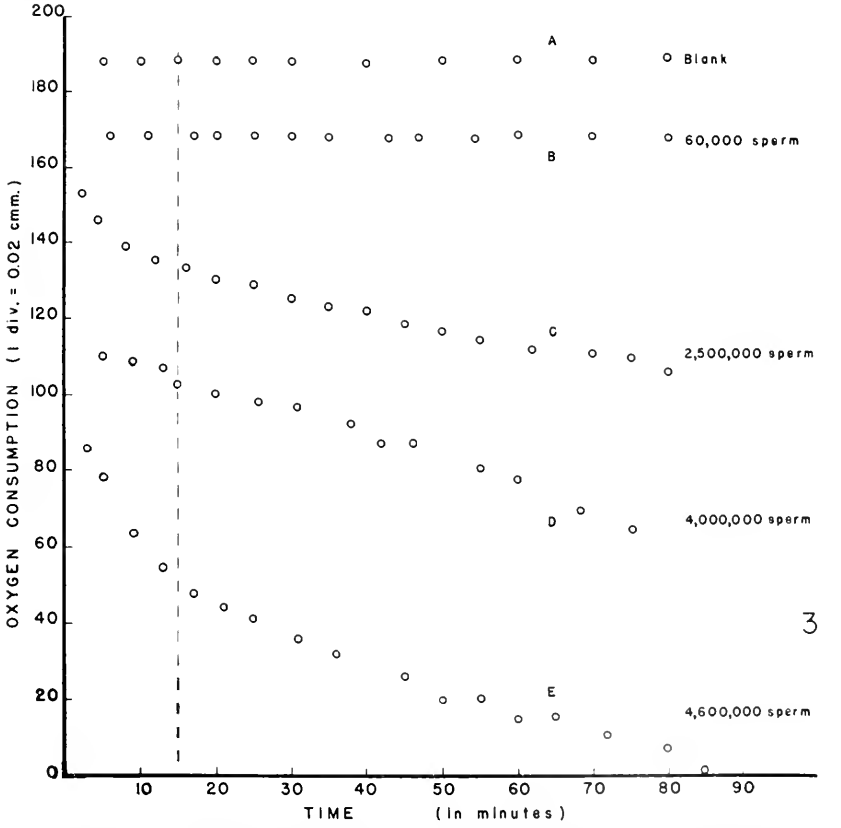

FIGURE 3. Oxygen consumption of fox seminal sperm during first 1½ hours after emission. *A*, a blank run with no sperm in respirometer. *B*, a run on a sample with very low sperm count (5,000,000/cc.). *C*, *D*, and *E* are determinations on samples of 2,500,000; 4,000,000 and 4,600,000 sperm, respectively. First 15 minutes a stabilization period.

continues steadily at a lower rate for one to two hours (Figure 3, E). The initial high oxygen consumption may be attributed to adjustment to temperature changes during transfer. But the regularity of the

initial increase, rather than a random increase or decrease, in oxygen consumption during the first few minutes seems to suggest a natural rapid initial respiration which might be brought about by the mixing of the sperm with prostatic secretions just prior to ejaculation. The absence of preliminary adjustment period in the blank would indicate that the apparent initial high oxygen uptake is characteristic of the sperm suspension and not of the apparatus.

Determinations of the oxygen consumption of sperm from six foxes are presented in Table I, expressed in cu. mm. oxygen consumed

TABLE I

Oxygen Consumption of Fox Sperm

Measurements based on first hour after stabilization period

Fox	cu. mm./10^8 sperm/hr.	Number of sperm in sample (0.02 cc.)	Remarks *
915 M	26.6	600,000	Uncentrifuged
191 A	26.7	3,500,000	Uncentrifuged
35 B	27.3	4,400,000	Uncentrifuged
35 B	19.2	4,600,000	Uncentrifuged
255 B	18.0	1,670,000	Centrifuged
161 B	20.8	2,500,000	Centrifuged
255 B	19.2	3,750,000	Centrifuged
255 B	17.5	4,000,000	Centrifuged
35 B	14.7	4,070,000	Centrifuged
35 B	5.4	10,000,000	Centrifuged; sperm motility sluggish, many free tails (late in season)

* A hand centrifuge (60 r.p.m. for 25 seconds) was sufficient to separate the sperm fraction from the oily secretation and the tissue debris.

per 100,000,000 sperm per hour (ZO_2 value of Redenz and of Henle and Zittle). Excluding the last determination, made on a sample with many immotile and broken sperm, the values lie between 19.2 and 27.3 for uncentrifuged suspensions, and between 14.7 and 20.8 for suspensions from which epithelial cellular debris was centrifuged off. The measurements cover the first hour after the stabilization period.

The amount of oxygen consumed, plotted against time, is shown in Figure 3, for three determinations. Included in the graph are a blank run (*A*) and a determination on a suspension with too few (60,000) sperm to measure any oxygen consumption (*B*). As the concentration of sperm increases, the slope of the curve becomes steeper.

An appreciable variation was found in determinations made on sperm from the same fox on successive days. A diminished respiratory

rate seemed to be correlated with a decrease in reproductive activity as indicated by the production of fewer sperm, a decrease in motility of the sperm, and a reduction in volume of ejaculate.

When the ejaculate was secured during the middle of the breeding period, the oxygen consumption of the sperm suspensions was proportional to the number of sperm present. Figure 4, based on the data in Table I, shows that with a threefold increase in sperm concentration

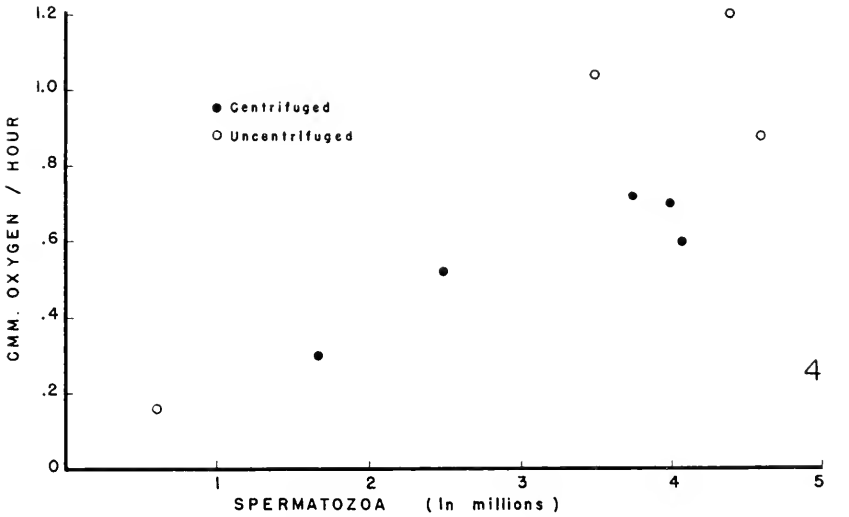

FIGURE 4. Rates of oxygen consumption of sperm suspensions of different concentrations during first hour after stabilization period. The oxygen consumed is proportional to the number of sperm present. The high values of two uncentrifuged samples due to contamination by cellular debris. All determinations made on 0.02 cc. samples.

among the centrifuged samples and an eightfold increase among uncentrifuged samples the increased respiration was proportional to the increased concentration.

Oxygen consumption of components of semen

Samples of semen frequently showed such unusually high oxygen consumption as to suggest the presence of respiring material other than sperm. The seminal fluid was centrifuged at low speed which was sufficient to isolate for measurement three fractions: (1) the lighter oily secretions derived mainly from the preputial glands, (2) the middle sperm-containing portion, (3) and the relatively sperm-free sediment consisting of dead and cornified cells and tissue debris, which in part was traced back to the epididymis in origin.

(1) The lipoidal secretions from the preputial glands had no detectable oxygen uptake.

(2) The middle portion, consisting of sperm and prostatic secretions, consumes oxygen. If the sperm were very few in number (5,000,000/cc. or less), or lacking altogether, as frequently is the case in animals at the close of the breeding season, the prostatic secretions showed no oxygen uptake. Samples with immotile and apparently dead sperm did not consume oxygen.

(3) The sediment in the bottom fraction respires at a high rate. This material may convey to entire semen a large oxygen consumption regardless of the number of sperm present. This sediment was traced back in part to the epididymis and it is probably comparable to the "epididymal secretion" of Henle and Zittle (1942) which showed a slight oxygen consumption and which augmented sperm respiration considerably.

Urine was tested to determine whether it might contribute anything as a contaminant to the total oxygen consumption of semen. No respiration was detected.

The point to be emphasized in this connection is that no oxygen consumption can be detected for semen without sperm, so long as the secretions are not contaminated by epithelial cells and debris.

Oxygen consumption of dog sperm suspensions

Several determinations were made on the ejaculates of a dog. The average oxygen consumption found of 21 cu. mm./10^8 sperm/hour is about the same as the fox. This is significant in the light of the fact that the seminal sperm of the dog are viable much longer than are those of the fox. Fox sperm did not retain motility longer than three hours after ejaculation; dog sperm were still motile 24 hours after ejaculation, although they were allowed to cool to room temperature (ca. 20° C.). The seminal fluid of the dog contains a negligible amount of tissue debris, and reliable results may be obtained on seminal sperm without centrifugation.

Discussion

A comparison of the respiratory determinations of fox and dog sperm with measurements of other investigators on other mammalian sperm is summarized in Table II. The environmental modifications to which most samples were subjected before and during observation may easily be responsible for some of the variations. The low rate of aerobic respiration of human seminal sperm after the first half-hour has been emphasized by MacLeod, who found practically no uptake

of oxygen when the sperm were suspended in Ringer or Ringer-glucose solution. However, when p-phenylene diamine (M/50) was added to the substrate the oxygen consumption was comparable to that for fox sperm (MacLeod, 1942).

The oxygen consumption of sperm may be expressed in terms of cc./gm./hr., although the calculation is only an approximation, based

TABLE II

Comparison of oxygen consumption determinations of suspensions of mammalian spermatozoa

Source	cu. mm./10^8 sperm/hr.	Authority
Rat, Guinea pig, Bull epididymis	0–38	Windstosser, 1935
Bull epididymis	7–30 (av. 18)	Redenz, 1933
Bull epididymis	11–27 (av. 15)	Henle and Zittle, 1942
Bull semen	7–10 (av. 9)	Henle and Zittle, 1942
Bull semen	8–28 (av. 19)	Lardy and Phillips, 1941
Boar semen	3–12.5*	Winchester and McKenzie, 1941
Human semen	0.3–3 (av. 1.8)	MacLeod, 1941
Human semen	18†	MacLeod, 1942
Human semen	15–17 (av. 16)	Shettles, 1940
Fox semen	14–30 (av. 19)	Bishop
Dog semen	17–26 (av. 21)	Bishop

* Higher values were consistently obtained with lower concentrations of sperm.
† A high oxygen consumption by the human sperm was obtained upon the addition of p-phenylene diaminé to the substrate.

on a rough measurement of the volume and the assumption that the specific gravity of the suspension is close to one. For fox sperm the values vary from 1.5 to 3.6, and for the dog between 4.8 and 5.4 cc./gm./hr.

Windstosser (1935) claimed to have demonstrated oxygen consumption by suspensions of immotile epididymal sperm. In the fox no oxygen consumption was detected in samples with immotile sperm, except where attributable to contamination. The possibility is not eliminated that immotile sperm consume oxygen, but if this is the case we were not able to measure it.

SUMMARY

A volumetric microrespirometer was used to measure the oxygen consumption of fox sperm obtained by electrical stimulation. Determinations were made on unmodified semen and on the components of semen during the first $1\frac{1}{2}$–2 hours following ejaculation. After an initial high stabilization period, lasting up to 15 minutes, the oxygen uptake of sperm suspensions averaged 19 cu. mm./10^8 sperm/

hour at 37°–38° C. The oxygen consumption was proportional to the number of sperm present over an eightfold range. Neither sperm-free semen, preputial secretions, prostatic fluids, nor urine consumed oxygen if not contaminated by tissue debris.

ACKNOWLEDGMENT

I am indebted to Dr. Laurence Irving, Dr. P. F. Scholander, and Dr. Robert K. Enders for counsel and for placing at my disposal the facilities of the Edward Martin Biological Laboratory, Swarthmore College, and to Mr. Charles F. Bassett, Director of the U. S. Fur Animal Station, Saratoga Springs, New York, for the facilities of the Station.

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A NOTE ON THE FREEZING-POINTS OF THE URINES OF TWO FRESH-WATER FISHES: THE CATFISH (*AMEIURUS NEBULOSUS*) AND THE SUCKER (*CATOSTOMUS COMMERSONII*)

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In his book, *Osmotic Regulation in Aquatic Animals*, Krogh (1939) comments on the scarcity of available data for the osmotic concentration of the urines of fresh-water fishes. Since such data are apparently not available for two of our common local forms, the catfish (*Ameiurus nebulosus*) and the sucker (*Catostomus commersonii*), we have been interested to make freezing-point determinations of their urines. During the urine collections, incidental observations on rate of flow were also included.

The fish, which were obtained locally, were kept in the laboratory fish tanks, and care was taken to use only fish in good condition. They were given no food during the experimental period. One series of determinations was made during the winter (November through February); the second, shorter series was made in May.

Urine was collected from the catfish through a small cannula tied into the urinary papilla and anchored by a stitch to the body wall. The clamping of a tube attached to the cannula prevented loss of fluid during the collection period; upon releasing the clamp, the urinary bladder could readily be emptied by pressure gently applied to the ventral body wall. In the case of the sucker, a similar cannula was used but the absence of a urinary bladder made necessary the attachment of a rubber balloon for collecting the urine.

By using a specially constructed container, freezing-point determinations could be made on amounts of fluid as small as 4 cc. A Heidenhain thermometer was used, graduated in 1/100° C. intervals, from which readings could be estimated to 2/1000° C. The Δ of the fresh-water medium was found to be .002° C.

Eleven urine samples from 5 catfish and 13 samples from 8 suckers were used for the determinations. The Δ values for the urines, together with the rates of urine flow, are summarized in Tables I and II. Corresponding to the relatively faster rates of flow the urine of the

catfish was extremely dilute, in comparison with the slower flow and more concentrated urine of the sucker. Although the data are not full enough to give definite information as to seasonal variations, they

TABLE I

Urine Flow and Δ of the Urine of the Catfish, Ameiurus nebulosus

Fish No.	Date	Body Wt., gm.	Collection Period, hrs.	Urine Flow, cc. per kgm. B.W. per hour	Δ of Urine, ° C.
1	Nov. 1	333	33	2.1	.034
	Nov. 2		14	2.7	.035
	Nov. 8		9	2.5	.030
				Av. 2.4	Av. .033
2	Nov. 22	241	17	1.7	.021
3	Jan. 9	...	...	...	.029
4	May 16	510	4	2.4	.019
	May 16		5.5	3.7	.021
	May 16		2.5	3.9	.023
	May 17*		10	3.0	.022
				Av. 3.25	Av. .021
5	May 17	335	7	3.2	.023
	May 17		4.5	3.6	.019
				Av. 3.4	Av. .021
Averages				(Winter) 2.1 (Spring) 3.3	.025

* Subsequent blood sample gave Δ serum value of .49° C.

suggest for both species a faster rate of urine flow in the spring than in the winter.

DISCUSSION

Our average Δ value of .094° for the urine of the sucker checks closely with Smith's (1932) figure of .09° for the fresh-water eel and rather well with the .07° and .08° which he obtained for the two ganoids, the bowfin *Amia* and the gar pike *Lepidosteus*, respectively. The distinctly lower Δ value of .025° for the catfish urine is doubtless correlated with its faster flow, although our fish were far from attaining the daily output of 300 cc. per kgm. of body weight that Marshall (1934) reports. The relatively fast urine flows in the catfish are of interest from the standpoint of the large glomerular filtering surface in this fish. Marshall and Smith (1930) have shown that the catfish renal

corpuscle is larger than that of many other fresh-water fishes: 99μ as against 60μ in the sucker. Also, studies made by Nash (1931) show that the catfish leads a group of eight fresh-water fishes studied with respect both to glomerular number and glomerular volume per unit

TABLE II

Urine Flow and Δ of the Urine of the Sucker, Catostomus commersonii

Fish No.	Date	Body Wt., gm.	Collection Period, hrs.	Urine Flow, cc. per kgm. B.W. per hour	Δ of Urine, ° C.
1	Jan. 17 Jan. 19	672	36 25	0.22 0.30 Av. 0.26	.047 .077 Av. .062
2	Feb. 2	895	24	0.26	.069
3	Feb. 2	850	24	(0.44)*	.086
4	Feb. 10 Feb. 10	1210	36 8.5	0.14 0.54 Av. 0.34	.120 .092 Av. .106
5	Feb. 10	1125	36	0.27	.181
6	Feb. 20 Feb. 21 Feb. 21	1007	15 16.5 10.5	0.35 0.26 0.42 Av. 0.34	.046 .062 .073 Av. .060
7	May 8 May 8	1079	18.5 8	0.38 1.6+ Av. 0.99	.107 .137 Av. .122
8	May 11	778	8.5	1.2+	.064
	Averages			(Winter) 0.29 (Spring) 1.10	.094

* Approximate only; omitted from average.

of body surface area. Unfortunately, Nash's figures do not also include values for the sucker.

Seasonal variations related to activity, nutritional status, and breeding should be investigated before the ranges of values for Δ and flow of urine can be definitely stated.

SUMMARY

1. Freezing-point determinations on the urines of five catfish, *Ameiurus nebulosus*, collected in winter and in May, ranged from −.019° C. to −.035° C., with an average of −.025° C.

2. Similar determinations on the urines of eight suckers, *Catostomus commersonii*, showed a range from −.046° C. to −.181° C., with an average of −.094° C.

3. The rate of urine flow was considerably higher in the catfish than in the sucker.

4. The possibility of seasonal variations in urine flow is suggested.

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INDUCTION OF TRIPLOIDY AND HAPLOIDY IN AXOLOTL EGGS BY COLD TREATMENT

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Recent investigations on polyploidy in three species of salamanders have shown that spontaneous deviations in chromosome number occur with surprising frequency among the larvae (Fankhauser, 1941b) and that it is relatively easy to induce triploidy experimentally by refrigerating eggs immediately after fertilization. This treatment presumably suppresses the second maturation division which is normally not completed until about one hour after laying (Fankhauser and Griffiths, 1939; Griffiths, 1941; Fankhauser, 1942).

The effects of the addition of a third chromosome set on development and growth have been studied in detail. Triploidy, as well as higher degrees of polyploidy, do not produce a significant increase in body size in salamander larvae, since the larger size of the individual cells is compensated by a corresponding decrease in cell number (Fankhauser, 1941a). On the other hand, triploidy was found to have a striking effect on sex differentiation; at the time of metamorphosis triploid testes appear normal, while triploid ovaries are much reduced in size and greatly retarded in differentiation (Fankhauser, 1940). Since in other animals triploidy affects the heterogametic sex only, this indicated that the female salamander produces two kinds of gametes, while the male is homogametic.

In view of these interesting and partly unexpected effects of triploidy in salamanders it seemed desirable to extend the work to still other species. Experiments with axolotl eggs had been planned for some time because axolotls may be raised and bred in the laboratory with comparative ease, and because a large amount of information is available regarding the development of the gonads under normal and experimental conditions. It is to be expected that the effects of polyploidy on sex can be analyzed more completely in this species than in those used before in which not even the normal process of sex differentiation had been described. The preliminary experiments to be reported in this paper were prompted by the recent investigations on the sex ratio in the progeny of sex-reversed Amblystoma females,

which produced convincing evidence that the female is normally heterozygous with regard to the sex chromosomes (Humphrey, 1942).

Materials and Methods

Eggs of the white (partial albino) variety of axolotls which are kept and bred in the Department of Anatomy of the University of Buffalo were used exclusively. The experiments were performed in Buffalo, and the embryos developing from the treated eggs, as well as untreated controls, were then shipped to Princeton for identification of the chromosome number of each animal and for the study of their later development.

The eggs to be refrigerated were removed immediately, or within a few minutes, after laying (which coincides with fertilization) and put in a container with ice water at + 1° to + 3° C., for from nine to 24 hours. Following this treatment they were transferred at once to dishes kept at room temperature and allowed to develop.

Shortly after hatching the posterior half of the tail of each larva was amputated and fixed in Bouin's or Navashin's fluid for two to four hours. These tailtips were stained in toto with Harris' acid haemalum and mounted, and the chromosomes counted in mitotic figures in the epidermis of the transparent tailfin.

In white axolotl larvae the fin surrounding the tail is practically free from melanophores which in normally pigmented larvae interfere with the microscopical study. However, when compared with tailtip preparations of larvae of *Triturus viridescens*, those of the axolotl larvae have several disadvantages: (1) many preparations contain very few mitotic figures; in some tailtips they are completely absent; (2) the diploid chromosome number is 28 against 22; (3) the arrangement of the chromosomes at metaphase is less regular and makes counting more difficult; (4) the cells are from 10 to 20 per cent smaller.

Results of Refrigeration Experiments

The results of the refrigeration experiments are summarized in Table I. The mortality among the treated eggs was high. Many eggs either did not cleave or divided abnormally and died at or before gastrulation. About 20 per cent of the eggs developed to hatching larvae, as compared with from 40 to 45 per cent in similar experiments with eggs of *Triturus viridescens*, and with 25 per cent in experiments with *Triturus pyrrhogaster* (Fankhauser, Crotta and Perrot, 1942).

Cytological examination of the tailtip preparations showed that the great majority of the larvae developing from refrigerated eggs—25 of 31, or about 80 per cent—were triploid. The absence of diploid

larvae in the experiments with eggs of spawning 56 indicates that eggs of different females differ in their reaction to the cold treatment, as had been shown previously for Triturus. The total percentage of triploid larvae obtained is higher than in the experiments with *Triturus pyrrhogaster*, but lower than in the original experiments of Griffiths with *Triturus viridescens*, in which almost 100 per cent of the larvae from treated eggs were triploid. In more recent experiments with

TABLE I

Effects of refrigeration of axolotl eggs on chromosome number of young larvae
Temperature: + 1° C. to + 3° C.*
Beginning of treatment: immediately or within a few minutes after laying (fertilization).
Duration of treatment: 9 to 24 hours.

Spawning number	Total number of eggs treated	Number of Embryos	Number of larvae tail-clipped	Chromosome number		
				triploid (3N = 42)	diploid (2N = 28)	haploid (N = 14)
54	22	5	5	3	2	—
55	66	6	6	3	3	—
56	66	29	20	19	—	1
Total	154	40	31	25	5	1
Controls (total)			148	1	147	—

* In the experiment with eggs of spawning 56 the temperature rose to + 6.7° C. at the end of the treatment.

eggs of the last mentioned species, performed by Miss Rita Crotta, this figure varied from 33 per cent to 100 per cent with eggs of different females.

The occurrence of a single haploid larva was not unexpected since several haploid larvae appeared in the more extensive refrigeration experiments with eggs of *T. viridescens* and *T. pyrrhogaster*. The mechanism which is responsible for the occasional production of haploids is unknown at present. The larva was easily recognized as haploid before tail-clipping. It showed symptoms that are typical for haploid amphibian larvae, viz., dwarfing, edema, and a very characteristic pigment pattern (Figure 6). The animal was preserved shortly after amputation of the tailtip. It should be mentioned that edema and reduced viability were also noted in some of the triploid and diploid larvae developed from refrigerated eggs and thus may not have been an effect of haploidy in this case.

The single triploid found among the controls demonstrates that occasional triploid embryos originate spontaneously in the axolotl as well as in the other species of salamanders studied. In *Triturus viridescens*, where a large number of larvae have been examined during the past five years, the frequency of spontaneous triploidy is slightly over one per cent (22 of 1635 individuals, or 1.36 per cent).

