THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

VOLUME LXXXI
AUGUST TO DECEMBER, 1941

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FIFTY-THIRD YEAR

I. TRUSTEES AND [illegible]

ERRATA

The editor wishes to call attention to the following errata in the June, 1941 issue of this journal:

Page 445, line 1: "591 mμ" should read "491 mμ."
Page 453, line 40: "testes" should read "tests" (= skeleton + skin).

I. TRUSTEES

EX OFFICIO

FRANK R. LILLIE, *President of the Corporation,* The University of Chicago.
CHARLES PACKARD, *Director,* Columbia University.
LAWRASON RIGGS, JR., *Treasurer,* 120 Broadway, New York City.
PHILIP H. ARMSTRONG, *Clerk of the Corporation,* Syracuse University Medical College.

EMERITUS

H. C. BUMPUS, Brown University.
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Ross G. Harrison, Yale University.
H. S. Jennings, University of California.
C. E. McClung, University of Pennsylvania.
T. H. Morgan, California Institute of Technology.
G. H. Parker, Harvard University.
W. B. Scott, Princeton University.

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R. Chambers, Washington Square College, New York University.
W. E. Garrey, Vanderbilt University Medical School.
S. O. Mast, Johns Hopkins University.
A. P. Mathews, University of Cincinnati.
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H. H. Plough, Amherst College.
W. R. Taylor, University of Michigan.

TO SERVE UNTIL 1943

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B. M. Duggar, University of Wisconsin.
L. V. Heilbrunn, University of Pennsylvania.
Laurence Irving, Swarthmore College.
J. H. Northrop, Rockefeller Institute.
W. J. V. Osterhout, Rockefeller Institute.
A. H. Sturtevant, California Institute of Technology.
Lorande L. Woodruff, Yale University.

TO SERVE UNTIL 1942

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E. R. Clark, University of Pennsylvania.
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E. N. Harvey, Princeton University.
M. H. Jacobs, University of Pennsylvania.
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Franz Schrader, Columbia University.
B. H. Willier, Johns Hopkins University.

TO SERVE UNTIL 1941

W. R. Amberson, University of Maryland School of Medicine.
W. C. Curtis, University of Missouri.
H. B. Goodrich, Wesleyan University.
I. F. Lewis, University of Virginia.
R. S. Lillie, The University of Chicago.
A. C. Redfield, Harvard University.
C. C. Speidel, University of Virginia.
D. H. Tennent, Bryn Mawr College.

EXECUTIVE COMMITTEE OF THE BOARD OF TRUSTEES

Frank R. Lillie, *Ex. Off. Chairman.*
Charles Packard, *Ex. Off.*
Lawrason Riggs, Jr., *Ex. Off.*

L. V. Heilbrunn, to serve until 1941.
A. C. Redfield, to serve until 1941.
P. B. Armstrong, to serve until 1942.
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The Library Committee

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C. C. Speidel.
A. H. Sturtevant.
William R. Taylor.

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The Supply Department Committee

Laurence Irving, *Chairman.*
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A. C. Redfield.
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The Evening Lecture Committee

B. H. Willier, *Chairman.*
M. H. Jacobs.
Charles Packard.

II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

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

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and suc-

cessors, 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 is 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 1940.

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 1940, 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 $807,499.95)	$856,629.50
	Interest in Real Estate	24,921.89
	Cash	30,648.97
		$912,200.36
Library Fund,	Securities (market $150,077.06)	$164,294.79
	Real Estate	20,000.00
	Cash	10,862.62
		$195,157.41

The income collected from these funds during the year was:

General Endowment	$35,674.49
Library	8,463.30
	$44,137.79

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

Arrears General Fund	$12,253.69
Arrears Library Funds	4,325.00
	$16,578.69
Arrears at the end of 1939	$15,322.81
a falling behind of	$ 1,255.88

General Biological Supply House, Inc.: The dividends from the General Biological Supply House, Inc. totalled $18,542.00, an increase of $3,556 over 1939.

Bar Neck Property: The rental from the Bar Neck property which is based on the net profit of the garage was $5,097.64, an improvement of about $1,500 over the prior year, during which the absence of the drawbridge adversely affected the business of the garage.

In addition, the notes given for the acquisition of the Bar Neck property are now paid off so that the entire income from this property can now be used for current expenses of the Laboratory.