Because of the cytological disadvantages of the axolotl tailtips mentioned before, no chromosome counts could be made in five preparations, and in many others the counts were approximate only. Most triploid metaphase plates were so crowded (see Figures 7 and 8) that not more than 35 to 37 chromosomes could be made out individually; the unanalyzable residue showed clearly that the actual number was somewhat higher than this, presumably triploid. In three triploid metaphase plates, however, exactly 42 chromosomes could be counted.

In view of the difficulties encountered in obtaining accurate chromosome counts, a widely used secondary criterion of polyploidy became more important, namely, the larger size of the triploid nuclei in interphase. Twenty epidermis nuclei, from each tailtip, from corresponding regions in the ventral fin, were drawn at a magnification of 630×, and the total area occupied by the 20 nuclei was determined with a planimeter. Since the epidermis nuclei in the fin are very flat discs, this area gives a fair measure of the nuclear volume. This is indicated by the fact that the average areas of 20 triploid, 20 diploid, and 20

PLATE I

FIGURE 1. Diploid larva from spawning 52, 13 mm. long, five days after tail-clipping; a new tailtip is regenerating. × 5.4.

FIGURE 2. Spontaneous triploid larva from same spawning, 12.5 mm. long; the melanophores on the head are slightly less numerous and more widely spaced than in the diploid. × 5.4.

FIGURE 3. The same diploid larva as in Figure 1, 7½ weeks later, 37 mm. long. × 2.4.

FIGURE 4. The same triploid larva as in Figure 2, 34.5 mm. long. The difference in the pigment pattern is more clearly visible than in Figures 1 and 2. × 2.4.

FIGURE 5. Triploid larva 56.2 developed from a refrigerated egg, shortly after hatching and amputation of tailtip. × 5.4.

FIGURE 6. Haploid larva 56.19, developed from a refrigerated egg, of same age as triploid 56.2, but before amputation of tailtip. The melanophores are small and numerous. × 5.4.

FIGURE 7. Triploid metaphase (42 chromosomes) in epidermis of tailfin, as seen in whole-mount of tailtip. The chromosomes are crowded, the nuclei large. × 627.

FIGURE 8. Diploid metaphase (28 chromosomes) from tailtip of control, in a slightly earlier stage of metaphase with less condensed chromosomes; for this reason the whole plate appears as large as the triploid plate in Figure 7. × 627.

FIGURES 9 to 11. Connective tissue nuclei in the fin of a triploid, a diploid, and a haploid tailtip. The differences in size and spacing of the nuclei are clearly marked. × 314.

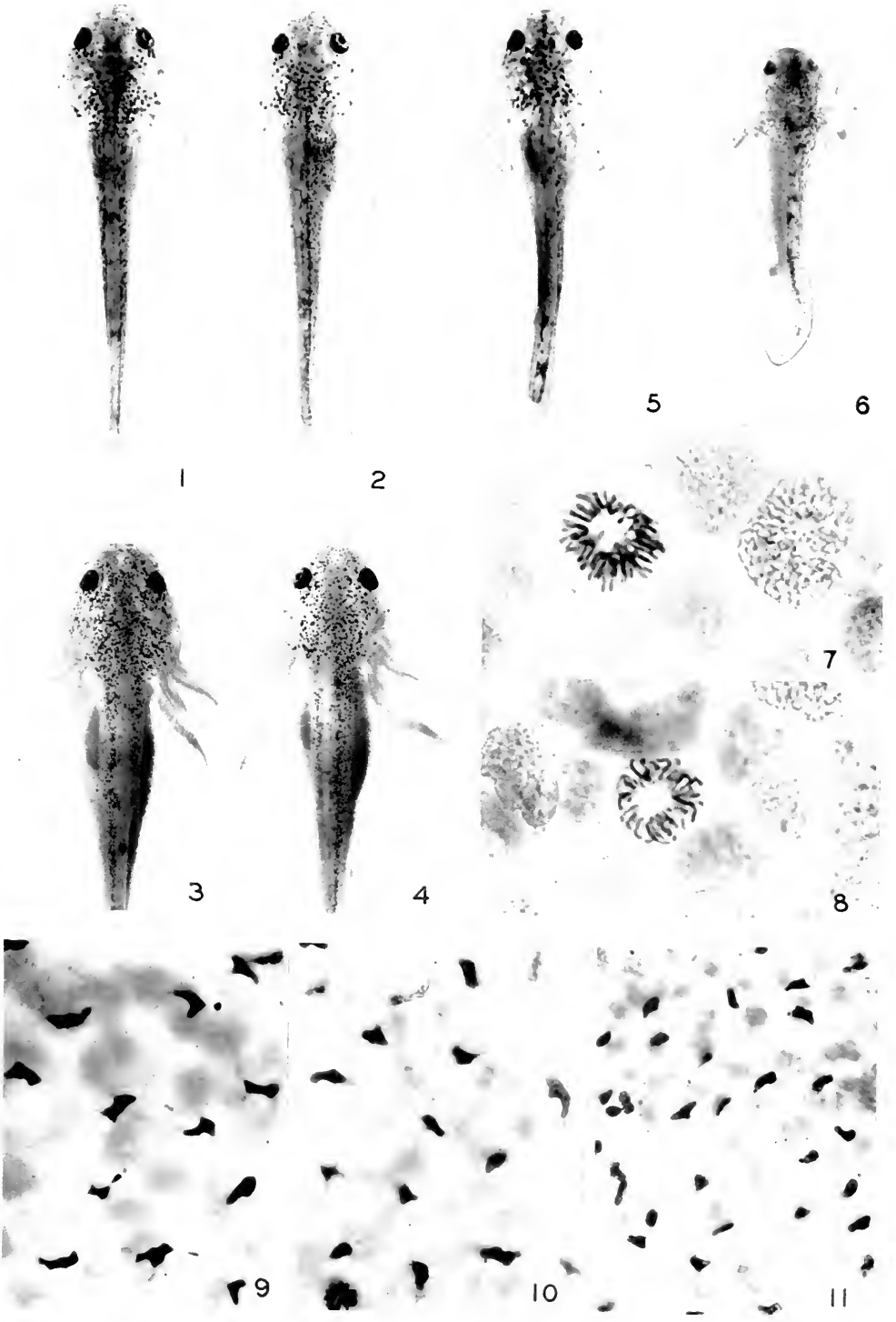

PLATE I

haploid nuclei differ almost exactly in proportion to the chromosome number (Table II). More important is the fact that although the area of 20 nuclei varies considerably between individual tailtips, the range of variation of the triploid tailtips does not overlap with that of the diploid. It is thus possible to identify a triploid tailtip from the size of the nuclei alone, without actual chromosome counts.

In many tailtip preparations red blood cells were present in capillaries in the fin. As many erythrocyte nuclei as possible were drawn at a magnification of 1433× and their greatest diameter measured. The average length of these nuclei from nineteen triploid tailtips was

TABLE II

Size of epidermis nuclei in tailfin as an index of polyploidy

	Area (in square inches) of 20 nuclei drawn at a magnification of 630×		
	Range	Average	Ratio (diploid = 2)
26 triploid tailtips	5.39 to 8.05	6.76	3.19
20 diploid tailtips	3.30 to 4.89	4.24	2.00
1 haploid tailtip	2.18	2.18	1.03

21.7 mm., the averages for the individual tailtips ranging from 20.5 to 23.5 mm. The average for four diploid tailtips was 18.2 mm., with a range of from 17.9 to 18.8 mm. The length of the erythrocyte nuclei thus offers another criterion of triploidy.

The connective tissue nuclei in the tailfin also are larger in triploid preparations and more widely spaced (Figures 9 to 11). This is easily detected when triploid and diploid preparations are viewed side by side with a comparison eyepiece. Because of their small size and irregular shape these nuclei are not favorable for actual measurements.

The classification of the larvae according to chromosome number would be greatly simplified if it were possible to recognize triploid individuals by their appearance under the dissecting microscope. In *Triturus viridescens* and *pyrrhogaster*, as well as in the lungless salamander, *Eurycea bislineata*, triploid larvae may be identified tentatively because of their usually striking pigment pattern. The individual melanophores are larger, but there are fewer pigment cells in corresponding areas (Fankhauser, Crotta and Perrot, 1942, Figure 2).

In axolotls of the white variety a number of melanophores are always present. However, they do not form a characteristic pigment pattern and can be used for a preliminary identification of triploid individuals only in some favorable cases. Following the classification

of the larvae by the tailtip method, the pigment pattern quite often served as a mark of identification (Figures 1 to 5).

Aside from the melanophore pattern, the general appearance of the nine triploid larvae which are surviving at present is normal. With one exception they are of normal size and grow at about the same rate as the controls. Seventeen of the triploid larvae developing from treated eggs were more or less abnormal and died early. However, this was true also of the five diploid larvae raised from refrigerated eggs. The abnormalities, therefore, are not necessarily connected with the change in chromosome number and must be attributed to some general harmful effect of the low temperature on the eggs, presumably on the egg cytoplasm.

Discussion

The observations reported in this paper are of general interest in several respects.

(1) They demonstrate the spontaneous occurrence of triploidy in a normal population of larvae in a fourth species of salamanders. It is noteworthy that this triploid axolotl larva developed from an egg laid spontaneously, while those previously reported in the other three species all came from eggs deposited following pituitary stimulation.

(2) The success of the three preliminary refrigeration experiments (Table I) shows that this method will be capable of producing a large number of triploid larvae, in spite of the high mortality among treated eggs.

(3) The normal body size and viability of triploid axolotl larvae confirm the observations made on the other species of salamanders. A compensation of the increase in cell size through a reduction in cell number seems to represent the typical response of the salamander embryo to an increase in chromosome number (cf. Fankhauser, 1941a).

(4) The existence of genetically different varieties of axolotls offers an opportunity to combine experimental induction of triploidy with cross-fertilization. For instance, reciprocal crosses between black and white individuals could be made and the resulting fertilized eggs refrigerated as spawned. This treatment would add a second set of maternal chromosomes and produce embryos in which either one or two of the three homologous chromosomes carry the "white" factor. It will be interesting to determine whether or not the single "black" factor in the last mentioned combination will be completely dominant over the two factors for white.

In general, the results obtained so far afford good reason to expect that it will now be possible to extend the investigations on polyploidy

beyond the period of embryonic and larval development and to combine them with breeding experiments, thus opening the way to a more detailed genetical analysis.

Summary

1. One hundred and fifty-four eggs of the white race of axolotls were refrigerated shortly after fertilization, at + 1° C. to + 3° C., for from nine to 24 hours, and raised at room temperature.

2. Thirty-one larvae were obtained from refrigerated eggs. Twenty-five of these were triploid, five diploid, and one haploid. The triploid larvae presumably arose from eggs in which the exposure to cold had suppressed the second maturation division. The origin of the haploid larva is unknown.

3. Of 148 control larvae, 147 were diploid and one triploid.

4. Seventeen of the triploid larvae, as well as the five diploid larvae which developed from refrigerated eggs, were more or less abnormal, presumably because of a general harmful effect of the low temperature on the eggs. This was also responsible for the high mortality among treated eggs before and during cleavage.

5. Seven of the remaining eight experimental triploids, as well as the single spontaneous triploid, are normal in size, appearance, and viability.

6. Experimental induction of triploidy in axolotls is of particular interest because it may make it possible to raise triploid individuals to sexual maturity and to carry out breeding experiments.

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EMBRYONIC TEMPERATURE TOLERANCE AND RATE OF DEVELOPMENT IN RANA CATESBEIANA [1]

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The frogs of eastern North America, like many other organisms, have definite breeding seasons. The chronological breeding sequence is, (1) *Rana sylvatica*, (2) *Rana pipiens*, (3) *Rana palustris*, (4) *Rana clamitans*, and (5) *Rana catesbeiana*. Some overlapping in breeding time occurs, especially in seasons marked by a rapid rise in temperature, but in general the species breed in the order given. Although our knowledge of the distribution of these forms is incomplete, it appears that the same sequence holds for northern distribution. For example, *Rana sylvatica*, which is the first to breed, ranges farthest north, and *Rana catesbeiana*, which is the last to breed, has the least extensive northern distribution (Table II).

A probable basis for this dissimilarity in breeding habits and geographic distribution is to be found in certain physiological differences among the embryos that restrict each species to certain definite temperature conditions. Two of the most important effects of temperature on poikilotherms are, (1) the limiting of the range of temperatures in which vital activities occur, and (2) in modifying the rate of metabolism at different temperatures in this range. Differences that may be thought of as adaptations to both of these effects are found in frogs. *Rana sylvatica* develops normally at temperatures from 2.5° C. to 24° C., and *Rana clamitans* from 12° C. to 32° C. Thus, the early breeding, northern *Rana sylvatica* has a range of embryonic temperature tolerance that is necessitated by the low temperatures of its breeding environment. The embryos of *Rana clamitans* similarly are adapted to the warmer pond waters present when they are developing. Marked differences in rate of development distinguish these frogs. *Rana sylvatica* has the more rapid rate of embryonic growth. *Rana clamitans* is 58 per cent slower at 20° C. Thus *Rana sylvatica*, by its rapid rate of growth, offsets in part the retarding effect on its embryonic metabolism of the cold water characteristic of its breeding environment.

These questions have been discussed previously (Moore, 1939) and it was shown that *Rana sylvatica*, *Rana pipiens*, *Rana palustris*, and *Rana clamitans* fall into the same sequence when a comparison is made

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of their order of breeding, northern distributional limit, embryonic temperature tolerance, and rapidity of development. No observations on temperature tolerance, and only fragmentary data on rate of development were available at that time for *Rana catesbeiana*. In some respects this species is the most interesting as it is the last to breed and has the least extensive northern distribution. The data on *Rana catesbeiana* now to be presented are, in a limited sense, a test of the validity of the correlations previously found for the other four species. To fit the general scheme *Rana catesbeiana* should have the slowest rate of development and the highest range of embryonic temperature tolerance.

In addition a comparison will be made of the rate of development and embryonic temperature tolerance of *Rana catesbeiana* from different latitudes. Data of this nature should throw some light on evolution in the genus Rana.

Materials and Methods

The adults used in these experiments were from three localities: Rossie, St. Lawrence Co., New York; Indian Lake, Hamilton Co., in the Adirondack Mountains of New York; Schriever, Terrebone Parish, Louisiana. Some indication of the climate of these regions may be gained from data in Climate and Man (Yearbook of Agriculture, 1941). At Schriever the average July temperature is 81.9° F. (27.7° C.) and the growing season (number of days between the last killing frost in spring and the first killing frost in the autumn) is 256 days. At Ogdensburg, which is near Rossie, the average July temperature is 70.2° F. (21.2° C.) and the growing season 152 days. Indian Lake, N. Y., has an average of 64.7° F. (18.2° C.) for the month of July, and a growing season of 85 days. As *Rana catesbeiana* occurs only in the region between southern Canada and northern Mexico it will be seen that the localities mentioned above are near the northern and southern limits of geographic distribution. If adaptive changes have occurred in temperature tolerance and rate of development in different regions, material from these localities should reveal them.

Eggs were secured by means of pituitary injections. At the time of first cleavage they were placed in waterbaths, incubators, and cold-rooms. The methods used in obtaining and handling the eggs were identical with those employed in previous experiments (Moore, 1939).

Temperature Tolerance and Rate of Development

1. *Rossie, New York.*—Two females from this locality were used. In the first experiment eggs were placed at 36.2 ± 0.6°, 31.5 ± 0.9°,

TABLE I

Development of Rana catesbeiana at different temperatures. Time in hours after first cleavage. Temperatures in Centigrade.

Stage	Louisiana 14.8 ± 0.2°	Rossie 1 18.7 ± 0.4°	Rossie 2 19.1 ± 0.07°	Indian L. 19.1 ± 0.07°	Louisiana 19.8 ± 0.5°	Louisiana 24.8 ± 0.8°	Rossie 1 27.0 ± 0.1°	Rossie 2 28.2 ± 0.2°	Indian L. 28.2 ± 0.2°	Louisiana 29.9 ± 0.4°
3	0	0	0	0	0	0	0	0	0	0
8	21		12½	12½	15					
9	35	23	23½	20	18½					
10	48	30½		23		11		12½		
11	60									11½
12			35	35	35	15–21			12½	15
13	83	42½	41½–47	41½–47			18	17½	18	
14	96–121	53½	59½		48–60		21	20–23	20	19
15		68		59½					23	
16	135½–139	77½	64	64		35	30½			
17	143–192		72½–95	72½–95	83			35	35½	
18	215–288	97–127½			87–114	48	42	41	41½	35–39½
19	307–359	141–151½	144–154½	144–154½	120½	60–65	53½	47	47	
20	371	162½	157	157	131	71	56	59½	59	48½

27.0 ± 0.1°, 18.7 ± 0.4°, and 11.4 ± 0.3° (the mean and standard deviation of readings taken in the water containing the embryos). At 36.2° cleavage occurred in the animal hemisphere, but not in the vegetal hemisphere, and all embryos died before gastrulation. At 31.5°, 27.0°, and 18.7° the eggs developed normally with no indication

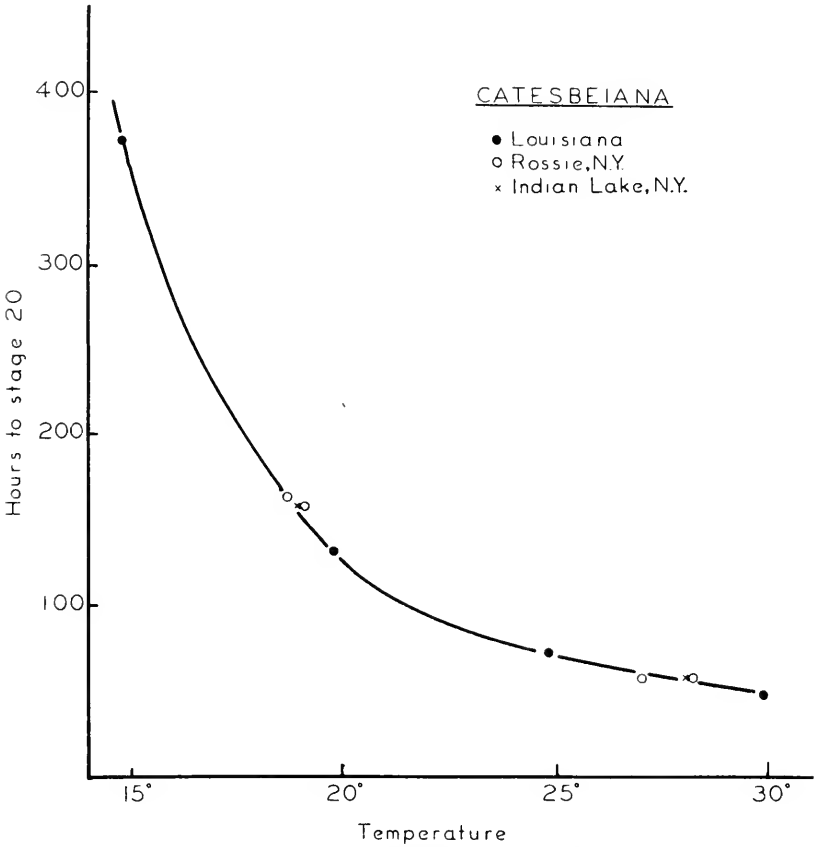

FIGURE 1. Hours required by embryos of *Rana catesbeiana* to reach stage 20 at several temperatures. Data from Table I.

of heat or cold injury. At 11.4° the eggs were definitely injured. Gastrulation was irregular and no normal embryos were secured.

In the second Rossie experiment eggs were placed at 36.4 ± 0.1°, 34.0 ± 0.0°, 28.2 ± 0.2°, 19.1 ± 0.07°, 13.9 ± 0.1°. At 36.4° and 34.0° the eggs were killed in the cleavage and blastula stages. Development at 28.2° and 19.1° was normal. At the lowest temperature, 13.9°, development was abnormal. Although some of the eggs de-

veloped to stage 20 (the stages of development are defined in Pollister and Moore, 1937), all showed injuries and abnormalities. This temperature is definitely near the normal zone and on the basis of previous experience it would be concluded that normal development would have been realized at 15°.

The rate of development was determined by examining the embryos at frequent intervals and noting the morphological stage to which they had advanced. The data in Table I represent the stage of development at the time of examination. In the case of stage 20 (beginning of gill circulation) the data represent the beginning of that stage. This stage was chosen for the accumulation of the most accurate data in order that a comparison could be made with the other species of frogs for which the stage 20 data is the best.

In the first experiment the rate of development was measured at 18.7 ± 0.4° and 27.0 ± 0.1°. At the lower temperature stage 20 was reached in 162½ hours after first cleavage, and at 27.0° in 56 hours. In the second experiment the eggs reached stage 20 in 157 hours at 19.1 ± 0.07°. At 28.2 ± 0.2° the beginning of stage 20 was missed and when examined at 59½ hours the embryos had apparently been in this stage for two or three hours. The time to reach stage 20 is indicated in Figure 1 where it will be seen that the points for both Rossie females fall on the same curve.

2. *Indian Lake, New York.*—The eggs from one female were used. This experiment was carried out at the same time and at the same temperatures as the second Rossie experiment. The temperatures employed were 36.4 ± 0.1°, 34.0 ± 0.05°, 28.2 ± 0.2°, 19.1 ± 0.07°, and 13.9 ± 0.07°. As in the case of the eggs from the second Rossie female no development beyond gastrulation occurred at 36.4° or 34.0°. At 28.2° and 19.1° the embryos were normal. At 13.9 all embryos were killed before gastrulation. It will be recalled that in the second Rossie experiment the eggs developed somewhat better at this temperature, although none were normal.

The rate of development was measured at 28.2° and 19.1°. As the second Rossie experiment was carried out concurrently a comparison of rate between these two groups of eggs could be made. This was done but no difference could be detected. At 19.1° stage 20 was reached in 157 hours after first cleavage (stage 3). The precise beginning of stage 20 was missed by several hours at 28.2°. When the eggs were examined at 59 hours they had apparently been in this stage for two or three hours.

3. *Schriever, Louisiana.*—Eggs from a single female of this locality were kept at 12.3 ± 0.1°, 14.8 ± 0.2°, 19.8 ± 0.5°, 24.8 ± 0.8°, 29.9

± 0.4°, 33.3 ± 0.1°, and 35.6 ± 0.1°. At 12.3° the eggs were killed. At 14.8° there were many deaths and irregularities but some embryos developed fairly normally. This temperature was very close, no more than one degree below the lower limiting temperature for this species. At 19.8°, 24.8°, and 29.9° development was normal. At 33.3° and 35.6° the embryos were killed.

The rate of development was measured at four temperatures. At the lowest temperature, 14.8°, the eggs were somewhat injured but the fairly normal ones reached stage 20 in 371 hours after first cleavage. At 19.8°, a temperature well within the normal range, stage 20 was reached in 131 hours. The times necessary to reach this stage at 24.8° and 29.9° are 71 and 48½ hours respectively.

From these experiments on *Rana catesbeiana* we can set the limits of temperature tolerance. The lower limiting temperature is close to 15° judging from the development of the Louisiana eggs at 14.8° and the Rossie and Indian Lake eggs at 13.9°. The upper limit is about 32°. Development was normal at 28.2°, 29.9°, and 31.5° and abnormal at 33.3° and 34.2°.

These experiments revealed no difference in temperature tolerance in eggs from the three localities. The Indian Lake eggs (the coldest region) were certainly no more resistant at low temperatures than eggs from the other localities. As a matter of fact the Rossie eggs developed more normally than those from Indian Lake at 13.9° (the slight difference between these two groups of eggs is undoubtedly due to individual variation). Similarly eggs from Louisiana (the warmest region) were no more resistant at high temperatures than those from Indian Lake, N. Y. The data on rate of development indicate that the embryos from the three localities develop at the same speed. The data in Figure 1 form part of the same smooth curve.

Comparisons with Other Species of the Genus Rana

With the data on *Rana catesbeiana* at hand, it is possible to compare the rate of development and temperature tolerance of the five common frogs of the northeast. Observations on the other four have been given previously (Moore, 1939) but since that time more data have been accumulated.[2] These new data have been incorporated in Table II

[2] In these later experiments the temperature was much more rigorously controlled by using water baths with mercury thermoregulators instead of incubators. As a result of these new experiments it is necessary to make a slight change in the value for the upper limiting temperature of *Rana clamitans*. Previously it was found that *Rana clamitans* eggs developed normally in incubators at 33.4°. As the eggs were in water at 20° when placed in the incubator, several cleavages would have occurred before the final temperature was reached. This leads to a slight error as the tem-

where the breeding season, limit of northern distribution, temperature tolerance, and rate of development for the five species of the genus are given. As predicted *Rana catesbeiana* has the highest range of embryonic temperature tolerance and the slowest rate of growth. It will be noticed however that *Rana catesbeiana* shows no increase over the 32° upper limit of *Rana clamitans*.

We can conclude from the temperature tolerance data in Table II, that the range over which development is possible varies so that each

TABLE II

Relation between the date for the beginning of the breeding season in the New York region, northern distributional limit, embryonic temperature tolerance, and hours to reach stage 20 at 20° C. in frogs

Species	Breeding season	Northern limit	Embryonic temperature tolerance		Hours to stage 20
			Lower	Upper	
R. sylvatica	Mid March	67° 30′	2.5°	24°	72
R. pipiens	Early April	60°	6°	28°	96
R. palustris	Mid April	51–55°	7°	30°	105
R. clamitans	May	50°	12°	32°	114
R. catesbeiana	June	47°	15°	32°	134

species is adapted to definite breeding conditions. A species such as *Rana sylvatica*, which has the lowest temperature tolerance range, breeds first in the spring and maintains itself in parts of northern Canada where no other species of the genus is found. If *Rana catesbeiana* were to breed at the same time as *Rana sylvatica* its eggs would be so injured by cold that they would not reach gastrulation. On the other hand if *Rana sylvatica* were to spawn at the same time as *Rana catesbeiana* its eggs would fare no better.

It will be noticed from the data in Table II that *Rana catesbeiana* has the narrowest temperature tolerance range, the interval over which normal development is possible being 18°. For *Rana clamitans* this value is 21° and for *Rana palustris*, *Rana pipiens*, and *Rana sylvatica* 23–24°. This significance of this narrow range of temperature tolerance in *Rana catesbeiana* is not apparent. In no event is a tendency toward stenothermy characteristic of southern or summer breeding forms. However, this narrow range is correlated with small variation of water temperature in the breeding ponds of *Rana catesbeiana*. Not

perature tolerance increases rapidly during early development. When eggs are placed in water baths the final temperature is attained rapidly. *Rana clamitans* eggs do not develop at 33.8° and the upper limit is 32°, under these conditions.

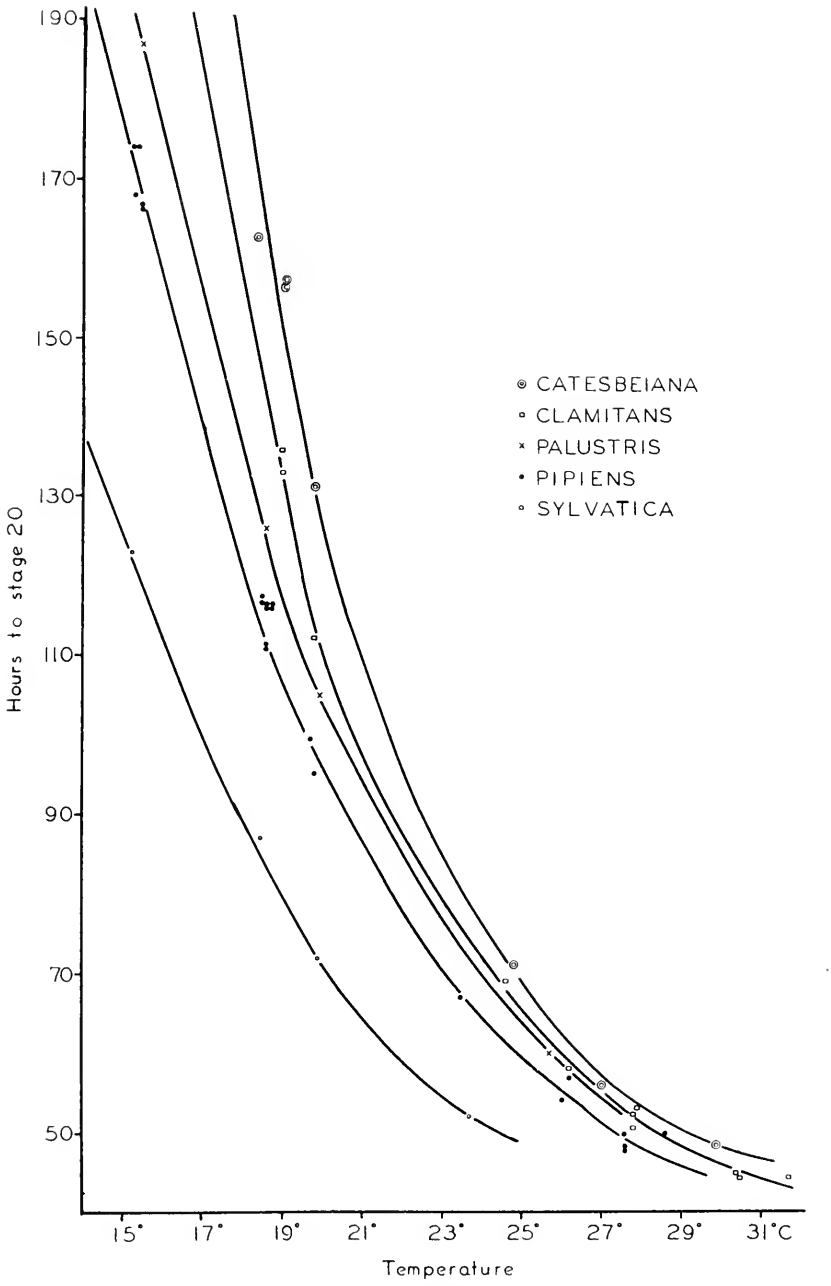

FIGURE 2. A comparison of rate of development in five species of the genus Rana. Data for *sylvatica*, *pipiens*, *palustris*, and *clamitans* from Moore (1939, and unpublished). Data for *catesbeiana* from Table I.

only does this species breed in the summer when water temperature varies less than in the spring, but in general it chooses the larger and deeper bodies of water that are more constant in their temperature than the smaller and shallower ponds, pools, and swamps where forms such as *Rana sylvatica*, *Rana pipiens*, and *Rana palustris* breed.

From Table II we can see that *Rana catesbeiana* requires the longest time to reach stage 20. That this species would have the slowest rate of development was not unexpected, as some incomplete data previously secured indicated this probable. Further, this might have been predicted on the basis of its breeding season and geographic distribution. A comparison of the rate of development over a wide range of temperatures is made in Figure 2. Little is known concerning the mechanism controlling the rate of development, but, in the final analysis, it is probably genetically determined. In certain cases it may be of considerable ecological importance. Thus we find that in general, species growing in cold regions, or in the cold months of the year, are characterized by a rapid rate of development or metabolism when compared with closely related forms in more temperate regions or breeding under warmer conditions (Fox, 1939 and earlier papers; Thorson, 1936; Moore, 1939). This rapid rate of metabolism in species characterized by low environmental temperatures enables them to offset to some extent the retarding effect of the cold surroundings on their vital activities.

Thermal Adaptation and Temperature Coefficients

Several investigators have noticed a correlation between the temperature coefficient (Q_{10} or b) and the temperature tolerance of the organism studied. Zawadowsky and Sidorov (1928) concluded from a compilation of published data on the Q_{10} for development and the upper and lower limiting temperatures for embryonic growth in several species, that the Q_{10} is greater in those forms more resistant to high temperatures. It was doubtful if much importance could be attached to this finding as the species belonged to such widely separated groups (nematodes, echinoderms, and amphibia). Therefore, these two investigators made a study on the effect of temperature on development in three ascarids and concluded that the correlation between Q_{10} and temperature tolerance suggested by their literature survey did not hold for these worms. Their choice of material for these studies was unfortunate as the difference in temperature tolerance among the three species used is so slight as to be questionable.

The following year Brown (1929) reported a correlation between the lethal temperature for cladocerans and the temperature coefficient of

their development. Those species which could withstand the highest temperatures were characterized by a Q_{10} of greater value than those species not so resistant to heat. *Moina macrocopa* with a lethal temperature of 48° had a Q_{10} of 2.39 (between 20° and 30°) and *Daphnia longispina* with a lethal temperature of 42° had a Q_{10} of 1.48. Brown gives data on seven species establishing a general relation between Q_{10} and temperature tolerance. However, the use of Q_{10} as a temperature coefficient for comparing species with different temperature tolerances is not permissible for the following reason. The Q_{10} is not constant in value but increases regularly with decreasing temperature (this is evident in the data given by Brown). Thus, if a comparison is made of the Q_{10} of the same temperature interval of two species that differ in temperature tolerance, this interval would cover a lower part of the temperature tolerance range (where the Q_{10} is normally greater) of the species more tolerant of high temperatures, and an upper part of the temperature tolerance range (where the Q_{10} is less) of the species more resistant to cold. Thus the latter species would apparently have a lower temperature coefficient even if the curves of the two were of identical shapes.

A more serious objection can be raised against Brown's conclusion that a definite relation exists between Q_{10} and temperature tolerance, namely he measured Q_{10} and temperature tolerance for two entirely different processes. His lethal temperatures were determined by placing *adult* cladocerans in water of various temperatures for one minute to test their power of survival, but his Q_{10} was for the time interval from the beginning of the first young instar to the end of the first adult instar. In most species the temperature tolerance of embryonic and adult stages is different, so unless it is shown that the cladocerans used by Brown have the same embryonic and adult temperature tolerance his conclusions are unjustified. It is therefore for two important reasons, the use of an unsatisfactory temperature coefficient, and the comparison of temperature tolerance and temperature coefficient for two different processes, that the conclusions of Brown are questionable.

In 1931 Bělehrádek placed the relationship of thermal coefficient and thermal adaptation on a somewhat more secure basis. His use of a temperature coefficient, b, which is constant over a considerable portion of the species temperature tolerance range, was a decided advance. When time-temperature data (such as those in Figure 2) are plotted on a log-log scale they form a straight line in many cases. The slope of this line is Bělehrádek's temperature coefficient b. By treating a considerable volume of data in this manner he showed the value of b was usually greater in those organisms more resistant to high tem-

perature. The processes analyzed were germination of seeds, amoeboid movement, muscular activity, heart beat, development, etc. His principal data for development come from Brown's studies on cladocerans, from which he computed *b* values. These species, their lethal temperatures, their Q_{10} between 20° and 30° as given by Brown, and *b* as computed by Bělehrádek are given in Table III. At first sight this

TABLE III

The lethal temperature, Q_{10}, and b for five species of cladocerans. Data from Brown and Bělehrádek

Species	Lethal temperature	Q_{10}	*b*
Moina macrocopa	48°	2.39	2.15
Pseudosida bidentata	48°	2.03	1.80
Daphnia pulex	44°	1.70	1.63
Simocephalus	43°	1.81	1.43
Daphnia longispina	42°	1.48	1.00

series appears as a striking confirmation of the postulated relationship between temperature tolerance and the temperature coefficient of development. It should be remembered, however, that a comparison is being made between the Q_{10} of rate of development of young stages and the temperature tolerance of adults. As mentioned before there is no justification for such a comparison. Further, it is difficult to understand how Bělehrádek arrives at some of his values for *b*. When the data for Moina are plotted on a log-log scale it is readily seen that they do not fall on a single straight line, but on two lines intersecting at 25°. The *b* values for these two lines are 2.3 and 1.6 (compare with values in Table III). The data for Pseudosida show such a scatter as to make them unsuitable for computing *b*. Bělehrádek's value of 1.80 is a rough average. The data for *Daphnia pulex* can be represented with a line of slope 1.6 to 1.8. The points for Simocephalus are even more scattered and there seems little justification for the assigned value of 1.43 since lines with slopes of 1.2 or 1.6 would be equally valid.

These data on cladocerans are the most extensive offered by Bělehrádek on a homogeneous group of animals purporting to show a relation between *b* and thermal adaptation in development. Even though it is not satisfactory, for the reasons given before, other data given by him covering a wide variety of processes in animals and plants seem to justify the conclusion that the value of *b* is greater in those species with increased resistance to high temperatures.

In view of past interest in the relation between thermal adaptation and the temperature coefficient it might be well to consider the data

for amphibia from this point of view. Observations on both rate of development and embryonic temperature tolerance are available. If the data on time to reach stage 20 (Figure 2) are plotted on a log-log scale the points in the central part of the temperature tolerance range can be represented by a straight line, but at high and low temperatures the points usually fall above this line (Moore, 1942, Figure 3). In Table IV a comparison is made of the embryonic temperature tolerance

TABLE IV

The lower limiting embryonic temperature, upper limiting embryonic temperature, and b for five species of the genus Rana

Species	Lower temperature	Upper temperature	*b*
Rana sylvatica	2.5°	24°	1.98
Rana pipiens	6°	28°	2.13
Rana palustris	7°	30°	2.30
Rana clamitans	12°	32°	2.60
Rana catesbeiana	15°	32°	2.88

and *b* for the central portion of the curve in frogs. This series is a striking confirmation of Brown and Bělehrádek's theory that the temperature coefficient increases with the adaptation of protoplasm to higher temperatures. Shorn of its specialized terminology this means that the rate of development in a species adapted to low temperature is less affected by a change in temperature than that of a species adapted to higher temperatures.

RATE OF DEVELOPMENT AND TEMPERATURE TOLERANCE IN RANA CATESBEIANA FROM DIFFERENT LATITUDES

The constant relation between rate of development and temperature tolerance on one hand and geographic distribution on the other suggests a causal connection between the two. It has long been held that temperature and moisture are the principal ecological factors limiting the distribution of amphibia. In the northeastern United States an abundance of water excludes moisture as a limiting factor. Temperature is thus left as that variable of the environment most important in controlling distribution. However, distribution is determined not by the environment alone, or by the organism alone, but by the interaction of the two. Our knowledge of the factors in the organism which are important in this connection is scanty. The difference in distribution between *Rana catesbeiana* and *Rana clamitans* obviously is not due to the presence of a dorso-lateral ridge of skin in the latter and its absence in the former. In fact the characters used in

taxonomy seem of little significance in this all-important interaction between the species and the environment which determines geographic distribution. Instead we must look for physiological differences among species that adapt each to a specific environment. In our northeast United States amphibia we must therefore search for physiological differences that are adaptations to temperature. The results so far obtained indicate that the embryonic temperature tolerance, rate of development, type of jelly mass determining the amount of oxygen available to the embryos, and perhaps egg size which is an indication of the amount of stored food material, are important in this connection (Moore, 1939; 1940; 1941; 1942).

It is necessary that physiological characters such as temperature tolerance remain fairly constant over the geographic range of the organism if they are to be controlling factors in distribution. If they were able to change easily, presumably any temperature region (with other environmental factors constant) could be colonized by any frog. Thus it may not come as a surprise that the temperature tolerance and rate of development of *Rana catesbeiana* are the same in individuals from the Adirondack Mountains and from Louisiana. These localities are near the northern and southern limits of distribution for the species and there is no evidence that in the former locality the eggs are more resistant to low temperature or in the latter locality more resistant to high temperature. With no change in temperature tolerance or other physiological adaptations we might expect a frog to be found in a fairly definite temperature region, and be excluded from colder environments in the north, and warmer environments in the south. The origin of a new population, race, or subspecies with the necessary physiological changes that would allow successful colonization of previously unfavorable regions would result in an extension of the distributional range of the species. Apparently no such change has occurred in *Rana catesbeiana* in the localities studied. These changes have occurred in *Rana pipiens* with the result that it has extended its range farther south than any of the five species found in the northeast (Moore, 1942 and unpublished).

Summary

A study of the embryonic temperature tolerance and rate of development of *Rana catesbeiana* has been made. The eggs develop normally between 15° C. and 32° C. and the time necessary to reach stage 20 at 20° C. is 134 hours. This species has the highest range of temperature tolerance and the slowest development of any of the five species of Rana common in the northeastern United States.

The significance of these results from an ecological point of view is discussed.

No difference in either rate of development or temperature tolerance of individuals from New York or Louisiana was found.

The relationship between the temperature coefficient (Q_{10} or b) of development and thermal adaptation is discussed. In frogs the value of b is greater in species adapted to high temperatures.

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SEXUAL DIMORPHISM IN THE PECTORAL FIN OF GAMBUSIA AND THE INDUCTION OF THE MALE CHARACTER IN THE FEMALE BY ANDROGENIC HORMONES[1]

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Sexual dimorphism has been found to occur among the poeciliid fishes in all of the fins except the pectoral. During a recent study of the fins of *Gambusia affinis* the writer discovered a peculiarity in structure in the pectoral fin and further study disclosed that normally it is confined to the male. Since the discovery of the character, Dr. Carl L. Hubbs has made a preliminary survey of the various Poeciliidae in the collections at the Museum of Zoology at the University of Michigan and has found that the character occurs in all the species of Gambusia, in Flexipenis and Heterophallus and to a slight extent in Belonesox. The character will be useful in taxonomic work.

The specimens used recently in a series of experiments conducted for the purpose of making a quantitative study of the effects of ethynyl testosterone and methyl testosterone upon the anal fin of the female of *Gambusia affinis* (Turner, 1942) were re-examined and it became apparent that the pectoral fin peculiarities of the male are induced in the female by the use of the androgenic hormones. The pectoral fins of the female specimens used previously were intact and since concentrations of hormone from 1 mg./2,000 cc. of water to 1 mg./20,000,000 cc. of water had been used it was possible to determine the effective limits of concentration for the development of the male type of pectoral fin in females. Some additional specimens were treated with hormone to secure a complete set of results.

It is the purpose of this paper first, to give an account of the morphogenesis of the pectoral fins of normal males and females, second, to describe the origin and character of the peculiar structure of the pectoral fin of normal males and third, to give an account of the concentrations of androgenic hormone required for the production in females of the male type of pectoral fin.

[1] This work was aided in part by a grant-in-aid from the Graduate School of Northwestern University.

The specimens used in the experiments were females 25 mm. to 27 mm. in standard length. All of the experiments were conducted at a temperature of approximately 23° C.[2]

Pectoral Fins of Juvenile Males and Females

The pectoral fins of male and of female specimens 13 mm. to 15 mm. in length are practically identical in structure (Figure 1). The fins contain 13 bony rays. Each ray is composed of an upper and a lower element which are fused together except at the base. At this stage each ray is segmented except for the basal third but an examination of a series of earlier stages reveals that the unsegmented portions are in reality parts of the rays which had been segmented previously but had undergone an extensive anchylosis of segments during their development. In indicating the number of segments in each ray the anchylosis has been taken into account and the total number of segments indicated for a ray in the tables includes the existing segments together with those formed previously and later obliterated by anchylosis. Rays 6 to 10 are bifurcated at this stage while the others are not. In later stages rays 4 and 5 become divided also while rays 1, 2, 3, 12, and 13 remain as single elements in all stages of development in both males and females. The ray-segment formula (Turner, 1941) for the fin at this stage is indicated in Table I, C, Anchylosis is most extensive in rays 3 to 7 at this stage, a little less than half of the length of each ray being solidified.

It is possible to distinguish the sexes in juvenile specimens by peculiarities of the anal fin (Turner, 1941). When the pectoral fins of large numbers of juvenile males and females, distinguished by the character of the anal fins, are compared it is found that the fifth rays of the pectoral fins of females are bifurcated while those of males are frequently undivided. However, there is a great deal of individual variation and hence this peculiarity is not reliable for distinguishing the sexes at this stage.

Morphogenesis of Pectoral Fin of Females

Development of the pectoral fin from the juvenile stage to an advanced adult stage in the female involves axial elongation and further segmentation of all of the rays, primary bifurcation of ray 4, secondary bifurcation of rays 6 to 9 and the dorsal branch of ray 10, and further anchylosis of the basal segments in all rays (Figure 2). The greatest axial elongation and addition of segments occur in rays 2 to 12 in which seven to eleven segments are added. The progress

[2] The writer is indebted to Miss Peggy Uddbom for efficient technical assistance.

of anchylosis of basal segments is greater in marginal rays than in central rays. In rays 4 to 9 the basal segments become obliterated by anchylosis for less than one-half of the lengths of the rays. In marginal rays anchylosis occurs over a greater length of the basal

TABLE I

Ray segment formulae for mature females, mature males and juvenile specimens. A. Mature 35 mm. female. B. Mature 24 mm. male. C. Juvenile male or female 15 mm. in length

A

Ray number	1	2	3	4	5	6	7	8	9	10	11	12	13
Segment number in secondary branches						6 5 4 4	5 5 5 5	4 4 4 4	3 3 4 3	3 3			
Segment number in primary branches				8 10	12 14	10 11	11 11	12 11	12 11	10 11	8 7		
Segment number in bases	6	21	20	14	12	11	11	12	12	10	10	15	9

B

Ray number	1	2	3	4	5	6	7	8	9	10	11	12	13
Segment number in secondary branches						4 3 3 3	5 5 3 3	4 3 4 3	4 4				
Segment number in primary branches				6 6	11 9	10 11	10 11	10 10	7 10	10 8	5 5		
Segment number in bases	5	8	22	16	14	12	11	10	11	9	11	8	3

C

Ray number	1	2	3	4	5	6	7	8	9	10	11	12	13
Segment number in primary branches						6 6	6 5	6 6	7 6	4 3			
Segment number in bases	4	11	13	15	15	12	12	11	9	10	9	7	3

portion, over two-thirds of the entire length being involved in ray 2. The ray-segment formula of a mature 35 mm. female is shown in Table I, A. In old females, 50 mm. in length, the anterior branch of ray 5 and the posterior branch of ray 10 are divided also and all rays have added more segments.

In each new bifurcation the new segments of a branch are somewhat shorter with the result that the rays which are branched most tend to contain the larger number of segments. In actual length, however, ray 3 which is undivided and ray 4 which contains only primary branching are as long as rays 7 and 8 which have both primary and secondary branching. The ray-segment pattern does not follow the actual fin contour which is controlled by the relative axial lengths of the rays. The arrangement of rays and segments and the contour of the anterior portion of the fin in an old female are represented in Figure 2.

Morphogenesis of Pectoral Fin of Male

The same process of axial elongation, segmentation, and bifurcation of rays and of anchylosis of the basal segments of the rays occurs during the development of the pectoral fin in the male. However, elongation and segmentation are not so extensive in the first and the last two rays. The ray-segment formula in a large adult male, 24 mm. in length, is represented in Table I, B.

A considerable degree of variation in the above formula is caused by the variation in the time of the onset of sexual maturity. Maturity may occur at a relatively early stage and, since at maturity, growth of the body and also elongation and segmentation of the rays are terminated, the small mature males have a smaller number of segments in the rays. Males may become mature when 16 mm. in length or maturity may be postponed until they are 28 mm. in length and the number of segments in the various rays will vary according to the length of the body. For example, ray 5 in a large 25 mm. mature male has thirteen segments in the primary branches while a small 19 mm. male has eight or nine segments in these branches.