Retirement Fund: A total of $3,710 was paid in pensions and $923.20 advanced from current funds in prior years was repaid. This fund at the end of the year consisted of:

Participations in mortgages	$ 8,154.39
Interest in Real estate	2,301.88
Cash	5,048.06
	$15,504.33

Plant Assets: The land (exclusive of Gansett and Devil's Lane Tracts), the buildings, equipment and library represent an investment of $1,867,005.60
less reserve for depreciation 564,225.03
or a net of $1,302,780.57

Income and Expenses: Income exceeded expenses (including depreciation of $25,648.22) by $8,035.14.

There was expended from current funds for plant account a net of $16,702.27 and in payment of note indebtedness $3,500, and $2,500 was transferred to the Reserve Fund.

The total damage caused by the hurricane on September 21, 1938, finally liquidated during 1940 was $30,152.47
of which 20,000.00
was met by the grant of the Carnegie Corporation (1939) and the balance of $10,152.47 was paid from current funds or charged off.

At the end of the year the Laboratory had no indebtedness on notes or mortgages. It owed on accounts payable $3,689.51, against which it had accounts receivable of $11,667.91 and cash in its general bank accounts of $13,359.26.

The Rockefeller Foundation made a grant of $110,400 for an addition to the library. During 1940, there was received $64,776.62 on this grant, of which $39,851.12 was expended in the year.

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, 1940

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		$1,107,357.77	
Securities and Cash—Minor Funds—Schedule II..		9,194.41	$1,116,552.18
Plant Assets:			
Land—Schedule IV	$ 111,425.38		
Buildings—Schedule IV	1,277,685.06		
Equipment—Schedule IV	179,181.15		
Library—Schedule IV	298,714.01		
	$1,867,005.60		
Less Reserve for Depreciation	564,225.03	1,302,780.57	
Cash in Building Fund		24,925.50	
Cash in Reserve Fund		2,524.65	1,330,230.72
Current Assets:			
Cash		$ 13,359.26	
Accounts-Receivable		11,667.91	
Inventories:			
Supply Department	$ 38,976.75		
Biological Bulletin	11,069.82	50,046.57	

Investments:			
Devil's Lane Property	$ 45,099.78		
Gansett Property	6,030.81		
Stock in General Biological Supply House, Inc.	12,700.00		
Other Investment Stocks	17,770.00		
Securities, Real Estate, and Cash —Retirement Fund—Schedule V	15,504.33	97,104.92	
Prepaid Insurance		3,445.81	
Items in Suspense		172.40	$ 175,796.87
Liabilities			
Endowment Funds:			
Endowment Funds—Schedule III	$1,105,900.37		
Reserve for Amortization of Bond Premiums	1,457.40	$1,107,357.77	
Minor Funds—Schedule III		9,194.41	$1,116,552.18
Plant Liabilities and Surplus:			
Donations and Gifts—Schedule III		$1,104,666.73	
Other Investments in Plant from Gifts and Current Funds		225,563.99	$1,330,230.72
Current Liabilities and Surplus:			
Accounts—Payable		$ 3,689.51	
Current Surplus—Exhibit C		172,107.36	$ 175,796.87

EXHIBIT B

MARINE BIOLOGICAL LABORATORY INCOME AND EXPENSE, YEAR ENDED DECEMBER 31, 1940

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 35,674.49		$ 35,674.49
Library Fund		8,463.30		8,463.30
Instruction	$ 10,283.63	9,670.00	$ 613.63	
Research	6,086.18	15,068.00		8,981.82
Evening Lectures	32.90		32.90	
Biological Bulletin and Membership Dues	8,834.37	9,725.93		891.56
Supply Department—Schedule VI	27,345.79	32,376.45		5,030.66
Mess—Schedule VII	25,081.97	24,227.77	854.20	
Dormitories—Schedule VIII	23,658.88	13,060.53	10,598.35	
(Interest and Depreciation charged to above 3 Departments—See Schedules VI, VII, and VIII)	24,040.85			24,040.85