During the period of development in which rays 3, 4, and 5 of the anal fin (Turner, 1941) are undergoing rapid elongation a similar process is occurring to a less extent in rays 2, 3, 4, and 5 in the pectoral fin. However, development of the anal fin is a little in advance of that of the pectoral fin. The effect of the rapid growth of the 3, 4, 5-ray complex in the anal fin is to subordinate growth in the other rays

Plate I

1. Pectoral fin of juvenile male or female, 15 mm. in length.

2. Portion of pectoral fin of an old female, 35 mm. in length. Distal two-thirds of rays 2 to 6 and the tip of ray 1 are shown.

3. Distal four-fifths of rays 1 to 6 in a mature 22 mm. male.

Rays are indicated by numbers in all figures. All drawings show the left pectoral fin from the posterior aspect. With the exception of Figure 4, rays have been separated for convenience in illustrating.

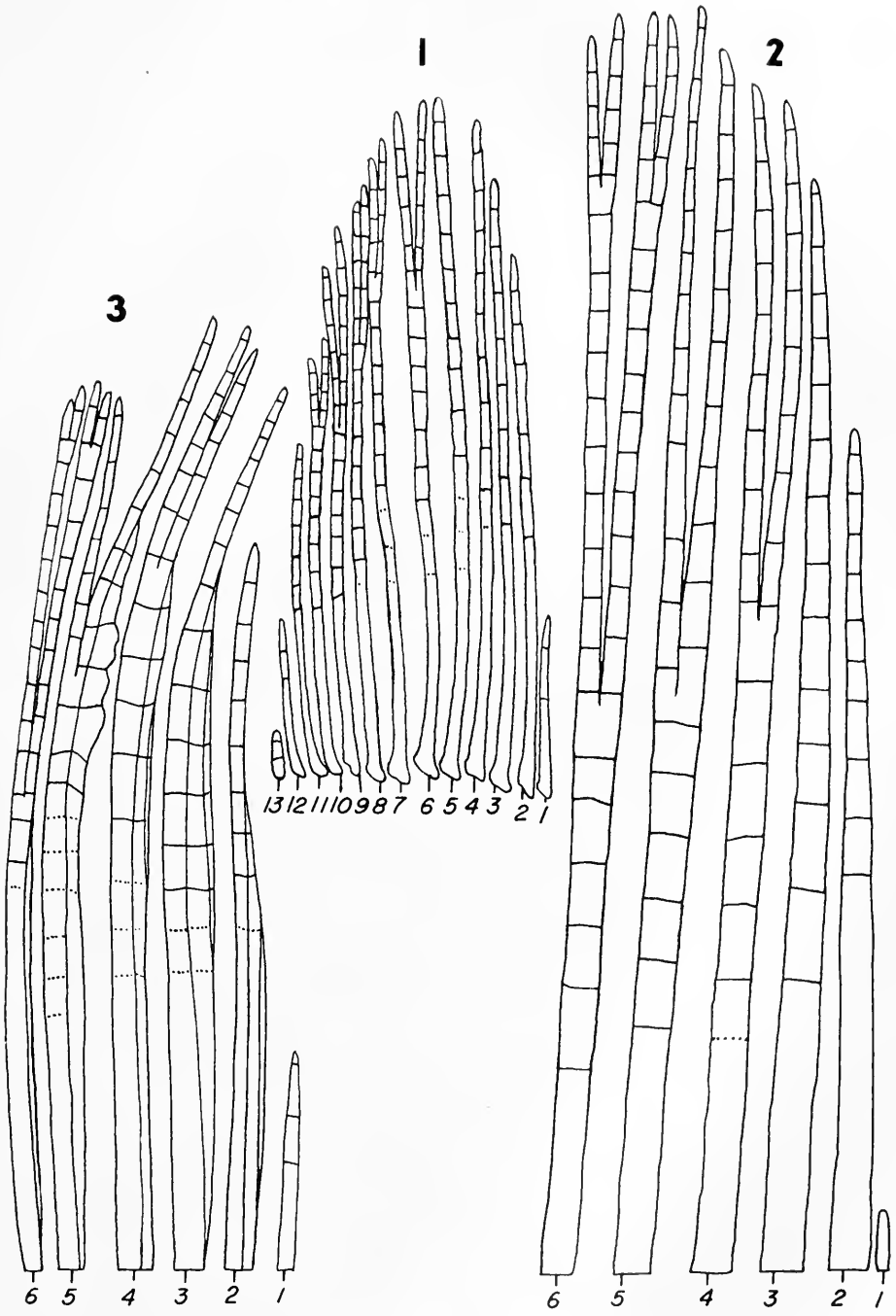

PLATE I

as well as to increase the length of the rays in the growing complex. This subordination of the growth of the rays not included in the rapidly growing complex is evident to some extent in the pectoral fin (compare rays 3 and 4 with 5 and 6 in Figures 2 and 3). Presumably the growth in rays 2, 3, 4, and 5 is a result of a susceptibility of the tissues of these rays to stimulation by a low concentration of hormone released from the developing testis. If a fish matures slowly as is usual in the large late maturing males, and a low hormone concentration is maintained over a relatively long period of time, growth in the susceptible rays is of longer duration and the differences between the lengths of the susceptible rays and the other rays is greater. In the small rapidly maturing males in which the maintenance of a low hormone concentration is of short duration and in which axial growth is quickly terminated by differentiation, the lengths of the susceptible rays are hardly greater than that of ray 6.

A second phase of dimorphism becomes apparent in the 19-segment stage.[3] In the fins of juvenile specimens each ray is composed of two bony elements which are separated at the bases but are fused together for the entire length of each ray so that they are indistinguishable. They may be separated by soaking the rays in 5 per cent potassium hydroxide. For convenience in description the normal position of the fin is disregarded and the fin will be described as though it were placed in a strictly horizontal plane. In this position each ray is composed of a dorsal and a ventral component which are fused together. From the 19-segment stage to the 26-segment stage dense, opaque tissue arises along the anterior margins of rays 2, 3, 4, and 5 and to a slight extent along the anterior margins of rays 6 and 7. Except in ray 2 only the ventral components are involved. New bony tissue is laid down upon the components of the rays. As a result the ventral components (both components in ray 2) are thickened while the dorsal components are not. Except for a short distance at the bases most of the lengths of the rays are involved. Distally the addition of new bone to the ventral components stops short of the ends of the rays by several segments. In ray 5 only the anterior branch is involved. The progress of the process is slow and at the 26-segment stage the rays involved have increased in width to the extent of about one-half of the original width. The thickening of the ventral components is accompanied by some degree of displacement of the components, the ventral member shifting into an anterior position for a distance equal to one-third of the width of the ray.

[3] Nineteen segments in the third (index) ray of the anal fin (Turner, 1941).

A third phase of differentiation begins at the 33-segment stage and progresses rapidly throughout the brief terminal period of transformation. The addition of new bony substance to the ventral components of rays 2 to 7, which up to this stage has occurred along most of the lengths of the unbranched bases, now becomes sharply localized. Beginning in an area at the point of the bifurcation of ray 5 and extending progressively to rays 4, 3, and 2 the rapid addition of new bone to each ray becomes so extensive that the newly formed bony shelf overlaps the ray anterior to it. As the shelf becomes wider it turns upward so that the area has an imbricated pattern (Figure 4). The area is quite restricted in ray 5 and covers only three or four segments of the anterior branch distal and basal to the point of the bifurcation of the ray but in rays 2, 3, and 4 the area covers a wider range of segments and is more basal in position (Figure 3). In ray 2 the dorsal as well as the ventral component is thickened. In a well developed mature male the anterior margin of the newly formed mass on ray 5 is bluntly serrated (Figure 4). During late differentiation of the fin, connective tissue forms between rays 2, 3, 4, and the anterior branch of 5 and binds the rays firmly together into a unit. The entire process of differentiation results in the partial separation of the unit from the rest of the fin and also the arching of the anterior portion of the fin.

Development of the pectoral fin in the male from the juvenile stage to completion may be summarized as follows: 1) There is axial growth, segmentation, and bifurcation of rays in the female pattern from the 12-segment to the 19-segment stage. 2) A slight separation and displacement of the dorsal and ventral components of rays 3 to 7 occurs and an addition of new bony substance to the anterior margins of the ventral components occurs at a later stage. In ray 2 new bone is added to both the ventral and the dorsal components. Rays 2, 3, 4, and the anterior branch of ray 5 grow and add new segments. 3) The process of addition of new bony substance becomes accelerated and localized in a small area within the former area involving ventral component modification. Dense connective tissue forms between the rays in the newly differentiated area and the distal parts of rays 2 to 5 are bent slightly in an anterior direction.

Modification of Female Pectoral Fin by Androgenic Hormones

Modification of the female pectoral fin proceeds in the male pattern when effective androgenic hormones are applied but the extent of the modification is limited somewhat in older specimens by the fact that the fin is already fixed in the female pattern.

The introduction of methyl testosterone into an aquarium and the maintenance for 30 days of a constant concentration of 1 mg./20,000,000 cc. of water induces in medium sized females the addition of new bone in the extensive area described in the development of the male fin. The area has the same limits and characteristics as those of the normal developing fin of the male (Figure 5). Axial growth of rays 2 to 5 does not occur at this concentration but at a concentration of approximately 1 mg./7,000,000 cc. there is some elongation. The localization and acceleration of new bone formation described under phase three in normal male development occurs at a concentration of approximately 1 mg./5,000,000 cc. The degree of differentiation at this concentration (Figure 6) is somewhat less than that in the fin of a fully differentiated male and increases in concentration do not produce further differentiation. Concentrations higher than 1 mg./5,000,000 cc. produce a condition at the ends of rays 2 to 5 in which segmentation is more rapid than axial elongation with the result that the terminal segments formed under these conditions are short.

Old females, 50 mm. in length, when treated with methyl testosterone at the most effective concentration produce modifications of the pectoral fin which are in general like those produced in medium sized females. However, there are some structural differences. In the old females ray 5 possesses secondary bifurcations in both of the primary branches before the hormone is administered. The first phase of modification in the direction of the male structure, namely, an elongation of rays 2 to 5 and the subordination of growth in the other rays is very slight. A number of thin segments are added to rays 3, 4, and 5 but due to the fact that the other rays have developed in the female pattern before hormone treatment is started they are not subordinated in growth. The second phase of modification, widening of the ventral components of rays 3 to 6 and of both components in ray 2 occurs in old females and is almost as extensive as in medium sized females. The localized widening of the ventral components of rays 3 to 5 occurs but it is not extensive and the position of the area is shifted axially.

PLATE II

4. Enlarged drawing of the imbricated area including parts of rays 3, 4, and 5 in a mature male.

5. Rays 2 to 8 of a medium sized female which has been treated with methyl testosterone at a concentration of 1 mg./20,000,000 cc. of water. Basal parts of rays are not shown.

6. Rays 2 to 5 of a medium sized female treated with methyl testosterone at a concentration of 1 mg./5,000,000 cc. of water. Basal portions of segments are not shown.

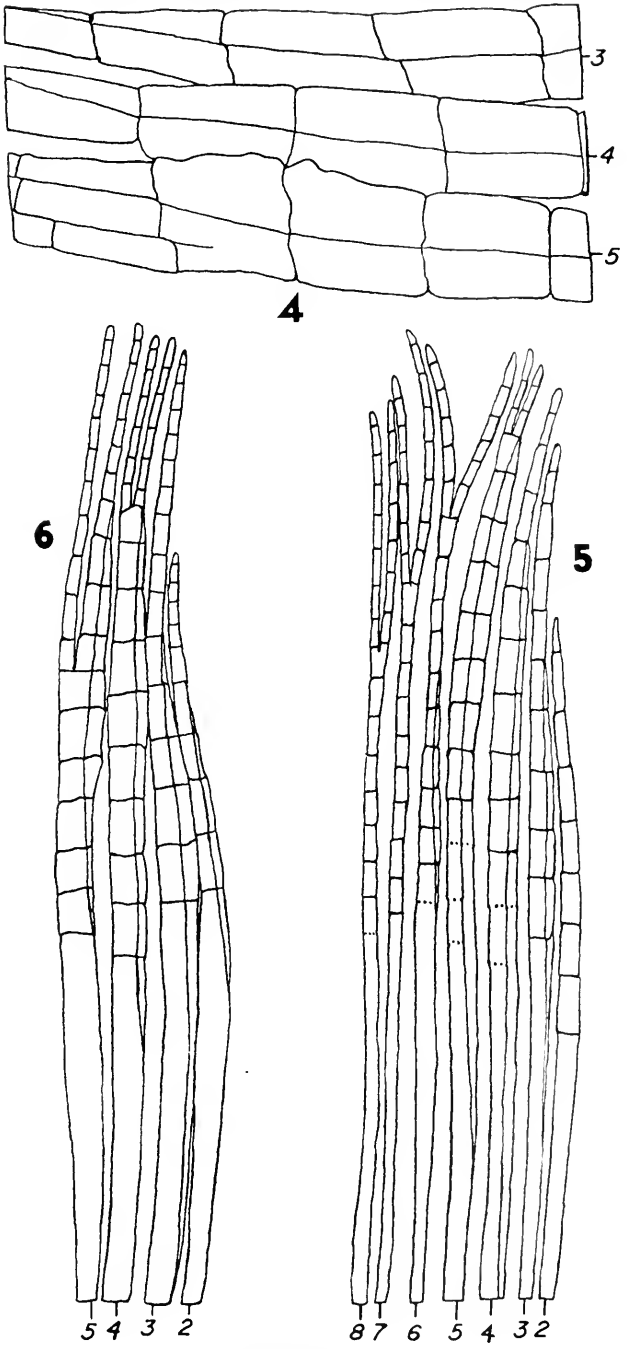

PLATE II

In normal mature males and in medium sized treated females the localized area includes that portion of ray 5 lying between a point about three segments basal to the primary bifurcation of the ray and four segments distal to the bifurcation. In old females the area includes that portion of ray 5 between a point three segments basal to the point of the primary bifurcation and 13 segments distal to the primary bifurcation. In normal mature males there is no secondary branching of ray 5 but in old females the secondary branching occurs and upon the anterior member of the anterior secondary branch the ventral component is widened and displaced for several segments. In comparing the position of the localized area in normal mature males and in old females treated with hormone it would appear that the area is oriented with reference to the point of primary bifurcation of ray 5 but that the position of the area is governed to some extent by the total length of the ray. Since in the mature male axial growth of the rays is terminalized when sexual maturity is attained, ray 5 does not increase in length after maturity. The influence of the length of the ray upon the position of the localized area is constant, therefore, and the area occurs in approximately the same position in all normal mature males. In old females, however, the ray has attained a much greater length before hormone treatment and the influence of the greater length is reflected in the shifting of the position of the localized area to a more distal position. The formation of a smaller localized area on the secondary branch can never occur in normal males because the branch is never formed. The formation of the area in treated females indicates a potentiality in males which is never realized because terminalization of growth prevents the development of the structure (secondary branch) which would become modified.

Comparison of Responses of Pectoral and Anal Fins

In the development of normal males the beginning of metamorphosis of the anal fin into a gonopodium is evident before there is any modification of the pectoral fin, but as metamorphosis of the anal fin proceeds the initial stage of the development of the pectoral fin occurs. The addition of new bony substance in the restricted area in rays 2, 3, 4, and 5 of the pectoral fin continues for some time after the gonopodial development is complete and the male is mature and reproductively functional. Apparently the structures of the anal fin which develop are susceptible to a slightly lower concentration of male hormone than any in the pectoral. The concentration required for the terminalization of development of the gonopodium is not

sufficient for the most extensive development of the pectoral fin and it is assumed that the augmented concentration required for the completion of development in the pectoral fin in normal males is furnished by the testis which continues its development after the gonopodium is completely developed.

It is not possible to produce in females, treated with different concentrations of hormone, development of the pectoral fin as extensive as that in old normal males. A concentration of 1 mg./5,000,000 cc. (methyl testosterone in water) produces maximal development of the pectoral fin in a female treated at a constant hormone level and concentrations high enough to produce early terminalization of growth and abnormalities in the differentiated structures of the anal fin (Turner, 1942) do not produce further development in the pectoral. The failure of further development in the pectoral fin of treated females may be due to the fact that the females were treated with a constant level of hormone concentration while in normal males the target organ is furnished with a continually rising concentration of hormone by the developing testis.

When ethynyl testosterone is employed, concentrations a little more than twice as great are required in order to produce the same effects as those secured with methyl testosterone but structural modifications secured by the use of ethynyl testosterone are identical with those produced by methyl testosterone.

The fact that females can be induced to develop the male characteristics in the pectoral fin indicates clearly that the female possesses the genetic factors for the development of the characters but, in the absence of an androgenic hormone, the development remains latent.

Sexual Dimorphism in Pectoral Fin of Cobitid Fishes

Vladykov (1935) has described sexually dimorphic structures in the pectoral fins in Cobitis, Misgurnus, Leptobotia, and Barbatula. In the males of different genera, from one to seven of the inner rays are enlarged. A circular bony plate occurs upon one or more rays at the bases of the fins in some species and in other species there is a conspicuous elongation of one or two of the inner rays. In all, the pectoral fin of the female lacks these features.

The differential elongation of some of the rays of the pectoral fin in the male, the thickening of specific rays and the local differentiations within the fin suggest that the situation is parallel to that in Gambusia. If females possess genetic determiners for the peculiarities but fail to develop them because of the lack of stimulation by an androgenic

hormone the characters could be brought out, presumably, by the application of androgenic hormones.

Summary

1. The pectoral fins of juvenile males and females in Gambusia are practically identical in structure.

2. In Gambusia and in a number of closely related genera a modification of the pectoral fin occurs in the male with the onset of sexual maturity. The structural peculiarities of the character consist of elongation and segmentation of rays 2, 3, 4, and of the anterior branch of ray 5, and the partial separation of the dorsal and the ventral components of these rays, a general widening of the ventral component and a development of an imbricated area by further widening of the ventral components within a localized area.

3. Females possess one or more genetic factors for the same structural characters and they can be induced to develop the characters by the application of methyl testosterone or ethynyl testosterone.

4. The first stages in the development of the characters are induced in medium sized females by treatment with methyl testosterone at a concentration of 1 mg./20,000,000 cc. of water. A concentration of 1 mg./5,000,000 cc. produces maximal development of the characters. Ethynyl testosterone produces the same results at concentrations a little more than twice as great.

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STUDIES ON THE LIFE HISTORY AND HOST–PARASITE RELATIONSHIP OF PELTOGASTER PAGURI

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Introduction

Peltogaster paguri, a rhizocephalan described one hundred years ago by Rathke from the coast of Norway, was the first species of the order to be reported from United States waters. A specimen was collected as early as 1866 by A. S. Packard, Jr., from Eastport, Maine. Whereas in European waters this parasite became the subject of a number of studies, in American waters it gained no further notice until the writer reported its rediscovery on the Maine coast and subsequently gave an account of its endoparasitic development and the rôle played by the complemental males in reproduction (Reinhard, 1939; 1942, 1942a). Concurrently, brief field notes on Peltogaster collected in the same locality were published by Walker and Pearse (1939).

In the present pages are gathered together biological and statistical notes, many being new facts about Peltogaster, others serving rather as a means of comparing the American race of this parasite with the European. The biology of *Peltogaster paguri* has been studied notably on the Channel coast of France since 1927 by Charles Pérez where the host is *Pagurus bernhardus*. On this side of the Atlantic, in an environment considerably more boreal, the parasite occurs on *Pagurus pubescens*. Such differences in host and geographical conditions should be reflected in modifications of the life history and host-parasite relationships. Moreover, to make this study as comparative as possible, comparable data for other Rhizocephala will be included wherever pertinent.

The field work on which this report is based was carried out at the University of Maine Marine Laboratory during the months of July and August, 1938 to 1941. During 1938 all the crabs were collected from nearby Googin's Ledge, where the parasite was first found by the writer on July 26 of that year. In succeeding summers attention was paid to other localities in Frenchman's Bay, and, contrary to the findings of Walker and Pearse, parasitized crabs were obtained from the

shore of Mount Desert Island and from the Lamoine shore, as well as from Racoon Cove, Skillings River, and Bar Island. Googin's Ledge, however, was favored for collecting purposes, since the percentage of infestation was higher there than elsewhere.

The host in all cases was the hermit crab *Pagurus pubescens* Kröyer, and no Rhizocephala were ever found on the other common hermit crab of this locality, *Pagurus acadianus* Benedict, although more than 300 specimens of this species were also examined.

The Percentage of Infestation

In Table I is given a summary of crabs examined with percentage of parasitism found in relation to locality and year. The entire collection of 3,092 crabs yielded 424 parasitized specimens or 13.7 per cent,

Table I

Percentage of parasitism of Pagurus pubescens with Peltogaster paguri: 3,092 crabs examined

Locality	Year	Number of crabs examined			Per cent parasitized		
		♂	♀	Total	♂	♀	Total
Googin's Ledge	1938	128	213	341	28.9	23.0	25.2
" "	1939	366	500	866	9.0	11.4	10.3
" "	1940	318	274	592	15.7	14.2	15.0
" "	1941	315	330	645	13.6	18.2	15.9
Other localities	1939	185	106	291	6.5	8.4	7.5
" "	1940	51	36	87	9.8	11.1	10.3
" "	1941	205	65	270	7.3	15.3	9.3

but of the 2,444 Googin's Ledge crabs 15.0 per cent were parasitized as compared with an infection rate of 8.6 per cent for the 648 crabs from other localities in Frenchman's Bay.

Although male and female crabs were about equally represented in the collections (1,568 males to 1,524 females) the latter were more frequently parasitized, the rate being 14.2 per cent in the case of males, 17.6 per cent in the case of females.

There is apparently in Rhizocephala a tendency toward more frequent parasitism of female than of male hosts. Extreme instances of this are reported by Pérez (1927, 1931) who found *Peltogaster paguri* parasitizing only females of *Pagurus* (*Eupagurus*) *bernhardus* at two different localities on the coast of France. More commonly the reported preponderance of infested females over infested males is on the order of the percentages given by Brinkman (1936) for *Munida sarsi*

parasitized by *Triangulus munidae:* 5.9 per cent of males and 8.3 per cent of females parasitized in one locality; 2.2 per cent of males as against 10.1 per cent of females in another locality. There are instances, however, of male hosts outnumbering female. At Roscoff, Pérez (1931a) found male *Pagurus* (*Eupagurus*) *cuanensis* more frequently parasitized by Gemmosaccus (formerly called Chlorogaster) than were the females, but the observed cases seem too few to be decisive.

The data presented in Table I show that at Googin's Ledge the percentage of parasitized females exceeded that of males in 1939 and 1941, while in 1938 and 1940 the reverse was true, but the data for other localities show female host preference for each of the three years. Taking the data all together, the percentage of parasitized females is approximately 12 per cent higher than that of males. Brinkman (1936) has suggested that the female host is a richer source of nourishment than the male and is especially more suitable for nourishment of the endoparasitic phase of the rhizocephalan, hence the preponderance of parasitized females. This explanation has much to recommend it, but the exceptions to female host preference cited above show that this principle does not always operate.

Number of Parasites per Host

As shown in Table II the total number of infested crabs amounted to 424, but 29 of these had more than one parasite. The term "scar"

Table II

Summary of collections of Peltogaster paguri from Frenchman's Bay

Number per host	Records	Per cent
One external sac	377	
One scar	18	
Total single infestations		93.1
Two external sacs	22	
Scar and external sac	4	
Two scars	1	
Total double infestations		6.4
Three external sacs	2	
Total triple infestations		0.5
	424	100.0

in this table refers to crabs that have lost the external portion of the parasite though they still contain the living roots of the parasite.

The incidence of double and triple infestations is much less than would be expected on the basis of chance, i.e., either concurrent or successive infestations by more than one cypris larva. This seems to indicate some resistance on the part of the parasitized host which tends to lessen the possibility of a second infestation. Probability alone would allow 58 double infestations and eight triple infestations in the population examined, whereas the actual figures are 27 and two respectively.

Conceivably, biotic resistance would operate less strongly to prevent duplicate simultaneous infestations than superimposed infestations separated by a considerable time interval. If two infestations are acquired at approximately the same time the resulting parasites should be of about the same size; an infestation acquired some months after the first would result in two parasites of very unequal size. It is significant that 14 cases of double parasitism belonged to the class in which both external parasites were of approximately the same size, while only eight cases belonged to the other class. In Triangulus and Lernaeodiscus, according to Brinkman (1936), double infestations with parasites of equal age, as measured by size, are likewise more common than those in which the parasites differ in size.

Position of Parasite on Host

As Delage (1886) and others have noted, the external sac of Peltogaster is attached to the left side of the host's abdomen, occupying the protected space which the unparasitized female crab utilizes for carrying her eggs. A position on the right side of the abdomen would be hazardous for the parasite since this side is pressed tightly against the columella of the shell in which the crab lives. On *Pagurus pubescens* I have found the parasite regularly attached to the third abdominal segment, that is, the one that bears the first pleopod in the case of the male (Figure 1), the second pleopod in the case of the female. It is distinctive of the genus Peltogaster that the long axis of the sac is

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Plate 1

Photographs of *Pagurus pubescens* Kröyer parasitized by *Peltogaster paguri* Rathke. Natural size.

Figure 1. A large male crab of 18-mm. carapace length bearing a still immature 8-mm. Peltogaster.

Figure 2. A 14-mm. male with a mature Peltogaster of average size bent in the characteristic U-position.

Figure 3. Two parasites on a 12-mm. female. Note that the smaller Peltogaster is exceptional in facing backwards.

Figure 4. Two parasites of approximately equal size (about 12 mm.) attached to a 14-mm. female crab.

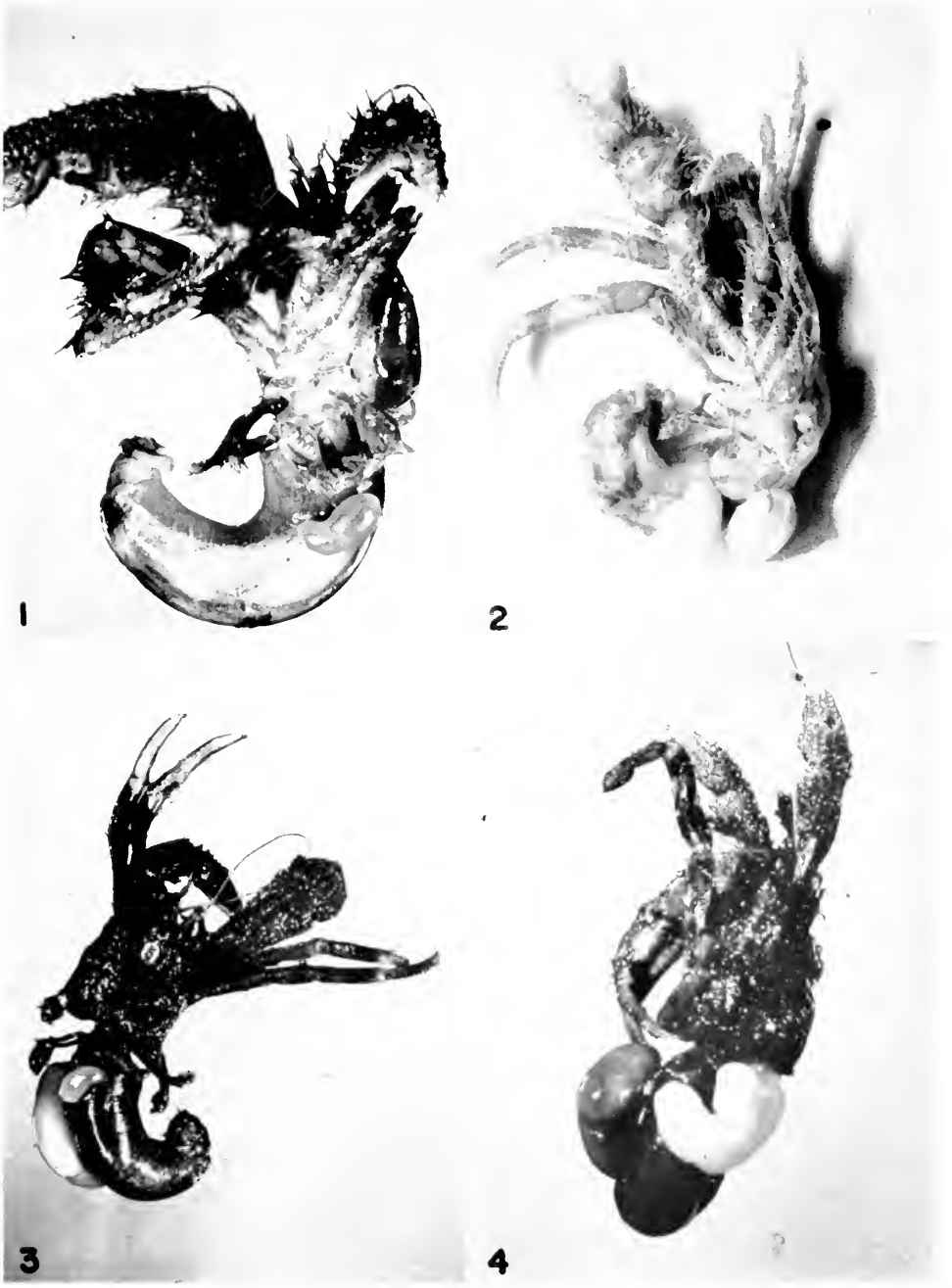

PLATE I

parallel to the long axis of the host and the mantle aperture is pointed forwards. Exceptions to the above generalizations occur primarily in multiple infestations.

Only one Peltogaster out of the 395 cases of single infestation occupied a position on the host that could be termed unusual. This Peltogaster, 10 mm. in length, was attached alongside the third instead of the second pleopod of a female crab. In no case was a Peltogaster found on the right side of the host's abdomen despite the fact that Walker and Pearse depict the animal in such a position without, however, suggesting either in text or legend that the figured specimen is remarkable. We must conclude therefore that the illustration belongs to the category of "typographical errors."

When two Peltogasters occur on the same host one occupies the normal position while the other is usually located dorsally on the same segment (Figure 4). There were only two cases in which both Peltogasters could be said to occur in approximately normal position. The dorsally located parasite, in one instance, had its long axis perpendicular to that of the host, with the mantle aperture to the left, and there were three cases where the dorsal Peltogaster was rotated through 180° so that the mantle aperture faced backwards (Figure 3).

Of the triple infestations, one consisted of a 2-mm. parasite in normal position with two others of approximately the same size arranged laterally in a line behind the first, but all three had the long axis diagonal to that of the host. The other case consisted of Peltogasters of different sizes: in normal position one of 14 mm., alongside but more dorsally one of 11 mm., and on the dorsum itself one of 3.5 mm. whose long axis was rotated 90° to the left.

Growth, Size, Color, and Sexual Maturity

It has been shown by Day (1935) and Foxon (1940) that the externae of *Sacculina carcini* reach breeding condition some six weeks or two months after their appearance. To determine the growth rate of Peltogaster, crabs with young externae were isolated in finger bowls and the size of the sac measured from time to time. A few of the experiments are here cited:

1. A male crab of 10-mm. carapace length which had a round, white, barely elevated patch on the abdomen, indicating the presence of an internans, was isolated on July 19. The Peltogaster emerged as an externa on July 30. By August 7 the sac measured about 2 mm. in length, and at the close of the experiment on August 19 the sac had reached a length of 3 mm.

2. A 4-mm. Peltogaster on a female crab of 10-mm. carapace length increased 2 mm. in length between August 7 and August 19.

3. A 4-mm. Peltogaster on a female crab of 16-mm. carapace length grew to a size of 9 mm. between July 19 and August 19.

The records from these experiments indicate that in the laboratory a young Peltogaster grows on the average approximately one mm. per week so that from six to seven weeks after eruption the external sac has reached sexual maturity.

The growth rate of the parasite, as well as the size it attains before reproducing, is dependent, to a large extent, upon the size of the host. Eggs are sometimes found in the mantle cavity of 6-mm. specimens when they occur on small hosts, while, on the other hand, very large hosts of 17- to 18-mm. carapace length have occasionally been found bearing 9-mm. Peltogasters which were not yet primiparous. On the crab of average size a Peltogaster of 7 to 8 mm. is sexually mature.

The age and sexual condition of the parasite may to some extent be gauged by the color of the living external sac. The colors and their significance are as follows:

White. The color of the newly erupted sac when only one to two mm. in length.

Red. The color of the immature sac. The ovaries have not yet ripened, and the coloration is that of the liquid contents of the visceral mass.

Apricot. The animal is mature and the color is due to ripe eggs or developing embryos.

Green. The mantle cavity is empty and the ovaries are spent. A Peltogaster rendered sterile by the presence of a female *Liriopsis pygmaea* may also be this color.

Brown. An occasional spent Peltogaster has this color which probably indicates a sac that is soon to drop off.

The size of adult Peltogasters when alive is difficult to measure exactly because of the curvature of the body and the expansions and contractions associated with ordinary respiratory movements. Moreover, when the mantle cavity is filled with eggs or embryos, the animal becomes greatly distended, and the same parasite measured after release of the nauplii will be noticeably smaller. Thus, a 15-mm. Peltogaster, after releasing nauplii, shrank to 11 mm., a 12-mm. parasite decreased to 10 mm. after shedding larvae, and a 13-mm. animal was reduced to 11 mm. Old parasites that can no longer produce eggs may live on for a time as "green spent" individuals and they likewise shrink in size. The green Peltogasters rarely measure more than 7 to 8 mm., whereas, a few weeks previously, when filled with

the last brood, many may have been in excess of 12 mm. in length. Hyperparasitism by an adult female Liriopsis also causes decrease in size.

Correlation Between Size of Host and Size of Parasite

Despite the factors just enumerated that cause marked size fluctuations, it is possible to say that the average size attained by adult Peltogasters is directly proportional to the size of the host. This is shown in Table III which gives a correlation coefficient of .601.

Table III

Correlation between size of crab and size of Peltogaster > 5-mm.

Peltogaster length in mm.	7.5	9.5	11.5	13.5	15.5	17.5	19.5	
21.5				1				1
19.5			1		3	2		6
17.5					1	1		2
15.5			3	9	6	1		19
13.5			5	4	3		1	13
11.5		13	24	9				46
9.5	1	20	27	7	1	1		57
7.5	17	20	13	1	1	1		53
5.5	4	12	2	3				21
	22	65	75	34	15	6	1	218

Crabs—carapace length in mm.

The very young Peltogasters are not included in this table because the primary concern here is with parasites of or near breeding age. It seems logical to conclude that this size relationship is based upon available food supply in the sense that the larger the host is, the larger can be the parasite which it is capable of supporting. This interpretation is in harmony with many records that could be gleaned from the literature (e.g., Boschma, 1928, p. 38) which show that on a host species of small size a particular species of rhizocephalan is apt to be dwarfed in comparison with the same species of parasite occurring on another and larger species of host. An alternative interpretation, that the Peltogasters on the larger hosts are older and therefore larger,

is unlikely in view of the fact that the size of the parasite when it becomes primiparous varies with the size of the host, as already shown.

To discover whether or not the size of the parasite is affected by the sex of the host a correlation table was prepared for the Peltogasters found on male crabs and another for those found on female crabs. A summary of these data is presented in Table IV which, as in the preceding, takes into account only the parasites greater than 5 mm.

TABLE IV

Relation between size of Peltogaster > 5-mm. and size of crabs with reference to sex of host

Crabs, carapace length	5.5	7.5	9.5	11.5	13.5	15.5	17.5
Average size P. on ♂ host	0	7.2	8.8	9.9	11.8	14.3	15.9
Average size P. on ♀ host	0	7.2	8.3	10.6	12.2	16.5	*

* Only one parasite was found on a female crab of this extreme size class; females of this size being of rare occurrence in the population.

It may be concluded from the above figures that the older female crabs in the size range from 11 to 16 mm. support larger parasites on the average than do males of the same sizes classes. Whether or not this is due to a difference in amount or quality of nourishment supplied by the different sexes is impossible to state with certainty at present.

AGE AT WHICH HOST BECOMES PARASITIZED

Charles Pérez, in a personal communication, suggested to the author that it would be interesting to determine the incidence of infestation in relation to the size of *Pagurus pubescens* in order to know at what approximate age these Pagurids are parasitized. Pérez informs me that on the coast of France *Pagurus bernhardus* is parasitized at a young age, whereas in the case of *Pagurus cuanensis* it is the adults that are parasitized. This latter host when adult is, however, of a size similar to that of young *P. bernhardus* at the age when *bernhardus* is parasitized.

My statistical data bearing on this question with reference to *Pagurus pubescens* is presented in Table V. Here it can be seen that fresh infestations with Peltogaster occur primarily in crabs of small and medium size, never in those of very large size. The greatest number of young infestations were found in adult crabs of 9 to 10-mm. carapace length, the size class which is most frequent in the population as a whole where it comprises about 40 per cent of all crabs. Because

of the small number of 5 to 6-mm. parasitized crabs the high percentage of young infestations in this class probably lacks real significance.

It would seem, therefore, that *Pagurus pubescens* presents an intermediate condition with respect to age at which parasitism takes place, since it is the young crabs and smaller adults that become parasitized. *P. pubescens* is actually a smaller species than *P. bern-*

TABLE V

Size at which crab becomes parasitized

Carapace length, mm.	Small		Medium		Large		Very large	
	5–6	7–8	9–10	11–12	13–14	15–16	17–18	19–20
Total parasitized crabs	4	27	93	97	40	18	6	1
Number with Peltogaster < 3 mm.	2	3	12	8	2	0	0	0
Per cent of parasitized having young infestations	50.1	11.1	12.9	8.2	5.0	0	0	0

hardus, but larger than *P. cuanensis*, so the hypothesis of Pérez that *Peltogaster paguri* attacks crabs of a definite size range irrespective of degree of maturity emerges strikingly confirmed.

REPRODUCTIVE CAPACITY

To ascertain the number of nauplii produced in a single brood, several lots were preserved for counting. Three 11-mm. Peltogasters yielded 9,800, 12,000, and 13,250 nauplii respectively, while one 12-mm. animal yielded 28,000. Data on the biotic potential of other Rhizocephala are non-existent.

Shortly after emission of the nauplii, the animal moults, usually casting off the external cuticle first, then the lining of the mantle cavity. A few hours later a fresh batch of eggs is released into the brood chamber. The interval between emission of nauplii and the passage of eggs into the mantle cavity for the production of the next brood was determined in ten cases, the average interval being 24 hours with a minimum of 12 hours and a maximum of 48 hours.

One of the important functions served by the moult is to free the vasa deferentia of the chitinous plug that bottles up the sperm. The manner in which the eggs are passed into the mantle cavity, the release of the sperm, and the adaptations connected with these processes have been well described by Smith (1906, p. 26). During ovulation the mantle aperture of Peltogaster is tightly closed, and the rhythmic muscular contractions of the animal cause the newly released eggs to be swished back and forth through the mantle cavity. The

jelly that later binds the eggs together has as yet not solidified, so that the ova are free to mix with the sperm as the contents of the cavity are churned together.

Numerous attempts were made to discover the time interval between successive broods but, although the Peltogasters kept under observation in aquaria promptly filled up with eggs after releasing nauplii, the embryos of the next brood never reached maturity and were aborted after various lengths of time. Judging from the degree of development attained before abortion took place it is possible to estimate the developmental period from fertilized egg to free-swimming nauplius to be approximately 30 to 40 days during the summer months. In *Sacculina carcini* the length of time between expulsion of one batch of nauplii and the next is stated to be greater than three weeks (Day, 1935) and perhaps six weeks (Delage, 1884).

Effect on the Host

The extensive literature dealing with the influence of Rhizocephala on the gonads and secondary sexual characters of host crabs, taken at face value, is full of conflicting results. But it is clear, largely from the work of Nilsson-Cantell (1926) and Brinkman (1936), that not only do various species of crabs react differently to the same parasite, but also the same species of host may be differently modified, depending on the species of parasite which infests it.

Pagurus pubescens, apart from the present investigation, has never been studied with reference to modifications caused by the presence of a rhizocephalan, but the effect of *Peltogaster paguri* on some other species of hermit crabs is known. Results of the published studies on *Peltogaster paguri* and closely related species, in so far as they deal with effects on the host, are presented for comparison in Table VI.

Briefly stated, the results of my own observations show that *Peltogaster paguri* causes complete degeneration of the gonads in the female *Pagurus pubescens* but does not cause castration of the male. Moreover, there is no appreciable modification of the secondary sexual characters in either sex.

To ascertain the effect on the gonads a large number of parasitized crabs were dissected in the fresh state. In addition, ten parasitized individuals of each sex were cut into serial sections and approximately the same number of normal individuals were sectioned for controls. Females bearing an external Peltogaster had ovaries so reduced that merely a trace remained which could be detected only with difficulty. Females, however, infected with the internal stage of the parasite, still had large ovaries with nearly as many eggs present as in the normal

TABLE VI

Effects of Peltogaster paguri and related species [1] *on various hosts. A tabulation of cases reported in the literature*

Parasite	Host	♂ Gonad	♀ Gonad	♂ Pleopods	♀ Pleopods	Authors	Remarks
Peltogaster paguri	*Pagurus bernhardus*	Atrophied	Atrophied (?)	Unmodified	Masculinized	Giard, 1887	Relatively few cases studied.
" "	" "			Unmodified	Masculinized	Guérin-Ganivet, 1911	Only six parasitized crabs examined.
" "	" "	Atrophied				Mouchet, 1931	No statement regarding external characters or effect on female.
" "	*Pagurus cuanensis*			Feminized	Unmodified	Nilsson-Cantell, 1926	Condition of gonads not noted.
" "	*Anapagurus chiroacanthus*			Unmodified	Unmodified	Nilsson-Cantell, 1926	Notes atrophy of male copulatory organ.
" "	*Pagurus pubescens*	Unaffected	Atrophied	Unmodified	Unmodified	Reinhard, this paper	
Peltogaster curvatus	*Pagurus meticulosus*	Atrophied	Atrophied	Feminized	Unmodified	Potts, 1906	
" "	*Pagurus prideauxii*	Atrophied	Atrophied	Feminized	Unmodified	Potts, 1906	
" "	" "	Atrophied				Mouchet, 1934	One specimen examined histologically.
Peltogaster sp.	*Pagurus samuelis*	Atrophied	Atrophied	Feminized	Masculinized	Shiino, 1931	Many cases, statistically treated, but species of parasite unfortunately not determined.

[1] References to "Peltogaster sulcatus" are omitted because this animal, now known as *Gemmosaccus sulcatus*, is a gregarious species, differing in a number of important respects from the genus Peltogaster.

animal. The male gonads, on the other hand, even when examined microscopically, showed no clear-cut evidence of degeneration and the spermatophores in the vas deferens appeared normal. Normal, active sperm were repeatedly extracted from the vasa deferentia of live parasitized males.

In number, shape, and ornamentation of the pleopods, as well as in position of the reproductive openings, the males and females of *Pagurus pubescens* are readily distinguished.

The male hermit crab possesses three pleopods while the female has four. No parasitized male with a supernumerary pleopod on segment 2 was found, but at least three normal males were noticed with this extra appendage. Two of these anomalous crabs were sectioned but the testes were found to be normal and there was no evidence of past or present infection with parasites.

In females, the rami of the pleopods are subequal in length, but in males the external ramus is considerably longer than the internal ramus. Since no feminizing of male pleopods or masculinization of female pleopods under the influence of Peltogaster could be detected by inspection alone, it was decided to try exact measurements in the expectation that the endopod-exopod ratio used by Shiino (1931) might show a difference between normal and parasitized animals. The pleopods of approximately 50 crabs of each sex, equally divided between normal and parasitized individuals, were examined statistically, but since no significant differences appeared to be materializing, this study was abandoned.

A further sex difference in the pleopods is seen in the bouquets of hairs that occur on the protopod. In many Pagurids it is stated that only the female pleopods have tufts of hairs on this portion of the appendage. The specimens of *Pagurus pubescens* that I examined show setae on the protopod of both sexes although these hairs are very numerous in the case of the female and sparse in the male. A comparison between normal and parasitized animals failed to show any increase in the number of hairs for parasitized males or decrease for parasitized females.

Finally, the tuft of setae that occurs on the outer margin of the endopod of a female abdominal appendage was used as a criterion. This cluster of hairs is normally absent in the case of the male. It occurred, however, on the pleopods of two unparasitized males, but on none of the parasitized specimens examined. It was never absent, so far as noted, from the pleopods of parasitized females.

The natural and not infrequent occurrence of intersexual and other types of anomalies in Crustacea makes it hazardous definitely to

ascribe such variations to the action of a parasite, when the condition is present in a particular host, unless sufficient cases are at hand to eliminate the possibility of coincidence. Among the crabs parasitized by *Peltogaster paguri* there is in my possession one female of 11-mm. carapace length that exhibits what is undoubtedly a very rare condition, the presence of a pleopod on the right side of the abdomen in addition to pleopods, normal in number and structure, on the left side. This extra appendage, well developed and female in type, occurs on the fourth segment. I see no reason, in view of the large number of parasitized females examined by me that proved to be unmodified, to ascribe this condition to the presence of Peltogaster.

Summary

Of 3,092 *Pagurus pubescens* examined, 13.7 per cent were found to be parasitized by *Peltogaster paguri*, the incidence of infestation being somewhat higher in female than in male crabs. Considerable biotic resistance on the part of the parasitized host is indicated by the small number of double and triple infestations.

The external sac, which reaches sexual maturity in from 6 to 7 weeks after eruption, produces from 10,000 to 28,000 nauplii, and successive broods may follow one another at approximately 30 to 40 day intervals. The average size attained by adult Peltogasters is directly proportional to the size of the host, female crabs supporting, on the average, larger parasites than do the males.

The age at which parasitism takes place depends on the relative size of the different host species, and is not necessarily related to the degree of host maturity.

Peltogaster causes complete degeneration of the gonads in the female *Pagurus pubescens* but does not cause castration of the male. Secondary sexual characters are not appreciably modified.

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SOME EFFECTS OF COVERING THE PERISARC UPON TUBULARIAN REGENERATION

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Recent experimental work on Tubularia has emphasized the importance of the uncovered ends of sectioned stems as loci of metabolic exchange. However, there have been some indications that the perisarc is not wholly impermeable. Thus Barth (1940) reports that as many as 50 per cent of stems shaken in Warburg manometers developed hydranth primordia even though both ends were ligatured. Although the stems in question had been subjected to environmental conditions which were hardly normal, the results suggested that the question of metabolic exchange through the perisarc should be investigated and its possible rôle in regeneration determined.

The problem was attacked by studying the effects of covering the middle of the stems for different distances, thereby reducing to a greater or lesser degree metabolic exchange through the perisarc without interfering with exchanges through the ends of the stems. As will be seen later, the findings show that, under conditions of normal (or at least nearly normal) oxygen and metabolite concentrations, varying the opportunities for metabolic exchange through the perisarc affects regeneration at the distal surface to a small degree. However, the effects of exchanges through the perisarc are definitely secondary as compared with those through the distal cut surface.