Dividends, General Biological Supply House, Inc.		18,542.00		18,542.00
Dividends, Crane Company		400.00		400.00
Rents:				
Bar Neck Property		5,097.64		5,097.64
Howes Property	117.80	160.00		42.20
Janitor House	24.07	360.00		335.93
Danchakoff Cottages	307.77	715.00		407.23
Sale of Library Duplicates		80.26		80.26
Apparatus Rental		1,226.53		1,226.53
Sundry Income		57.50		57.50
Maintenance of Plant:				
Buildings and Grounds	25,121.47		25,121.47	
Chemical and Special Apparatus Expense	15,833.22		15,833.22	
Library Expense	7,675.89		7,675.89	
Workmen's Compensation Insurance	538.14		538.14	
Truck Expense	466.96		466.96	
Bay Shore Property	77.40		77.40	
Great Cedar Swamp	19.20		19.20	
General Expenses:				
Administration Expense	12,426.64		12,426.64	
Endowment Fund Trustee and Safe-keeping	1,014.45		1,014.45	
Interest on Notes—Payable	87.50		87.50	
Bad Debts	228.66		228.66	
Reserve for Depreciation	25,648.22		25,648.22	
	$166,870.26	$174,905.40	$101,236.83	$109,271.97
Excess of Income over Expense carried to Current Surplus—Exhibit C	8,035.14		8,035.14	
	$174,905.40		$109,271.97	

EXHIBIT C

Marine Biological Laboratory, Current Surplus Account, Year Ended December 31, 1940

Balance, January 1, 1940		$163,206.29
Add:		
Excess of Income Over Expense for Year as shown in Exhibit B	$ 8,035.14	
Reserve for Depreciation Charged to Plant Funds	25,648.22	
Transfer from Reserve for Repairs and Repacements on account of Hurricane Water-Damage	1,072.68	$ 34,756.04
		$197,962.33

Deduct:			
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,			
Buildings	$ 1,426.71		
Equipment	5,465.65		
Library	10,104.08	$16,996.44	
Less Received for Plant Assets Disposed of	$ 162.43		
Loss on Equipment Charged Off Due to Hurricane Water-Damage	131.74	294.17	
		$16,702.27	
Payment of Plant Note—Payable		$ 3,500.00	
Transfer to Plant Reserve Fund		2,500.00	
Pensions Paid	$ 3,710.00		
Less Retirement Fund Income	557.30	3,152.70	$ 25,854.97
Balance, December 31, 1940—Exhibit A			$172,107.36

Respectfully submitted,

LAWRASON RIGGS, JR.,
Treasurer.

V. REPORT OF THE LIBRARIAN

The $18,850.00 appropriated for the Library by the Marine Biological Laboratory for 1940 was expended before the end of the year for books, $766.28; serials, $2863.57; binding, $957.55; express, $73.97; supplies, $268.85; salaries, $7200.00; back sets, $2653.35; sundries, $11.48; and a reserve fund of $3977.18 was retained to pay for current serials and back sets, ordered, but not yet received on account of the European war conditions. A balance of $77.77 reverted to the Laboratory plus $80.26 from the sale of duplicates by the Library. An examination of our record over the past decade shows a total of $5970.67 that has so reverted, $4419.49 from the ten yearly budgets assigned by the Executive Committee to Library expenditures and $1551.18 from the Library's sale of duplicates. This is an average reversion of $597.00 for each of the ten years. The explanation to account for a variation from year to year from overspending to underspending is too various to give here, but in general, it is due to caution taken to buy only under advantageous conditions. By calling attention to the sum that has reverted to the Laboratory during this ten-year period, it is the hope of the Librarian that on the occurrence of a very favorable opportunity for buying, the Laboratory will meet this opportunity by placing the necessary sum at the dis-

posal of the Library. It would, in fact, be a fine idea to begin a reserve fund for this purpose.

The Woods Hole Oceanographic Institution appropriation, which is outright and is carried over from one year to the next, was, for 1940, $600.00, plus $82.42 remaining from 1939. Of this $555.11 was expended and reported to the Director. A delayed set ordered from England in August at 50 pounds has arrived in March 1941, before this report goes to press.