Material and Methods

The colonies from which the stems were selected were collected from the Oceanographic wharf at Woods Hole, Massachusetts, and from the University of Maine Marine Laboratory float at Lamoine, Maine.[2] The stems were selected for similarity with respect to diameter and general appearance. The distal cut in all cases was made 5 mm. behind the hydranth.

Capillary tubing with outside and inside diameters of 2.5 mm. and 1.0 mm. respectively was cut into lengths of 2.5, 5, 10, and 20 mm. for

[1] Now in the Division of Anatomy of the University of Tennessee.

[2] The work at Woods Hole was performed while enjoying laboratory facilities financed by the Faculty Research Fund of the University of Michigan.

the various experiments performed. These were attached with plasticine to one edge of strips of window glass 2 cm. wide and 20 cm. long which in turn were fastened to the bottoms of 10-inch finger bowls. Each finger bowl contained two glass strips spaced about 5 cm. apart and on each strip were 13 of the tubes (Figure 1). The finger bowls, filled with sea water to a point where the tubes were just covered, were placed on a water table with temperatures recorded several times a day.

The first ten stems to develop the constriction separating hydranth from stem were measured and the remaining three were discarded. All stems were removed from the tubes when the first primordium was measurable. This was found necessary because disturbances in the water produced in removing the first stems sometimes caused others to shift their positions in the tubes.

In this connection it should be noted that although they could not be given the benefit of circulating water, the stems nevertheless re-

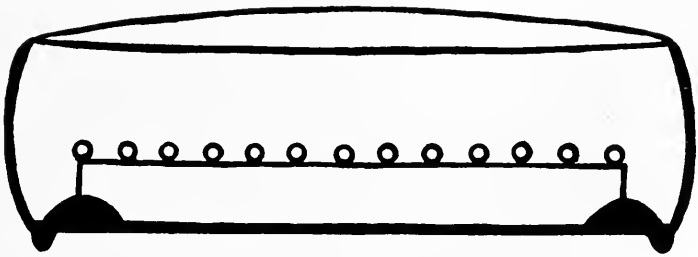


FIGURE 1

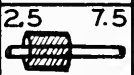
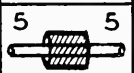
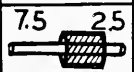
generated under favorable conditions. In a preliminary experiment a control set of stems lying on the bottom of the container required 1.5 hours longer to develop and averaged 6.8 units shorter than did an experimental lot in tubes which covered 50 per cent of the stem. It may be noted that in the case of the stems lying on the bottom the opportunities for metabolic exchange were limited along one surface by contact with the substrate, while those in the tubes, surrounded by water on all sides and located close to the surface, were regenerating under no such limitations.

The favorable conditions under which the experments were carried out were reflected by the unusually long primordia that developed. A number of the stems were found to have primordia 2.5 mm. or more long and one of 2.8 mm. length was recorded.

Experimental

Barth (1938a) reported an experiment in which a collar placed near or over the distal end of a tubularian stem was found to prevent hy-

TABLE I

		Distal Hydranths				Proximal Hydranths			
Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6	Col. 7	Col. 8	Col. 9	Col. 10
Expt.	Diagram	Percent	Diameter*	Time	Length*	Percent	Diameter*	Time	Length*
Lot 1		100	25.9	64.6 (55-72)	51.9 (65-35)	90	20.1	66.7 (55-77)	50.0 (75-28)
Lot 2		100	25.1	43.6 (42-45)	68.6 (81-51)	60	19.5	66.1 (64-68)	36 (46-32)
Lot 3		100	25.1	42.3 (41-44)	86.6 (102-58)	50	18.8	84.6 (67-96)	31.6 (37-22)
Lot 4		100	25.6	41.8 (41-44)	87.7 (103-78)	50	21.2	78.8 (67-97)	35.2 (51-22)

15 mm. stems with 5 mm. covered by glass tubes. Temperature range 13.4° to 14.6° C. All stems were removed from tubes after 41½ hours.

* 1 unit = 0.445 mm.

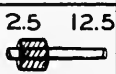
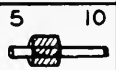
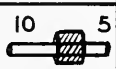
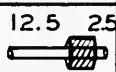
dranth regeneration at that surface. Therefore, before studying the problem mentioned above, it was necessary to determine how far from the distal end a tube, of the diameter and thickness employed, must be placed in order to eliminate its action in reducing exchanges through the end of the stem. To determine this, two experiments were performed (Table I). In the first experiment four lots were cut. In Lot 1 the distal end of each stem was arranged flush with one end of the tube. In Lot 2 the distal ends of the stems projected 2.5 mm.; in Lot 3, 5 mm.; and in Lot 4, 7.5 mm. from the ends of the tubes.

From the tabulated results it may be seen that with respect to regeneration of the distal hydranth there was no appreciable difference in mean length or mean time between Lots 3 and 4. Between Lots 2 and 3, however, there was a distinct increase in mean length and between Lots 1 and 2 there was both an increase in length and a decrease in time (columns 5 and 6). That the stems in Lot 1 regenerated at all may be attributed to their removal from the tubes after $41\frac{1}{2}$ hours. On the other hand, it should be noted that regenerative processes were not completely stopped during the period that the ends were covered. Subtracting the time they were covered (41.5 hrs.) from the mean time of measurement of the primordia (64.6 hrs.) we find that after removal from the tubes primordia regenerated in only 23.1 hours. This is approximately 19 hours less than were necessary for regeneration in Lots 3 and 4. Thus it would appear that the earlier steps in the regeneration process were passed through while they were still in the tubes.

Referring to the second half of the table we see that interfering with metabolic exchanges at the distal surface allows an increased percentage of proximal hydranths to regenerate (column 7). These hydranths appear to be larger and to develop more rapidly than when the distal hydranth has not been inhibited in this manner (column 9) although, because of the greater variability characteristic of the proximal hydranth, the latter findings are not as clear cut as in the case of the distal hydranths.

In the first experiment the time of constriction was slightly less and the length of the primordia slightly more in Lot 4 than in Lot 3. The experiment was repeated with longer stems which permitted placing the collars at greater distances from the distal end. This was done in order to determine if the difference was real, and if so, whether it was a result of the proximity of the tube to the proximal end or of the distance from the distal. In Experiment 2 (Table II) 20 mm. stems were used and the collars were placed 2.5 mm., 5 mm., 7.5 mm., and 12.5 mm. from the distal end. In this experiment, performed in Maine,

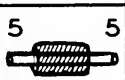
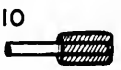
TABLE II

Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6	Col. 7	Col. 8	Col. 9	Col. 10	Col. 11
Expt. 2	Diagram	No. Measured	Av. Diameter ★	Av. Time (Hours)	Av. Length Primordia ★	Expt. 3	No. Measured	Av. Diameter ★	Av. Time (Hours)	Av. Length Primordia ★
Lot 1		8*	32.0	39.3 (37–42)	82.6 (67–103)					
Lot 2		10	27.3	39.3 (37–42)	90.9 (76–104)	Lot 1	10	25.4	58.0 (58)	106.0 (93–118)
Lot 3		9*	26.8	39.2 (37–42)	91.1 (71–101)	Lot 2	10	26.4	58.1 (58–59)	104.2 (98–113)
						Lot 3	10	25.5	58.2 (58–59)	105.7 (94–119)
Lot 4		10*	30.1	34.4 (37–41)	92.8 (78–104)	Lot 4	10	26.1	55.5 (55–57)	109.3 (101–123)

20 mm. stems with 5 mm. covered by glass tubes.
Experiment 2. Temperature range 13.6°–14.6° C. All stems removed from tubes after $37\frac{1}{2}$ hrs.
Experiment 3. Temperature range 12.8°–14.0° C. All stems were removed from tubes after $58\frac{1}{2}$ hrs.
* Because of vibrations of the building 5 stems in Lot 1, 4 in Lot 3, and 2 in Lot 4 shifted position and therefore were discarded.
★ 1 unit = 0.445 mm.

jarring of the building resulted in shifting the position of a number of the stems. In Lot 1 five, in Lot 3 four, and in Lot 4 two stems had to be discarded for this reason. The variation in average diameter of the stems which were measurable also detracts from the value of this experiment. However, the results were essentially the same as in Experiment 3 with reference to the effective inhibitory range of the collars. In both Experiments 2 and 3 there was no difference in time or length of the primordium between the 5 mm. and 7.5 mm. lots and in Experiment 3 increasing the distance to 10 mm. likewise did not increase regeneration. This shows that the increased regeneration in the 7.5 mm. lot of Experiment 1 was not the result of the greater distance from the collar

TABLE III

Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6
Expt. 4	Diagram	No. Measured	Av. Diameter*	Av. Time (Hours)	Av. Length Primordia*
Control		10	31.6	40.4 (38-35)	84.7 (62-95)
Experi-mental		10	31.5	39.3 (38-41)	91.7 (83-99)

20 mm. stems with 10 mm. covered by glass tubes. Temperature—13.6°-16.5° C.
* 1 unit = 0.445 mm.

to the distal end. On the other hand, it should be noted that in Lot 4 of all three experiments the proximal ends of the pieces were 2.5 mm. from the collar, and in each case they showed increased distal regeneration over Lot 3. Since it was shown by Experiments 1 and 2 that exchanges through the distal ends were influenced by collars 2.5 mm. from the distal ends, it appeared highly probable that a similar condition would obtain with respect to the proximal end.

The question of the possible beneficial effect upon distal regeneration by interfering with exchanges through the proximal cut surface was investigated next. Isolation of the distal from the proximal surface by a ligature is known to increase distal regeneration (Barth, 1938b). However, it does not necessarily follow that inhibiting proximal regeneration without stopping circulation would produce the same effect. A priori it might be predicted that the oxygen deficiency and increased

metabolite concentration which might develop at such a covered end might be distributed by the coelenteric currents and produce inhibition at the opposite end.

In Experiment 4 20 mm. pieces were placed in tubes 10 mm. long. In the control lot the stems projected 5 mm. from each end and in the experimental lot the proximal ends of the stems were arranged flush with one end of the tube (Table III, column 2). A glance at columns 5 and 6 of Table III shows that the experimental treatment resulted in an actual increase in distal regeneration with respect to length of the primordia and time at which constriction was first visible.

In this experiment the total amount of stem covered did not influence the result since it was the same in both experimental and control lots. Likewise the result cannot be attributed to the increased distance from the distal end of the stems to the tubes, since in the previous experiment it was found to have no effect between 5 mm. and 10 mm. Therefore, it is concluded that the increased regeneration of the experimental over the control lot was the result of covering the proximal end. It also lends added support to the conclusions arrived at from a comparison of Lots 3 and 4 of the first three experiments, namely, that the increased distal regeneration observed in Lot 4 of each experiment was the effect of the nearness of the collar to the proximal surface, thereby reducing in some manner its inhibitory effect upon distal regeneration.

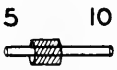
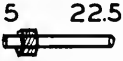
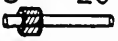
By allowing 5 mm. of stem to project from the ends of the tubes it was now possible to test the effects of covering various amounts of perisarc. In Experiment 5 a comparison was made between stems covered with 5 mm. and with 10 mm. tubes (Table IV). No difference in time of constriction was observed (column 5), but with respect to length, covering an additional 5 mm. of stem resulted in a decrease of 6 units (0.126 mm.). This represents a decrease of 8.6 per cent over the 5 mm. covered stems.

The experiment was repeated (Experiment 6) with tubes 2.5, 5, 10, and 20 mm. long. In this case the stems were longer (30 mm.) and larger, the temperature was lower (temperature range 13.8° to 16° compared with 18.0° to 18.6° C.) and the primordia which developed were larger. It is not possible to say how the differences in the two experiments correlate with the differences in the result, but it is readily observed that in Experiment 6 the tubes were far less effective in reducing the size of the regenerate.[3] In Experiment 5 doubling the area

[3] When saturated, seawater at 18° C. has about 7 per cent less oxygen, about 11 per cent less "free" carbon dioxide and about 2 per cent less "combined" carbon dioxide than it does at 14° C. (calculated from Table 235 in "Properties of Ordinary Water Substance," N. E. Dorsey, 1940, Reinhold Publishing Corp., 667 pp.). It is therefore apparent that stems regenerating at lower temperatures find it easier to

covered resulted in an 8.6 per cent decrease in average length of the primordia. In Experiment 6, on the other hand, it took a ratio of four to one to produce an equivalent reduction. Increasing the length of

TABLE IV

Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6
Expt. 5	Diagram	No. Measured	Av. Diameter*	Av. Time (Hours)	Av. Length Primordia*
5 mm.		10	24.9	28.8 (28–31)	81.7 (68–92)
10 mm.		10	25.0	28.8 (27–31)	75.7 (47–101)
Expt. 6					
2.5 mm.		10	29.9	40.2 (39–42)	102.7 (92–110)
5 mm.		10	30.0	40.5 (39–42)	99.9 (92–111)
10 mm.		10	30.1	40.5 (40–42)	94.0 (89–101)
20 mm.		10	30.6	40.7 (40–42)	93.0 (70–108)

Experiment 5. 20 mm. stems with the distal 5 mm. projecting from the ends of the tubes. Temperature range 18.0°–18.6° C.

Experiment 6. 30 mm. stems with the distal 5 mm. projecting from the ends of the tubes. Temperature range 13.5°–16.0° C.

*1 unit = 0.445 mm.

obtain oxygen and to eliminate carbon dioxide than at higher. In addition, lower temperatures reduce the respiratory requirements and the speed of synthesis of new tissues. All these factors would tend to decrease the effectiveness of tubes at lower temperatures, but at present it is not possible to say which are of major and which of minor significance.

the collar from 2.5 to 10 mm. produced an 8.5 per cent reduction in length and from 5 to 20 mm. gave a 7.9 per cent decrease. Doubling the surface covered gave only the following percentage reductions: from 2.5 to 5 mm.—2.7 per cent; from 5 to 10 mm.—5.9 per cent; from 10 to 20 mm.—1.1 per cent. An eight-fold increase in area covered was sufficient to decrease the length by only 9.4 per cent (from 102.7 to 93.0 units). It is of interest that in the 20 mm. lot, two-thirds of the entire surface area of the perisarc was covered. When such is possible with so little effect upon time and primordium length it is obvious that under the conditions of the experiment the metabolic exchange through the perisarc is definitely subordinate to that through the ends of the stem.

Discussion

These experiments were designed to determine whether and to what extent metabolic exchange through the perisarc affects the time of appearance and the length of the regenerating hydranth primordium. Because of the necessity of exposing at least 5 mm. of stem at each end of the tubes, it was not possible to test short, i.e. very young, stems. However, with this limitation, it was noted that even when large areas of the stem were covered there were relatively slight inhibitory effects. In one experiment there was a decrease of only 9.4 per cent in length when the glass tubes covered two-thirds as compared with one-twelfth of the area of the stem, an eight-fold difference (calculated from Experiment 6). In another (Experiment 5) run at a higher temperature and with shorter stems, doubling of the area covered gave a 10.2 per cent decrease in primordia length.

On the other hand, the ends of the stems showed marked susceptibility to the presence of the tubes. A regenerative decrease of similar order of magnitude as above was produced by arranging the stems with 2.5 mm. of the distal ends extending beyond the tubes. In this case the reduction in length was 20.8 per cent and 9.1 per cent (calculated from Experiment 1, Lots 2 and 3, and Experiment 2, Lots 1 and 2 respectively). Complete inhibition when stems are left with their ends flush with the tubes has already been reported (Barth, 1938a; Rose and Rose, 1941) and was confirmed by my own unreported preliminary experiments. In the present experiments the 40.0 per cent decrease in length of the hydranths of Lot 1 over Lot 3 in Experiment 1 represents an incomplete recovery following removal from the tubes.

The effects on time of regeneration were generally so slight that they do not warrant any discussion excepting the comment that it appears questionable whether data on tubularian regeneration should be recorded as "regeneration units (R. U.)" calculated from a formula in

which length is divided by time (Barth, 1938b) and based on the implied assumption that length and time are inversely related to each other. The present experiments cast doubt on the validity of this assumption, since in some cases length varied while time did not. A case in which such a relationship cannot hold was recently reported in abstract by Moog (1941) who found that stems at lower temperatures required longer to regenerate but produced larger primordia than did those at higher temperatures.

It should be pointed out that the tubes interfered with diffusion out of as well as into the stems. Barth who used somewhat similar tubes (1938a) failed to recognize this possibility and attributed the inhibition produced entirely to oxygen deficiency. Similarly in the experiments of Zwilling (1939), in which regeneration followed the localized removal of perisarc, it appears highly probable that the operation allowed the escape of inhibitors as well as the entrance of oxygen.

The necessity of oxygen for regeneration has been clearly demonstrated (Torrey, 1912; Barth, 1937, 1938a, 1940; Miller, 1937, 1939; Rose and Rose, 1941). However there is also growing evidence of inhibitory substances which must be allowed to escape from the stem in order for regeneration to proceed at a maximal rate. A thermally unstable inhibitor has been found by the Roses. Miller (1939) found carbon dioxide to be a powerful inhibitor, which even when mixed with oxygen in proportions of 10 per cent to 90 per cent would completely prevent regeneration. The fact that carbon dioxide combines with water to produce carbonic acid would suggest that the inhibitory action of the gas might be a result of the increased acidity of the solution. The possibility of inhibition by acidity was first suggested by experiments of Loeb (1904) concerning which he made the following statement: "One gains the impression that in the tubularian stem an acid is formed which delays growth if it is not neutralized" (p. 147). This suggestion seems to have passed unnoticed by later experimenters. Child (1931) found evidence of inhibitory action by lowered pH and Komori (1933) reported an experiment in which distal ends exposed to pH 6.0 failed to form hydranths while ten of the thirteen proximal ends of the same pieces exposed to pH 8.45 did. Work this past summer (Miller, 1942b) has shown that there actually is an increase in acidity of the coelenteric contents of stems enclosed in tubes used in the experiments reported in this communication.[4] Whether or not this acidification may be attributed entirely or largely to carbon dioxide accumu-

[4] The accumulation of pH lowering substances in tubes in which stems have remained for 96 hours has just been reported, as well as further evidence on the inhibitory action of low pH. (Goldin, A., 1942. *Biol. Bull.*, 82: 243–254.)

lation has not been determined, but it appears likely that it plays a major rôle.

Already reported experiments have indicated that the oxygen content of the middle of the coelenteron is not affected by increasing that of the external environment even though regeneration was stimulated by the treatment (Miller, 1940). When these findings are considered in the light of the fact that the oxygen consumption of the tubularian stem varies with the oxygen tension (Barth, 1940), it becomes evident that any oxygen that penetrates the perisarc is utilized by the subadjacent ectoderm before reaching the entodermal ciliary mechanism or the coelenteric contents. Likewise, it follows that any localized diminution of oxygen in the milieu, such as would occur within the tubes, would be matched by a corresponding decrease in oxygen uptake by these same cells, again without affecting the coelenteric contents. It is therefore suggested that the inhibitory action of the tubes, when placed with at least 5 mm. of stem projecting from the ends, was not the result of anoxemia.

On the other hand, an increase of acid producing metabolites has been noted in stems placed in the tubes (Miller, 1942b) and acidity is known to inhibit tubularian regeneration. It is therefore tentatively suggested that the degree of reduction in length of primordia of stems regenerating in the longer tubes represents the extent to which the elimination of inhibitors is accomplished by the unbroken perisarc.

Summary

1. Pieces of Tubularia stems were placed in glass tubes with outside and inside diameters of 2.5 mm. and 1 mm. respectively in order to determine the rôle in regeneration played by metabolic exchange through the perisarc.

2. Using tubes of the same length it was found that when the distal ends of the stems projected less than 5 mm. from the tubes regeneration was decreased at the distal surface. When the proximal ends projected less than 5 mm. from the tubes there was an increase in distal regeneration.

3. Using tubes of different lengths (with at least 5 mm. of stem projecting from either end) slight decreases in length of the regenerate were observed with increasing length of the tubes.

4. It was concluded that the powerful effects observed when the distal ends of the stems projected less than 5 mm. from the tubes represent the limitation of diffusion of inhibitors out of as well as of oxygen into the stems. On the other hand, in the light of other experiments, it was suggested that the effects obtained by covering the middle of the

stems represent the extent to which inhibitors of regeneration are eliminated through the unbroken perisarc rather than any induced alteration of the oxygen content of the coelenteron.

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THE NUTRITIVE REQUIREMENTS OF TENEBRIO MOLITOR LARVAE [1]

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A comprehensive review of the literature on invertebrate nutrition was published by Trager (1941). Our interest was stimulated by the possibility that the nutritive response of an insect, caused by a specific biological substance, might be used as a method of assay that could be applied to small quantities of human blood, urine, and necropsy tissue. We have worked intensively upon the problem for several years and our initial studies, started in 1931, were devoted to selecting the insect and the nutritive response best suited for this type of investigation. We chose the growth response of *Tenebrio molitor* larvae for several reasons. First, the larvae attain mature size without access to water which would allow yeast and bacteria to grow in the diet and vitiate results. Second, the larvae are docile, hardy, and large enough to be weighed without escape or injury. Third, the imagos are sufficiently prolific that large numbers of immature larvae of the same age and weight are available for use.

Experimental Methods

A sufficient number of imagos are collected in early summer to insure enough larvae to carry out the planned experiments. We obtained more than 500,000 larvae in 10 weeks from 3,000 beetles, with the following technique: the bottom of a five-gallon can is covered with paper and two pieces of paper folded as fans are placed in the can. Two hundred grams of beetle diet, consisting of white flour and 5 per cent brewer's yeast, is scattered over the fans which are covered with cardboard. A moistened bath sponge, on a glass plate, is placed on the cardboard to supply water for the beetles. Fifteen hundred beetles are placed in each can. The procedure is repeated daily in order to maintain a constant supply of immature larvae of approximately the same age. The growth of stock larvae can be arrested either by feeding them an insufficient diet or by non-hygroscopic refrigeration and such larvae are available throughout the winter months.

[1] This investigation was supported by a Landon Research grant.

The Kerr one-half pint fruit jar is ideal for a feeding cage. The dry dietary ingredients can be sealed in the jar with three or four marbles and numerous jars placed in a rotating device which mixes the diets thoroughly. The diets can be kept sealed from contamination until the experiment is started, when marbles are removed, larvae added, and wire screen and cloth substituted for the self-sealing lid. This makes it possible to study many variables with the same larval colony, groups of which can be fed the different diets at the same time.

The experimental procedure is simple. Immature larvae of the same age and weight are divided into groups of 25. Each group is fed 15 grams of diet [2] and each diet is fed to three groups to obtain a triple check on results. After a sufficient feeding period each group is weighed and the average weight of surviving larvae computed. At ordinary laboratory temperature and humidity alterations in growth are apparent after four to six weeks but maximal alterations are seen after 10 to 12 weeks.

Preliminary Experiments

Early studies, carried out before crystalline nutritive elements were available, demonstrated that larval growth was influenced by many physical and dietary factors and numerous experiments were done to establish a method of procedure that would control the different variables. It was observed that growth was modified by alterations of temperature and humidity; age, refrigeration, and state of nutrition of test larvae; the subjection of the diet to excess heat or chemical manipulation; the growth of yeast or bacteria in the diet; and the relative proportion of fat, carbohydrate, and protein in the diet. Optimum growth occurs when the diet contains not less than 50 per cent carbohydrate and not less than 15 per cent or more than 25 per cent protein. Apparently fats are not required and when present in excess of 3 per cent inhibit growth. These latter observations confirm the findings of Lafon and Teissier (1939) who studied the effect of qualitative and quantitative additions of lipids, carbohydrates, and proteins to the diet of molitor larvae. Tables I and II present some pertinent findings of additional experiments. The results appearing in Table I represent typical observations made at different times when molitor larvae were fed various diets. Animal tissues added to the diets were dried at 70° C. and powdered.

Table I shows that neither white wheat flour nor an orthodox vitamin-free diet allows appreciable growth but optimum growth oc-

[2] Fifteen grams of diet, adequate in nutritional factors, is sufficient to allow 25 larvae to attain adult size, although to carry this number through the life cycle requires 25 to 30 grams.

curs when either of these diets is supplemented with yeast. The results indicate that the vitamin-free diet and yeast causes better growth than white flour and yeast, suggesting that some ingredient of the vitamin-

TABLE I

The effect of different diets upon growth of molitor larvae. Each diet was fed to 75 larvae for 12 weeks. Average initial weight was 2.5 to 3 mg.

	Number of surviving larvae	Average weight (Milligrams)
White wheat flour	42	9
[1] Vitamin free diet	27	6
Whole wheat flour	57	31
White wheat flour and beef blood (5%)	30	4
White wheat flour and beef muscle (10%)	55	20
White wheat flour and beef liver (10%)	73	71
White wheat flour and brewer's yeast (10%)	69	92
Vitamin free diet and brewer's yeast (10%)	60	142

[1] The vitamin free diet contained casein 20 per cent, corn starch 74.5 per cent, Osbourne and Mendel salt mixture 4 per cent, cholesterol 1 per cent and crisco 0.5 per cent.

TABLE II

The influence of different percentages of five separate strains of brewer's yeast on growth of molitor larvae. Each diet was fed to 75 larvae for 83 days. The average initial weight was 2.5 mg.

Per cent of yeast in the diet [1]	Strains of brewer's yeast [2]									
	No. 1		No. 2		No. 3		No. 4		No. 5	
	Surviving larvae	Average weight (mg.)	Surviving larvae	Average weight (mg.)	Surviving larvae	Average weight (mg.)	Surviving larvae	Average weight (mg.)	Surviving larvae	Average weight (mg.)
0.5	47	22	44	23	53	19	38	17	41	14
1.0	52	40	42	40	60	24	43	23	42	19
1.5	62	53	54	48	60	37	52	30	40	23
2.0	56	74	40	68	51	49	48	35	37	27
2.5	54	95	49	80	61	64	50	42	38	37
3.0	58	107	54	92	57	85	53	49	53	43
5.0	47	135	43	126	54	109	54	94	47	78
10.0	58	132	57	129	57	112	52	132	52	105

[1] Control groups fed only white wheat flour averaged 9 mg. in weight.
[2] Supplied by Anheuser-Busch Biological Laboratories, St. Louis, Missouri.

free diet modifies growth. Whole wheat flour allows moderate growth, indicating that the pericarp supplies growth factors. With a basal diet of white flour, skeletal muscle causes a moderate response comparable to larvae fed whole wheat flour. Liver causes better growth

than muscle, which signifies that it contains more growth factors. The addition of blood to the diet retards growth. Yeast causes better growth than liver, indicating that it contains more growth factors or that liver contains substances which inhibit growth. The effect of adding different tissues to a vitamin-free diet has not been studied. A basic diet of white flour was used in preliminary studies because it more nearly imitated the natural food of this insect and survival on inadequate diets was observed to be better.

The observations appearing in Table II were made on a colony of molitor larvae fed white flour supplemented with different percentages of five separate strains of brewer's yeast. The table is presented to show that average growth in a controlled experiment is remarkably constant and the uniformity of results indicate that larvae do not select food particles but eat indiscriminately of the diet.

Table II shows a direct correlation between concentration of yeast and growth response, optimum growth occurring when the diets contained 5 per cent to 10 per cent yeast. The strains are numbered in the order of their ability to stimulate growth and the findings demonstrate the sensitivity of larvae to minor changes in the diet introduced by using different strains of yeast.

The above studies show that molitor larvae will attain mature size on a dry diet incompatible with the growth of yeast and bacteria but, in order to control the different variables, each experimental colony must consist of larvae of the same age, weight, and state of nutrition, started on the various diets at the same time, and kept in the same environment. Moreover, the diets should receive identical chemical and physical manipulation and contain the proper balance of fat, carbohydrate, and protein for optimum growth. Each diet should be fed to 75 or more larvae since there is marked variation in individual growth. When this procedure is followed the only factors which modify growth are the nutritive elements in the diet. However, before larval growth could be used to assay the nutritive elements it was necessary to establish the specific influence of a given factor. This proved to be difficult and was accomplished only after most of the crystalline B components were made available.

Experiments with Synthetic Diets

These studies were undertaken in an attempt to ascertain which of the known purified and crystalline vitamins influenced growth when added singly to a purified diet,[3] which ones stimulated growth by acting

[3] The basal diet contained vitamin free casein 20 per cent, corn starch 74.5 per cent, Osborne and Mendel salt mixture 4 per cent, cholesterol 1 per cent and crisco 0.5 per cent.

in conjunction with other vitamins, and which ones failed to affect growth entirely. We have carried out several experiments on this problem, extending the study to include each new crystalline vitamin when it was available. The source and quantity of vitamins added to the diets used in all experiments appear in Table III.

TABLE III

The quantity and nature of vitamins added to the diets

Nature of the vitamins [1]	Amount per gram of diet [2]
Vitamin A	
Natural purified vitamin from fish liver oil	40 units
Vitamin D_2	
Irradiated ergosterol	7 units
Vitamin E	
Synthetic alfa tocopherol	33 micrograms
Vitamin K	
Crystalline 2-methyl-1, 4-naphthoquinone	20 micrograms
Vitamin C	
Crystalline l-ascorbic acid	100 micrograms
Crystalline choline hydrochloride	100 micrograms
Crystalline thiamine hydrochloride	10 micrograms
Crystalline riboflavin	10 micrograms
Crystalline pyridoxin	10 micrograms
Crystalline calcium pantothenate	10 micrograms
Crystalline nicotinic acid	30 micrograms
Labco flavin free rice polish concentrate	
Rich in thiamine, pyridoxin, nicotinic acid, and filtrate factors	50 milligrams
Brewer's yeast. The yeast assayed 50 International B_1 units and 50 Bourquin-Sherman G units per gram	50 milligrams

[1] Solutions of crystalline vitamins of known concentration were mixed with the vitamin free diet until a syrup resulted. This was desiccated and powdered in a ball mill.

[2] The quantity of vitamins added to the diets was arbitrary. Five per cent yeast causes optimum growth and the amount of vitamins present in this amount of yeast is much less than the quantity of purified vitamins added to the diets.

Experimental procedure: Various diets, incorporating singly and collectively the purified vitamins, were fed to groups of 75 larvae for 12 weeks. For the sake of brevity only the pertinent results of composite experiments will be presented. These appear in Table IV.

Table IV shows that no vitamin, by itself, promotes growth when added to a vitamin-free diet. Furthermore, vitamins A, D, C, E, K, and choline, failed to influence growth when added to diets containing all the synthetic B vitamins. Moreover, no combination of the B vitamins (thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic acid) affect growth appreciably unless all five are present.

Finally, growth on diets containing the five B vitamins is only one-third that of larvae fed the vitamin-free diet supplemented with liver or yeast. Table IV also shows that the only vitamin which increased the incidence of survival was riboflavin.

The above findings demonstrate conclusively that molitor larvae require thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic

TABLE IV

The vitamin requirements of molitor larvae. Each diet was fed to 75 larvae for 12 weeks. Average initial weight was 4 to 5 mg.

Diet (Vitamins added to a vitamin free basal diet)	Number of surviving larvae	Average weight (milligrams)
Vitamin A	21	7
Vitamin D	27	6
Vitamin C	19	7
Vitamin E	18	8
Vitamin K	22	7
Choline	23	6
Thiamine (B_1)	27	7
Riboflavin (B_2)	60	8
Pyridoxin (B_6)	21	8
Nicotinic acid (N. A.)	17	8
Calcium pantothenate (C. P.)	25	7
A, D, C, E, K, and choline	22	9
B_1, B_2, B_6, and N. A.	62	13
B_1, B_2, B_6, and C. P.	59	12
B_1, B_2, N. A., and C. P.	65	14
B_1, B_6, N. A., and C. P.	24	11
B_2, B_6, N. A., and C. P.	69	14
B_1, B_2, B_6, N. A., and C. P.	73	36
A, D, C, E, K, choline, B_1, B_2, B_6, N. A., and C. P.	68	36
Brewer's yeast	73	114
Rat liver	67	102
Vitamin free basal diet	25	7

acid, however, these vitamins could only stimulate growth by acting in conjunction with each other. It was thought that superior growth on yeast or liver might be caused by phosphorylated flavin and the influence of lactoflavin phosphate [4] was investigated to test this concept. Two experiments were carried out with the same controlled colony of larvae; in the first, graded amounts of lactoflavin phosphate and riboflavin were each added to an unsupplemented vitamin-free diet, and in the second, the two flavins were added to the basal diet further supplemented with vitamins A, D, C, E, thiamine, pyridoxin, nico-

[4] The lactoflavin phosphate was obtained through the courtesy of Dr. R. Kuhn of Heidelberg. The preparation was made according to the method published by Kuhn, Rudy, and Weygand (1936).

tinic acid, and flavin-free rice polish concentrate as a source of pantothenic acid. The various diets were each fed to 75 larvae for 81 days and the results appear in Figure 1.

Figure 1 shows that results with the unsupplemented vitamin-free diet were entirely negative and confirm previous observations that riboflavin can only stimulate growth by acting in conjunction with

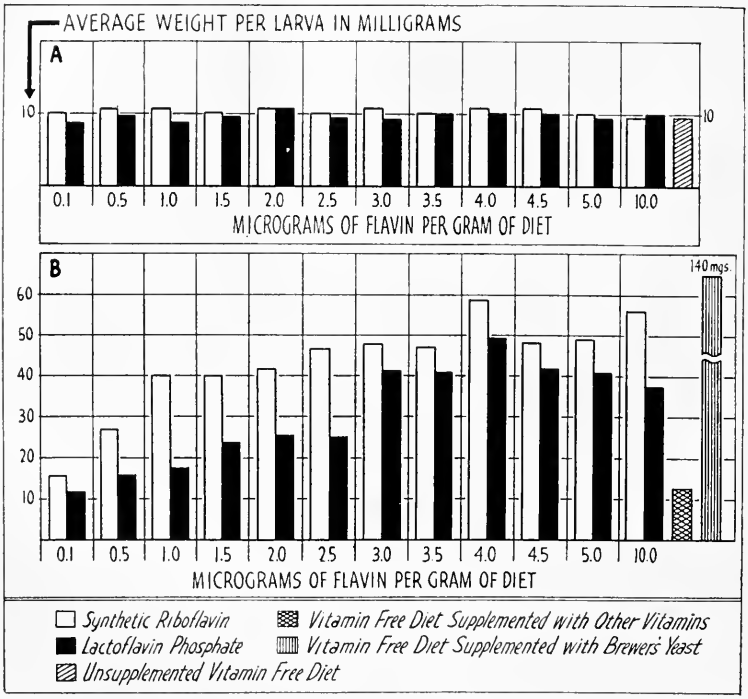

FIGURE 1. The comparative effect of synthetic riboflavin and lactoflavin phosphate upon growth of molitor larvae. Each diet was fed to 75 larvae for 81 days. The average initial weight was 4.9 mg.

A. An unsupplemented vitamin free diet was used.

B. The basal diet was supplemented with vitamins A, D, C, E, thiamine, pyridoxin, nicotinic acid and flavin free rice polish concentrate.

other components of the vitamin B complex. The findings demonstrate that lactoflavin phosphate possesses no merit over synthetic riboflavin in this respect.

In the second experiment results were different. When the basal diet was supplemented with the other essential B vitamins the influence of riboflavin is apparent. The figure shows that maximal growth was caused by approximately one microgram of riboflavin and 2.5 to 3 micrograms of lactoflavin phosphate, per gram of diet, demonstrating

that flavin phosphate, together with the other essential vitamins, causes no better growth than riboflavin. Indeed, the results suggest that excessive amounts of lactoflavin phosphate retard growth. Additional observations, not included in this report, show that diets containing as much as 75 micrograms of riboflavin per gram cause no inhibition, therefore the retarding effect of lactoflavin phosphate appears to be due to the phosphoric acid radical. The lesser potency of lactoflavin phosphate is logically explained on the basis of inert phosphoric acid content. The study was not designed to measure precisely the amount of flavin required for maximal growth but Figure 1 shows that larvae are sensitive to minute amounts and it appears that maximal growth results when the diet contains one to 3 micrograms per gram.

The experiments show that superior growth on diets containing yeast (See Table IV) is not due to the fact that zymoflavin occurs as the phosphoric acid ester and the finding indicates that yeast and liver contain larval growth factors additional to the vitamins investigated.

Comment

The vitamin requirements of several orders of insects have appeared in the literature but reports are so variable that no positive conclusions can be drawn as to which factors are essential. Apparently the only crystalline vitamins required are included in the vitamin B complex but there is no agreement as to specificity. Some of the confusion may be due to the different orders investigated. Subbarow and Trager (1940) reported studies with mosquito larvae that are sufficiently related to our findings to warrant comment. They state that these larvae require thiamine, riboflavin, pyridoxin, pantothenic acid probably glutathione and nicotinic amide, and certainly other unknown substances present in yeast and liver extract. Although we used a different experimental approach on an unrelated order of insect, we obtained identical results. It appears, therefore, that all insects probably have similar basic nutritive requirements and conflicting results of others are possibly due to the common practice of using highly purified diets in nutritional experiments. The failure of a given dietary factor to influence the nutritive response, when added to such diets, has frequently been interpreted as evidence that the test animal either does not require the vitamin or can synthesize it. We believe our studies with molitor larvae demonstrate the fallacy of this concept since no vitamin, by itself, had any effect and growth was not appreciably stimulated until thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic acid were all present. Even then

growth was less than one-third optimum and it is possible that with more complete diets the specific effect of additional crystalline vitamins could be demonstrated. Moreover, our studies were limited to larval growth and additional vitamins are probably necessary for other nutritive responses such as survival, metamorphosis, pupation, and fertility.

It is apparent that the nutrition of insects is extremely complex and that numerous factors, known and unknown, are required for optimum effects. However, with the rapid advances being made in the chemistry of nutrition and biology, it appears entirely possible that they may ultimately be reared on completely synthetic diets. Our studies still leave much to be learned but we believe they are sufficiently complete to demonstrate the cardinal advantages of molitor larvae as test material for this major problem. In some of our experiments more than 100 variables were investigated with a single colony of thousands of larvae, of the same age and weight, which were started on the various diets at the same time. Furthermore, it is possible to carry out such experiments with maximal economy of time, space, and expense.

It is interesting to speculate on the reason why so many of the B vitamins had to be in the diet before growth occurred and why other purified vitamins caused no effect. It is perhaps significant that most of the B vitamins are now known to function by entering into cellular enzyme systems, and it may be that insects require only the "enzymatic" vitamins. If this is true, thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic acid, as well as additional factors required for optimum growth, function in this manner. It is possible, therefore, that insects may have unique importance as test material in the elucidation of this problem.

Although larval growth is dependent upon the interrelationship of many factors, the effect of any one factor is concise and specific provided the complementing factors are added to the basal diet. Molitor growth, therefore, can be used as a biological assay for any isolated factor whose specific influence can be demonstrated. At present these factors include thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic acid. These assays can be both qualitative and quantitative on any material that does not contain the unknown factors required for optimum growth. Such assays may be of practical interest to the synthetic chemist who is interested in studying the biological activity of compounds which are related to the above vitamins. Furthermore, they may be important to the pharmacologist who is interested in the control assay of pharmaceuticals consisting of the above nutritive factors.

Summary

1. A standard experimental method is presented which can be used to investigate factors that modify the nutritive responses of molitor larvae.

2. Larvae failed to grow when fed a vitamin-free diet of casein, fat, carbohydrate, salt mixture and cholesterol but, when this diet was supplemented with yeast or liver, optimum growth occurred.

3. When the above diet was supplemented, individually, with vitamins A, D, C, E, K, choline, thiamine, riboflavin, pyridoxin, nicotinic acid and pantothenic acid, negative results were obtained. However, when added collectively to the diet, approximately one-third optimum growth resulted.

4. When different combinations of the above vitamins were added to the diet, no appreciable growth occurred unless the diet contained thiamine, riboflavin, pyridoxin, nicotinic acid and pantothenic acid. Conversely, if any of these five factors was omitted, results were essentially negative. Vitamins A, D, C, E, K and choline had no effect.

5. On the assumption that optimum growth on yeast might be caused by phosphorylated flavin, lactoflavin phosphate was investigated. The phosphorylated flavin caused no better growth than synthetic riboflavin, indicating that yeast contains larval growth factors additional to the above vitamins. Biotin, paraminobenzoic acid, folic acid and inositol were not studied.

6. Molitor growth is suggested as a method of biological assay for thiamine, riboflavin, pyridoxin, nicotinic acid and pantothenic acid.

Acknowledgment

We are deeply grateful to Merck and Company, and Eli Lilly and Company, who generously supplied us with purified nutritive factors when only a small amount of such material was available.

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THE CILIARY TRANSPORT-SYSTEM OF ASTERIAS FORBESI

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Certain persistent tubular structures derived from the anterior and right-posterior enterocoels of the larvae of echinoderms, and traditionally termed 'perihaemal systems,' seem questionably so-named; for they assuredly do not in major manner function as haemal, or blood, systems do in other phyla. If they play any part as a transport system serving nutritional, respiratory, and excretory ends, it must be a minor contribution indeed; but exclusive of the echinoderms, even animals of relatively minute size, e.g., tiny annelids, or semi-visible arthropods of different sorts, are generally equipped with pulsatile or tubular organs, or both, of constant anatomical make-up and relations which serve the function of transport. In short, it is probably true that echinoderms are the largest animal organisms lacking the homologue of a vascular system as that designation is customarily used.

In most if not all echinoderms, media serving respiratory, excretory, and nutritional ends are propelled by cilia on external, in coelomic, and in other, e.g., endodermally lined, spaces. In as much as the circulation paths thus established by ciliated tracts are for the most part very definite and constant as between individuals of the same species, the mapping of these paths is a procedure in concrete anatomy.

It should be mentioned at once that Gemmill (1915) published the results of his study of ciliary tracts in several asterids common in English waters, but apparently did not use *Asterias forbesi*. Furthermore, Gilsen, who published his extensive paper on echinoderms in 1924, covering all classes of that phylum, did not include this species; also, so far as ciliation is concerned, his account is limited to ciliation of external epithelia. Other less extensive studies are cited later in this narrative.

The purpose of the present paper, therefore, is to record observations on the cilia-produced flow on external and internal epithelia of the starfish most frequently employed as a study-type in all grades of educational institutions east of the Rocky Mountains in the United States. A detail of the mechanism serving certain physiological processes in this asterid may thus be more available.

Methods

All conclusions as to flow-direction were made by use of carmine suspension in sea-water, introduced to the area under observation from a small-bore pipette, and following its movement with a binocular dis-

secting microscope. It was assumed that the action of cilia was not modified by extirpation of the area on which they were located.

Ciliary tracts on external surfaces [1]

1. General aboral areas:

Echinoderm skeletons are mesodermal, and ectodermal epithelium completely covers the body exterior, though often reduced on tips of spines. While the epithelia of spines show their more-or-less individual ciliary equipment (described later), the composite directional flow on the body surface as a whole is shown in Plate I, Figure 1; it is centrifugal from the central disc (anal area) to the distal ends of the rays, with currents running laterally (inter-radially) from the axial mid-lines. The only areas not conforming to this rule are those in the angles of the inter-radial surfaces; here the flow is centripetal, with the obvious result that gonadal products emerging in those angles are thrown into the open, not into the substrate.

2. Spines and branchiae:

Cilia on aboral spines and branchiae uniformly beat from the bases to the distal ends on all sides, save that on all there is deviation enough (rarely with spiral tendency) so that there is a resultant mass-effect on the ray-surface-as-a-whole of a sort already mentioned.

3. Pedicellariae:

Figure 1 indicates the ciliary direction-map for a pedicellaria, the forceps and scissors types being similar. A specially adapted area is

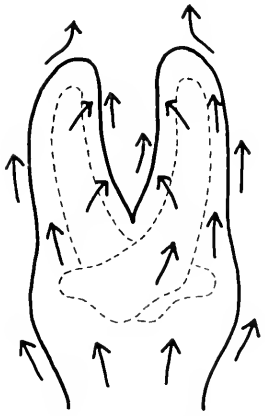

FIGURE 1. Ciliary flow on surface of a pedicellaria.

[1] Photographs reproduced in this paper were made by Arthur L. Princehorn, head of Photographic Service in Oberlin College.

that on the apposed sides of the jaws; cilia sweep suspended particles onto the inter-jaw ridges, i.e. onto the biting area, on which in turn the flow is distad.

4. The madreporite:

Madreporitic surfaces show currents which, in the main, flow toward the aboral disc-center, with minor flows toward adjacent rays. About 50 per cent of specimens show considerable streaming in all

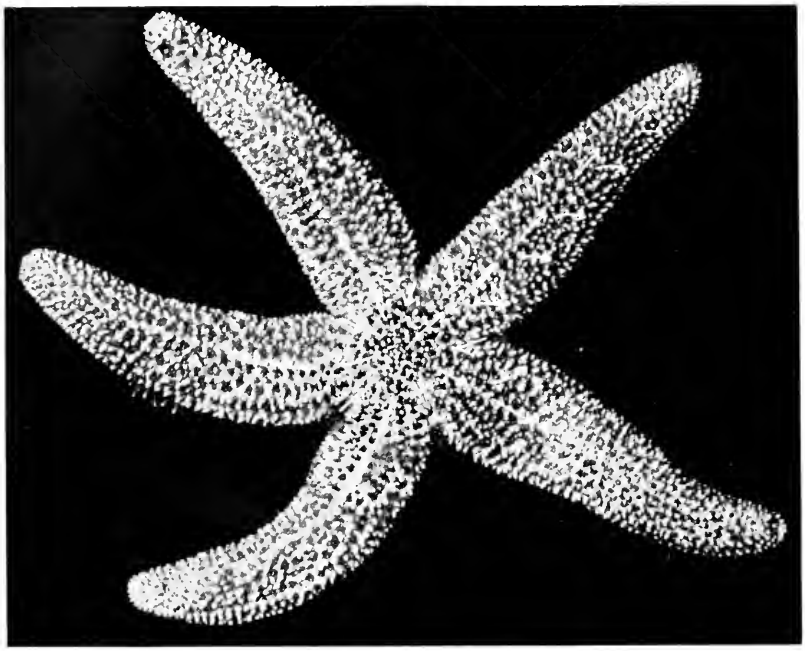

PLATE I, FIGURE 1: Direction of ciliary currents on external aboral surface.

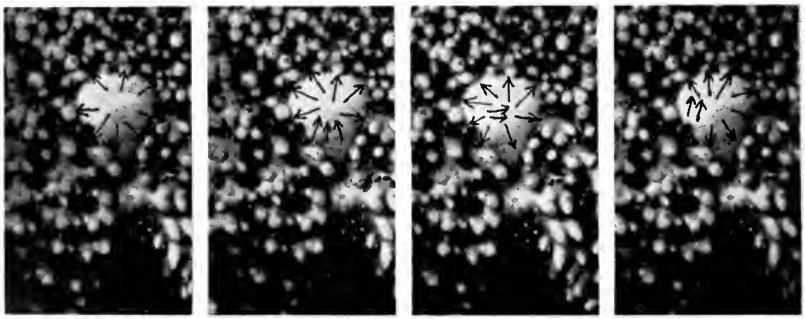

PLATE I, FIGURE 2: Various flow-patterns on madreporic plates.