In connection with plans made for extending the Library volumes into the addition (the moving will be completed before the end of April, 1941), a count of periodical sets gave the interesting figure of a total of 2366 titles. (These will require the shelving space of the entire top floors, now the fourth and third, and the new section of the reading-room floor, now the second floor. Books will remain in the old part of the second floor and the reprints will extend throughout the first floor.) Of these 2366 titles, only 1257 are received currently; 422 are subscriptions, 403 (15 new) of the Marine Biological Laboratory and 39 (0 new) of the Woods Hole Oceanographic Institution; 609 are exchanges, 544 (6 new) with the "Biological Bulletin" and 65 (1 new) with the Woods Hole Oceanographic Institution publications; 195 are gifts to the former institution and 11 to the latter. An unusual number of books, 142, was bought by the Marine Biological Laboratory Library as selected by the investigators from a list accumulated by the Librarian throughout the previous five years, while the Woods Hole Oceanographic Institution purchased 12; authors presented 8 books to the Marine Biological Laboratory and 2 were received on exchange; publishers gave 43, and the Woods Hole Oceanographic Institution received 2 books from publishers. The record for the filling in of back sets shows 22 completed; purchased by the Marine Biological Laboratory, 15, and by the Woods Hole Oceanographic Institution, 2, by exchange with the "Biological Bulletin," 3, by exchange of duplicates 1, and by gift, 1; partially completed back sets total 49; by purchase, for the Marine Biological Laboratory, 18, for the Woods Hole Oceanographic Institution, 2, by exchange with the "Biological Bulletin," 3, by gift to the former institution, 2, to the latter, 1, and by exchange of duplicates, 23. Reprint additions number 3528; 1614 current of 1939, 637 of 1940, and 1277 of previous dates. The present holdings of the Library number 47,697 bound volumes and 116,305 reprints.

VI. THE REPORT OF THE MANAGING EDITOR OF THE BIOLOGICAL BULLETIN

The *Biological Bulletin* is the property of the members of the Corporation of the Marine Biological Laboratory. It is the one tangible return which they receive in exchange for their membership fees, whether they return each year to the laboratory or not. It seems proper that you should receive, at least from time to time, some report on its progress, its policies and problems.

The annual reports of the Laboratory contain only occasional references to the *Bulletin,* recording appointments to the editorial board and such matters of fact. Fortunately there is preserved in the Tenth Report, for the years 1903–1906, a prospectus issued in 1902 announcing the resumption of publication of the *Bulletin,* which had been interrupted for a year. With the completion of the eightieth volume, after thirty-nine years of continuous publication, it is not unfitting to reexamine this prospectus and see to what extent its promise has been realized.

The *Biological Bulletin* was preceded by the *Zoological Bulletin,* of which two volumes were published in 1897 and 1898 under the editorship of Professor C. O. Whitman, then Director of the Laboratory, and Professor W. M. Wheeler. It was intended to be a companion journal to the *Journal of Morphology* and to take in shorter papers with simple illustrations, where relatively rapid publication of original contributions was desirable. Following the completion of two volumes, it was succeeded in 1899 by Volume I of the *Biological Bulletin.* The title page of the new *Biological Bulletin* shows it to have been edited by the Director and members of the staff of the Marine Biological Laboratory. Volume II was completed in June, 1901.

After the lapse of a year, Volume III appeared, bearing the title: *Biological Bulletin of the Marine Biological Laboratory, Woods Hole, Mass.,* thus clearly establishing the relation of the publication to the Laboratory. The treasurer's report for 1902 shows also that for the first time the relationship was a material one. The editorial staff consisted of E. G. Conklin, The University of Pennsylvania; Jacques Loeb, The University of Chicago; T. H. Morgan, Bryn Mawr; W. M. Wheeler, The University of Texas; C. O. Whitman, The University of Chicago; E. B. Wilson, Columbia University; and Frank R. Lillie appeared for the first time as Managing Editor. Dr. Lillie continued to edit the *Bulletin* for 25 years, when in 1927 he was succeeded by Dr. Carl R. Moore. With two other members of the original staff he still serves as a member of the editorial board.

The prospectus announcing the resumption of publication stated that:

"The Bulletin will be published as heretofore, under the auspices of the Marine Biological Laboratory, and its scope will include Zoology, General Biology, and Physiology. It will contain original articles in these fields and also occasional reviews, and reports of work and lectures at the Marine Biological Laboratory. Preliminary statements of important results will be made a special feature. The Bulletin will be open, as heretofore, to contributions from any source."

I think it is fair to say that the scope of the *Bulletin* still includes Zoölogy, General Biology, and Physiology and that the character of its contents remains essentially unchanged except as it reflects changing emphasis and new developments within these fields. The question of scope is, nevertheless, one of the most perplexing problems of policy with which the managing editor must deal. The objects of the Laboratory and the curiosity of its clientele are broad, and it seems proper that the contents of the *Bulletin* should reflect this catholic interest. It is not undesirable in this day of specialization that one journal at least should present a rather broad cross-section of biology as a whole. On the other hand, practical considerations require some limitation, even within the fields specified in the prospectus, and there is no easier way for an editor to dispose of an unwelcome manuscript than to rule it inappropriate. There is also an evil temptation which besets every editor to allow the selection of papers to reflect too closely his own particular interests and prejudices. I think that in the selection of papers, the guiding principle should be to produce a journal which will interest as widely as possible those who support it with their subscriptions. These are primarily the members of this Corporation, and secondarily, the biological libraries throughout the world. The character of both of these groups argues for as great a diversity as possible. Worthy papers differ greatly in the breadth of their interest. Many papers contain material of value to specialists which are of little interest to other biologists. Where specialists maintain their own journals, as is true, for example, in mammalian physiology, or genetics, it seems preferable that such papers should be published in these places. For many years it was the policy of the *Bulletin* not to publish taxonomic papers since taxonomy was not a part of the program of the Marine Biological Laboratory. However, I feel that today those who are still following the older disciplines deserve some encouragement and that the policy of the *Bulletin* should be more lenient, at least in connection with papers dealing with the local fauna and with groups of organisms which are not covered by specialized journals.

It is significant that the founders of the *Biological Bulletin* did not call it the *Marine Biological Bulletin,* though it was published under the auspices of a marine laboratory. It is evident that they came to Woods Hole not so much to study the sea, but because they found there good material for the study of more general problems, and that they intended that the *Bulletin* should represent all aspects of biology. In this characteristic there has been no change. It is, however, natural that the journal should deal to a large extent with the biology of marine organisms since these are the creatures on which many of your studies are made, and it seems reasonable to assume that you will be interested in papers of all sorts dealing with these organisms and with the waters in which they live. Thus the *Bulletin* should retain a distinctly salty flavor and should welcome especially work done at other marine stations.

The prospectus states that the *Bulletin* will be open to contributions "from any source." An examination of 778 papers published between 1930–1940 shows that 29 per cent originated from this Laboratory; 22 per cent were contributions from other marine laboratories, including 9 per cent derived from the Woods Hole Oceanographic Institution These included contributions from: the Mt. Desert Island Biological Laboratory, the Scripps Institution of Oceanography, the Duke University Marine Laboratory, the William Kerckhoff Marine Biological Laboratories of the California Institute of Technology, the Hopkins Marine Station, the U. S. Bureau of Fisheries, the Atlantic Biological Station at St. Andrews, the Chesapeake Biological Laboratory, the University of Washington Oceanographic Laboratories, and the New York Aquarium; the Naples Zoölogical Station, the Marine Biological Laboratory at Plymouth, the Bermuda Biological Station, the Bergens Museum Biologiske Stasjon, the Pacific Biological Station at Nanimo, and the Misaki Marine Biological Station at Kanagawa-ken. The remaining 49 per cent came from various university laboratories and may in part have been based on work done here or at other marine stations. This distribution conforms well to the principles discussed above.

While the bulk of the material in the *Bulletin* consists of original articles, some attempt was made a few years ago to print the lectures given at the Laboratory. Because of the difficulty in securing the manuscripts and also because of the general pressure for space, this practice was discontinued. The publication of reviews has not been made a feature of the *Bulletin* and there is probably no reason why it should in view of the special journals now devoted to this purpose. An occasional paper bringing together material of particular local interest would be of value to workers in the Laboratory. I have in mind as an example Dr. Harvey's paper on "Physical and chemical constants of the egg of

Arbacia punctulata" (*Biological Bulletin,* April, 1932). The promise that preliminary statements of important results would be made a special feature of the *Bulletin* is now fulfilled by the publication of abstracts of the papers delivered at the scientific meetings during the summer.

The prospectus continues:

"There is in America no journal that takes the place of the *Biologisches Centralblatt* or the *Anatomischer Anzeiger* in Germany, although there is abundance of material to support such a publication. It is hoped that the Bulletin may occupy this field, and meet the need for rapid publication of results; the editors, therefore, undertake to issue one number each month, making two volumes a year, if the material offered is sufficient."