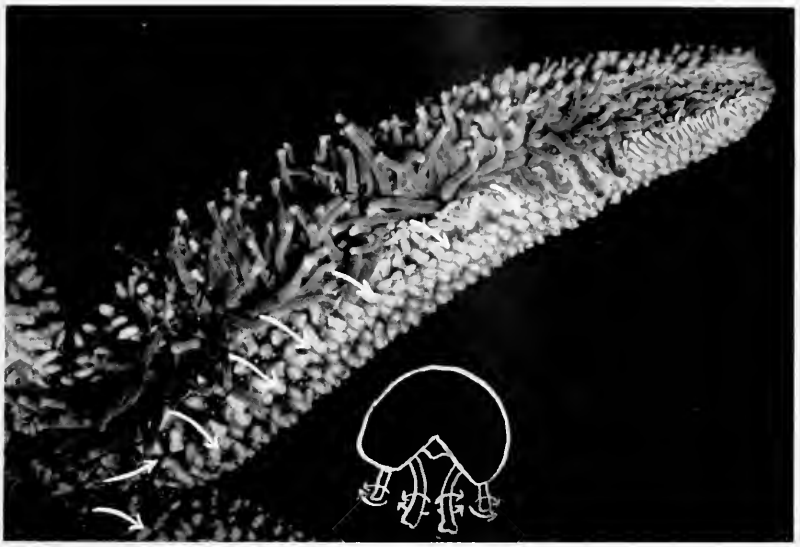

PLATE I, FIGURE 3: Ciliary currents on oral surface of one ray. Insert is diagram of cross-section of ray to show flow-direction on tube-feet and marginal spines.

directions from the porite center; as madreporitic plates themselves have a varied structural pattern, this ununiformity is not surprising. Plate I, Figure 2 presents instances of observed variations. Cilia in the pore-canals of a plate have a positive outward beat; it is this fact, presumably, which seems to have influenced Hartog (1887) to believe erroneously that a considerable portion of cilia in the stone-canal itself beat similarly.

5. General oral surface:

The ciliary-flow picture of a sample ray of this area is shown in Plate I, Figure 3; currents run centrifugally from the median oral region, but take an inter-radial direction from the ambulacral grooves, tube-feet, and adjacent ray surfaces.

6. Spines and branchiae (oral aspect):

On the lateral ray areas these structures are equipped like those on the aboral surface, viz. currents run distally. Those on the margins of the ambulacral grooves, however, are clothed with cilia which sweep laterally, i.e. at right angles to the long axis of spine or branchia, transversely around each side toward the inter-radial space. This description holds true for ciliation on either side of the groove (Plate I, Figure 3).

7. Tube-feet:

On these the set-up is the same as just mentioned for spines and branchiae on ambulacral margins, i.e. at right angles to long axis around toward the inter-radial space. Either side of the groove has a flow pattern which is the mirror image of that on the other side of the axial midline (Plate I, Figure 3).

8. Ambulacral groove:

Ciliation in the bottom of the groove is very weak indeed, with uncertain tendency to carry particles centripetally. The flow on either side of the midline is strongly toward the inter-radial space of its side. Neighboring cilia on tube-feet, unless these are completely eliminated, confuse observations.

9. Perioral membrane:

Cleared of near-by structures, all currents here flow toward the mouth and thus contribute, though in immeasurable degree, to nutritional intake.

Physiological Adaptations of External Ciliation

1. Material in suspension tending to settle on the surface and interfere with respiration or excretion is swept away by the shortest path; anal expulsions are similarly dispersed.

2. Cilia on the madreporite beat in such manner as to prevent debris from clogging the entrance to the water-vascular system.

3. Materials handled by pedicellaria cilia are brought from lateral contacts into their 'bite' area.

4. Cilia in ambulacral grooves, on tube-feet, and on groove margins propel the flow inter-radially, save in the midline of the groove where movement is uncertainly oralward.

5. Centrifugal flow, plus that in aboral direction in inter-radial angles, prevents gonadal products from settling on the relatively congested oral surface, where they might be engulfed in the food-flow, find poor oxygen supply, or be buried in the substrate.

Ciliary Tracts on Internal Surfaces

A. The major perivisceral coelom and organs covered by its peritoneum.

Food absorbed through gastric walls (if this occurs) or their diverticula, as well as oxygen from branchial structures, depends entirely, or very nearly so, on ciliary propulsion for distribution to tissues not parts of those systems. Every coelomic surface is ciliated. For the rays, in general, all flow is centrifugal (distad) except that on the

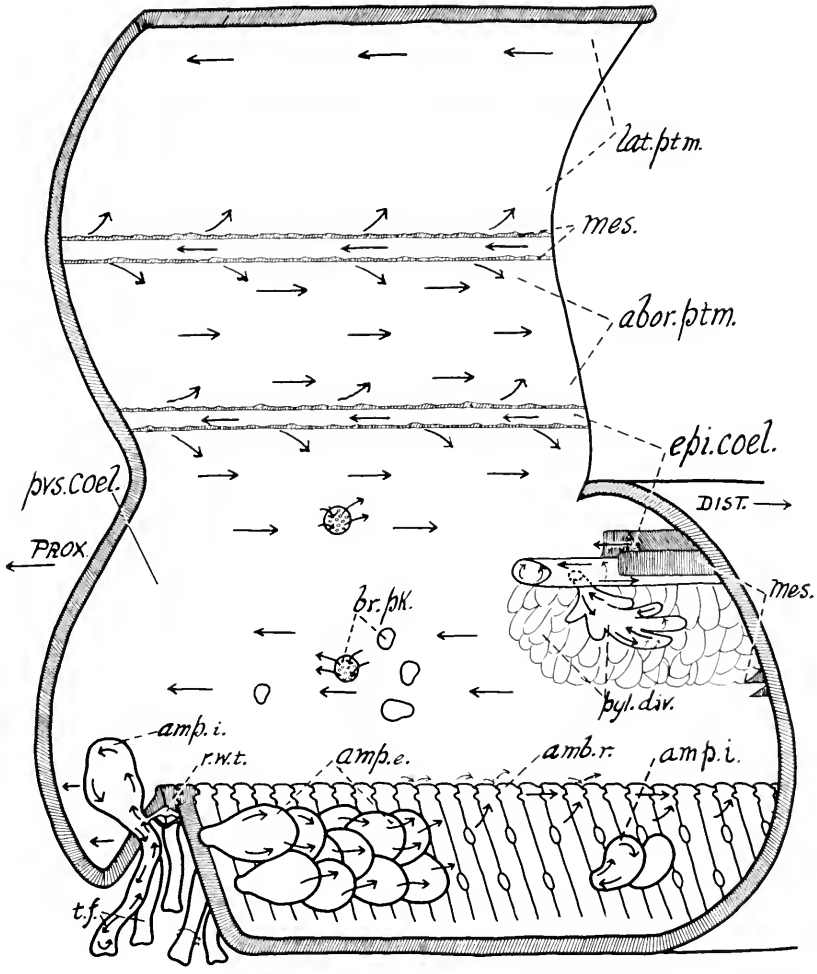

FIGURE 2: Stereogram of structures in opened ray of starfish, with direction of ciliary flows on same:

abor. ptm.	aboral peritoneum
amb. r.	ambulacral ridge
amp. e.	ampulla (exterior)
amp. i.	ampulla (interior)
br. pk.	branchial pocket
DIST.	distal direction
epi. coel.	epigastric coelom
lat. ptm.	lateral peritoneum
mes.	mesentery
PROX.	proximal direction
pvs. coel.	perivisceral coelom
pyl. div.	pyloric diverticulum
r.w.t.	radial water-tube
t.f.	tube-foot.

lateral (inter-radial) peritoneum. A certain degree of movement of coelomic contents is effected by the slow bending of rays but such would seem to be quite incidental. Certain details as to perivisceral coelomic ciliation are:

1. On the aboral body-wall the direction is distad, save in the narrow epigastric spaces between the mesenteries supporting the pyloric diverticula, where it is centripetal; there is some laterad flow from the inter-radial member, and mediad flow from medial member of these mesenterial pairs (Figure 2, and Plate II, Figure 1).

2. The cilia-propelled flow on the pyloric diverticula ('hepatic lobes') is the same on both oral and aboral surfaces, viz. distally along the median axial line of each of the two lobes; also distally along axes of the lateral lobular components and on each of their out-pocketings (Plate II, Figure 2).

3. Cilia on the cardiac stomach retractor muscles beat centrifugally (Plate II, Figure 3).

4. A strong centripetal flow occurs on the epithelial lining of the inter-radial body wall (Plate II, Figure 3; Figure 2).

5. On the ambulacral ridges the flow runs distally, the stream dipping into and out of the inter-ossicular depressions (Figure 2).

6. On the lateral walls of ambulacral ridges the flow is from the ampullary area toward the crest of the ridge (Figure 2; Plate II, Figure 3).

7. The currents on the peritoneal covering of the gonad on the aboral aspect are distad on the main axis and on the subdivisions of the organ as they extend toward the ambulacral ridge (Plate II, Figure 3). On the oral surfaces ciliary propulsion is centripetal (proximad), the reverse of that on the aboral area, thus supplementing that on the inter-radial lining of the ray coelom.

8. The flow on all coelomic surfaces of the stomach (cardiac and pyloric) is aboralward from the perioral region; that on the perioral membrane is weak and irregular.

9. Several branchiae arise externally from each internal branchial pocket; the flow into and out of these pockets conforms, as to direction, with that on the area where each is located (Figure 2).

10. Each branchia has a densely ciliated lining, the flow on all sides being toward the distal end; the stream out of a branchia is down its axial center, forced thus by inflow on all sides.

11. The junction between the rays and central disc, with the inter-radial pillars, ambulacral ridge origins, and Tiedemann's bodies, presents a somewhat complex current pattern, easiest appreciated from a

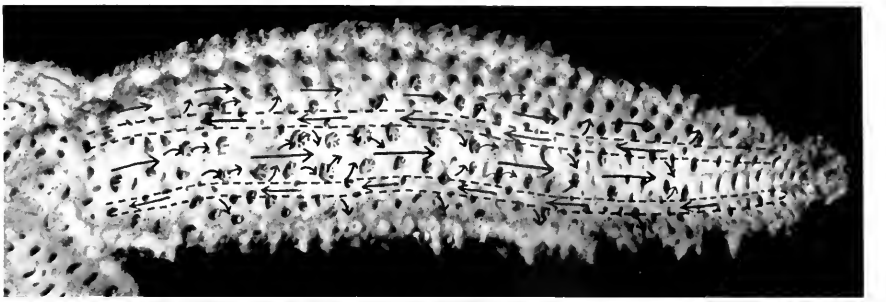

PLATE II, FIGURE 1: Currents on peritoneal lining of aboral body-wall of one ray. Dotted lines indicate attachments of mesenteries supporting pyloric diverticula. Currents dip into and out of branchial pockets.

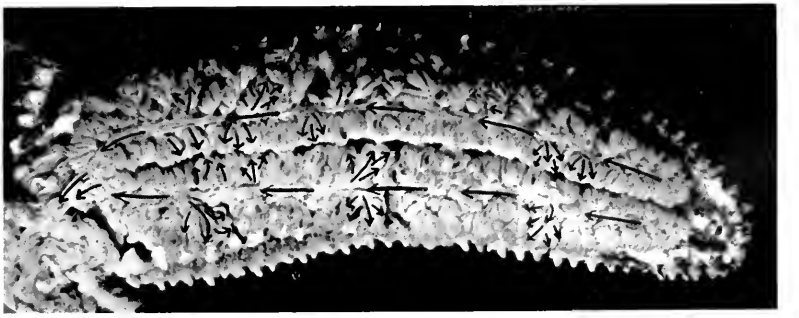

PLATE II, FIGURE 2: Aboral surface of pyloric diverticula in one ray. Currents are distad on all diverticular components.

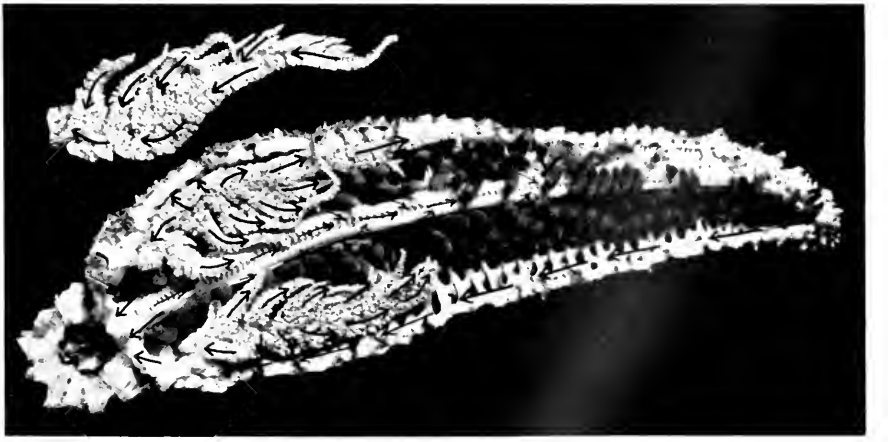

PLATE II, FIGURE 3: Ciliary currents on surface of structures in single ray with diverticula removed. The smaller figure shows the oral aspect of a gonad.

diagram (Figure 3). Direction flow on each side of an ambulacral ridge, i.e. between it and the ambulacral (inter-radial) pillars, is centripetal. On each Tiedemann's body the aboral ciliation beats toward the inter-radius to which it is adjacent. As this flow passes

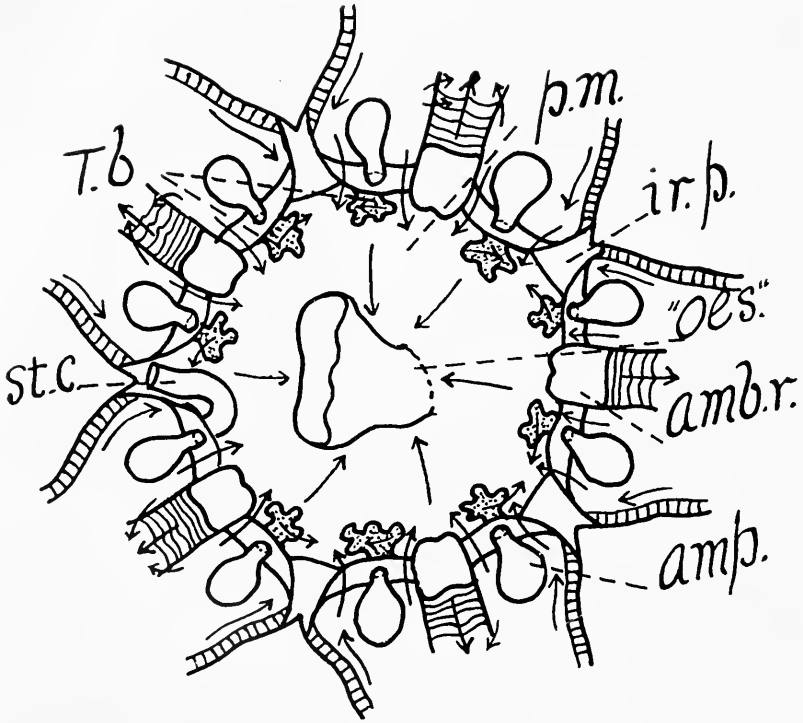

FIGURE 3: Diagram of structures located at margin of perioral membrane, and bases of rays, internally; direction of ciliary currents on same indicated.

amb. r.	ambulacral ridge
amp.	ampulla
ir. p.	inter-radial pillar
"oes."	oesophagus
p. m.	perioral membrane
st. c.	stone canal
T. b.	Tiedemann's bodies.

equatorially around the body, its direction is reversed on the oral aspect.

12. Currents on the peritoneal fold surrounding the stone canal and axial organ run across the long axis of these organs, in counter-clockwise direction when viewed from what might be termed the gastric side of these structures; however, at the junction of this fold with the body wall there may be currents running oralwards.

B. The peritoneum of the epigastric coelom.

1. The central area of this space, as exposed by removal of the pyloric stomach, shows a rather unexpected ciliary behavior. Viewed from its interior and looking aboralward, currents run in counter-clockwise direction, the flow on the rectal caecum supplementing the rest, i.e. going in the same direction. The flow into and out of branchial pockets conforms to this directional pattern also. There are minor deviations on small areas but the composite effect is as mentioned.

The writer is at a loss to account for this exception to what is otherwise, for the most part, quite a symmetrically ordered anatomy. However, one may appeal to the fact that the epigastric coelom, though centrally located in adult starfish, arises embryologically from the right posterior enterocoel, and explain this circular movement as a one-way ciliary beat modified when incorporated into a middle, polar position. No such one-way retention of current direction is found in the adult disposition of tracts on the left posterior enterocoelar (major perivisceral) peritoneum, however.

2. Ciliary movements on the oral face of the rectal caecum are noted above; those on the aboral surface are mixed, but with a tendency to flow distally on each lobe.

3. In the extensions of the epigastric coelom between the two mesenteries suspending each main lobe of the pyloric diverticula the contained fluid is swept strongly centripetally (Figure 2; Plate II, Figure 1).

C. Lining epithelium of the water-vascular system.

1. Stone canal: All epithelial surfaces here show a strong inflow current, if any. As is well known, a prominent infold extends most of the length of the canal along one side, its free edge giving rise to symmetrically arranged, coiled extensions which in cross-section present a double volute pattern. No positive movement of suspended particles in contact with the convex faces of this bifid structure appears, but their concave surfaces (which are continuous with the rest of the canal peritoneum) present a strong inwardly-going (oralward) current.

The fact that the stone-canal and sieve-plate are the main structural features between the ambulacral system and the exterior has always presented a temptation to assume that both inflow and outflow of water is effected by a mechanism in the stone-canal and its associated structures. However, it has long and repeatedly been recognized (Ludwig, 1890; Cuenot, 1891; McBride, 1896; Gemmill 1914) that the ciliated surfaces present only inward (oralward) currents. It seems highly probable, therefore, that outflow from the water-vascular spaces is

brought about by spasmodic contractions of the muscular walls of the tube-feet and associated ampullae.

2. The ampullae and tube-feet: All epithelia lining these structures are strongly ciliated for carrying water into and out of their cavities. By reference to Figure 2, the facts are at once appreciated: from the radial canal the current runs *into* the ampullae on the aboral side; *outward* on the oral area; *into* the tube-feet on the side toward the ambulacral groove margins; *out of* the tube-feet on the side toward the ambulacral midline.

D. Endodermally lined spaces.

1. From oral to anal ends of the alimentary system the epithelial cilia beat aboralward, as would be expected.

2. The ducts from the pyloric diverticula show a two-way flow, i.e. centripetal on the aboral area, centrifugal on oral surfaces (Figure 2). This is further true of ducts in the subordinate spaces of the organ. This parallels Gemmill's (1915) observations on several asterid forms and what Irving (1924) noted in our west coast Patiria. The diverticula are the main places of food absorption as well as of enzyme secretion, as long ago noted by Cuenot (1901) and Cohnheim (1901), and more recently proved by Jordan (1913), van der Heyde (1923) and Irving (1924); and this equipment of cilia seems perfectly adapted for bringing food from the stomach to the place of its subsequent digestion and utilization. Minor hoop-like tracts connect the in-coming (oral) currents with the out-going (aboral) in every diverticular lumen (Figure 2).

3. The endothelium of other alimentary diverticula, in particular the rectal caecum, is so given to mucus secretion that no distinct ciliary movement is detectable in it. Filling and emptying is rhythmical (Budington, 1936), the former accomplished by (a) relaxation of its own walls, and (b) the pressure of fluids in the pyloric stomach, thence through the intestine and into the caecum. Emptying of the rectal caecum is due to muscular contraction of its own walls.

Discussion

As already mentioned, thorough observation of asterid ciliation on several types was made by Gemmill (1915). He worked on *Asterias rubens*, *Astropecten irregularis*, *Porania pulvillus* and *Solaster papposus*. It has been my privilege to examine these same forms, also *Luidia sarsi*, *Asterina gibbosa*, and *Henricia sanguinolenta*. As would be anticipated, there is extensive agreement as to fundamental ciliary flow pattern in particular species belonging to the same genus; but to illustrate what seem species differences in the genus Asterias, the following table of

contrasts between *A. rubens* (following Gemmill's account) and *A. forbesi* is offered. Except for the items mentioned, they agree closely though not absolutely.

Area (external)	*A. rubens*	*A. forbesi*
Inter-radial aboral surfaces of rays	Confused	Strongly inter-radial
Aboral aspect of disc	Confused	Strongly centrifugal
Perioral membrane	Centrifugal	Centripetal
Madreporite	Toward center	Generally away from center, with tendency toward aboral flow all over; much variation
Gills and spines	Spirally to free ends	Same result but lack spiral feature
Tube-feet	Toward free end spirally	Same result but lack spiral feature
Pedicellaria	To free ends irregularly	Same but with bilateral flow into bite area
Area (internal)		
Lateral ambulacral ridge	Inward or outward, to or from midline	Toward midline only
Gonads	From attached to free extremities	Same on aboral surfaces; reverse on oral surfaces

In the writer's opinion, however, to use ciliary tracts as serious consideration in species identification would be an unwarranted procedure, for, as Kellogg (1915) pointed out in extensive studies of ciliation in lamellibranchs, variations of considerable degree occur commonly within a given species. As a criterion in generic determination, nevertheless, the major ciliary tracts would be as significant for comparison as would any other structural feature.

In its major aspects, then, the transport-system in asterids, while unlike that of metazoa at its own or higher levels, is definitely organized in an essentially constant pattern. No single central propelling organ is present. Instead, a myriad of cilia move environing fluids over ecto-, meso-, and endodermic epithelia in a manner which is comparable, physiologically, to that accomplished by arteries, capillaries, and veins in a majority of higher metazoa. The tracts herewith described as loci of centrifugal currents may easily be compared to arteries: likewise, centripetal flow-tracts may be thought of as analagous to veins.

Functionally the metabolic story in higher animals involves anatomical centralization of external respiration in gills or lungs, of excretion in tubular organs occurring singly or in masses (kidneys), of nutritive absorption in a centrally located food tube. In echinoderms the tissues accomplishing respiration and excretion are markedly non-

centralized; in large measure the same is true of the tissues serving food absorption, for the pyloric caeca, where food dialyzes into the coelom, may well be rated as diffuse also. In consequence, the transfer of anabolic and katabolic substances in asterids is not so much more complicated than in coelenterates and platyhelminths as one might assume. Diffusion through intercellular spaces, abundant in parenchymatous tissues, is adequate for the lower phyla mentioned; with greater specialization of anatomical features and the development of coelomic cavities, heavy ciliation externally and internally in asterid echinoderms is an efficient substitute for any more elaborate system for transport. Its fixed organization into a genetically repeated pattern of tracts makes it as definite as is the tubular vascular system, and other structural constants, in many other invertebrate and all chordate animals.

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SERIAL LIST

A SERIAL list of the holdings of The Marine Biological Laboratory Library will be published as a separately bound supplement to the February issue of The Biological Bulletin. This supplement, covering approximately 80 pages, will list with cross references the 2258 titles of journals in the Library. Titles are listed alphabetically to conform to the arrangement of the stacks in the Library, and hence should serve as a guide book to the Library itself, as well as an aid in securing microfilm copies of articles. Each subscriber will receive one copy of the list, and extra copies may be obtained at cost from The Marine Biological Laboratory provided orders are received before February 1.

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Beginning with the February issue, THE BIOLOGICAL BULLETIN will be printed in a new format. By greatly increasing the number of words set per page, there will be a considerable saving of material and labor during the present period of emergency.

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