At the time the *Bulletin* was founded, the American journals participating in its field were: the *Journal of Morphology,* the *American Journal of Physiology,* the *Annals of Botany,* the *American Naturalist,* the *Botanical Gazette* and the *Journal of Comparative Neurology.* The establishment shortly thereafter of the *Anatomical Record,* the *American Journal of Anatomy* and the *Journal of Experimental Zoology,* and the subsequent appearance of many others has largely eliminated this special raison d'être. The hope that the *Bulletin* might meet the need for rapid publication of results has not always been fulfilled, for it was recorded in the twenty-seventh report, for the year 1924, that ". . . lately, in common with many other biological research journals, it had been falling behind in promptness of publication so that about a year elapsed between the receipt of manuscripts and their appearance in print." In the hope of correcting this situation the *Bulletin* was enlarged to 900 pages and the subscription price increased from $6.00 to $9.00 per year, and the fees of members of the Corporation were increased proportionately. This change reduced the time required for publication to at most six months. At the present time the *Bulletin* is run on the theory that the only way to ensure prompt publication is to accept for publication no more papers than one can afford to publish at once. A journal operating on a fixed income has no more chance of catching up with an accumulation of papers it cannot afford to publish at once than does a man in like circumstances who has allowed himself to get some months behind in paying his bills. It is better to require a few papers less suited to one's purpose to find a publisher elsewhere than to delay the entire stream of publication chronically. During the past 11 years no paper has remained in the editor's hands more than two months before going to the printer except under special circumstances, as when occasionally delays occur in securing the necessary reports from

referees. In order to facilitate this practice, it is customary not to arrive at a decision concerning the disposition of any paper until the accumulated material can be considered together at the time of going to press.

The *Bulletin* was originally offered at a price of $3.00 per volume of 300 pages. It is interesting to note that this is exactly the cost to the subscriber per page of the present issues. Because of the low price, the length of articles was originally limited to 25 pages and lithographic prints were excluded. The cost of illustrations above $10 was charged to the author. These limitations are no longer exactly exercised though, naturally, longer papers must be discouraged if opportunities for publication are to be widely distributed. The criterion is that no paper should be longer than is necessary to adequately present its contribution and short and long papers alike should be scrutinized from this point of view. This is sometimes an unwelcome task, but on the whole, I have found our contributors uncommonly coöperative and goodnatured. They can usually be made to appreciate that concise presentation is read with understanding. It is no longer the practice to charge authors for a part of the cost of necessary illustration provided they are content with line cuts and halftones and comply with the general principles discussed above.

The prospectus closes:

"The *Bulletin* will undoubtedly meet a real need; but the responsibility for its success rests with American biologists, and the editors, therefore, confidently appeal to them for their support. This can most practically be given in the two forms of subscriptions and contributions to its pages." The need for the *Bulletin* and its success, as well as the support which it has received from American biologists, is amply attested by its contents. The Index which was published at the completion of Volume LX listed approximately 1200 titles. An index which is now being prepared of the last 20 volumes records an additional 660 titles of original articles and 652 titles of abstracts, making a total of some 2500 contributions. An examination of the original articles appearing between 1930–1940 showed that 40 per cent of these were written by members of the Corporation. The list of Corporation members contains very few productive workers in fields appropriate to the *Bulletin* who are not contributing to its pages.

On the financial side, the *Bulletin* is supported in three ways. Of 1100 subscriptions, 300 go to members of the Corporation in return for their membership fees. The remaining subscriptions are divided about equally between paid subscriptions from libraries and exchanges. The treasurer's reports show that with this support the *Bulletin* just about

breaks even or sometimes shows a small profit. This is due in a sense to the accountant's art and requires a word of explanation. The income from exchanges represents a transfer of Library funds to the *Bulletin* in payment for issues used to secure exchanges. Thus, in a sense, the Library helps to subsidize the *Bulletin* and the sum involved does not represent cash income. In return, however, the Library receives 656 items in exchange, or approximately one-half of its current list of periodicals. It is this fortunate association of the *Bulletin* with the Library which enables it to make ends meet. The large number of exchanges greatly widens the distribution of the papers published in the *Bulletin*.

Respectfully submitted,

ALFRED C. REDFIELD,
Managing Editor.

VII. THE REPORT OF THE DIRECTOR

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

Gentlemen: I present herewith a report of the fifty-third session of the Marine Biological Laboratory, for the year 1940.

1. *Present Conditions.* The grievous conditions which are now disturbing the world have thus far affected but little the activities of this Laboratory. In the season just past investigators occupied almost all of the available space, and students filled the courses. The attendance was practically equal to that of 1937, the largest in the history of the institution. Except for an occasional lack of current foreign journals, and of scientific apparatus usually purchased abroad, we continued our work in 1940 without serious difficulty. This fortunate state of affairs cannot be expected to continue. Evidences of the change are already apparent. During the season of 1941 several of our investigators will give up their usual lines of research and will work at home on problems connected with national defense, while others will carry on similar research at Woods Hole. Apparatus, formerly obtained without delay, is now difficult if not impossible to secure. Thus far, the Government has made no request for space and facilities to be devoted to defense measures. We shall continue our usual activities of instruction and research so long as no emergency arises which might temporarily interrupt them.

2. *The Library Addition.* We may congratulate ourselves that in spite of the uncertainties which have surrounded us during the past year, we have completed one part of the extensive building program which was

discussed at the last Trustees' meeting. The generosity of the Rockefeller Foundation made possible the immediate erection of the much-needed extension of our Library. It will be recalled that, under the terms of the gift of $110,400, the Laboratory accepted the responsibility of obtaining from other sources at least $25,000 to be used primarily for filling gaps in the present files of journals. A most liberal interpretation of these terms permitted us to proceed with actual construction before this sum was in hand.

Work was begun in August, 1940 under the direction of the architect, Mr. F. V. Bulfinch. On his advice, the Executive Committee voted to give the contract for the building to the Sawyer Construction Company of Boston, without waiting for bids to be submitted by other companies. The wisdom of this action was at once apparent, for excavation began immediately, even before the architects' plans were fully completed. The structural steel and other essential materials were obtained quickly and without difficulty. Had we waited six weeks or more for competitive bidding to be completed, it is very probable that their delivery would have been greatly delayed, if not indefinitely postponed. The construction company awarded contracts, after competitive bidding, for much of the inside work, such as the plumbing, heating, electrical installations, and the book stacks. The building was enclosed before cold weather set in, and was heated satisfactorily by means of a new oil furnace, a part of the Library project. It may be added that those parts of the Brick Building where heat was required were maintained at a comfortable temperature. The final cost of the building and its equipment was substantially less than the first estimates of the architect and the contractor. With the balance we have been able to rebuild the Eel Pond wall, considerably damaged by the building operations, install an electric book lift in the shaft provided in the old Library but never used, pave the road and parking spaces close to the new wing, and re-grade the lawn.

Those who use the Library will find many improvements over the old conditions. The journals and books are now spread out over three floors only, an arrangement which greatly reduces the amount of stair climbing. In the new wing there are many well-lighted desks, some of which will be reserved for those who spend all of their time among the books, and others which will be for general use. In the basement will be housed, temporarily it is hoped, apparatus for sterilizing glassware, for distilling water and for other services which heretofore have been widely scattered throughout the Brick Building. There are also two dark-rooms.

The space now available will serve the rapidly growing Library for many years. For this happy situation we are deeply indebted to the Rockefeller Foundation which now, as in the past, has so greatly furthered the work of the Laboratory. To the Library Committee and Mrs. Montgomery must be given credit for the planning of the book stacks, the amply spaced study tables, and other features which all readers will appreciate. Finally, thanks are due to Mr. Bulfinch and Mr. Sawyer, who have so successfully designed and constructed the building of which we may well be proud.

We take even greater satisfaction in its contents. The Library is an indispensable part of our equipment. Although the number of volumes is not large, the selection of books and serials has been made with such skill that we now have on our shelves practically all of the important journals, and we are adding to the number every year. In the Annual Report of 1889, a committee consisting of E. S. Minot, W. T. Sedgwick, and C. O. Whitman, made a double appeal ". . . first, to the public, for $1,500 to complete the more important sets of journals, and secondly, to all biologists, for reprints of their articles. We do this with confidence, because we believe that nowhere in this country would such a library be more useful or valuable than in the Marine Biological Laboratory." At that time the library consisted of 343 volumes, 23 reprints, and 23 serials, of which many were not current subscriptions. From this modest beginning it has grown in importance until it is now one of the most important biological libraries in the world. The increase in the number of volumes, serials, and reprints during the past twenty years is shown in the following figures, taken from the Librarian's reports.

	Volumes	Serials	Separates
1920	10,243	153	8,532
1925	15,000	500	25,000
1930	31,510	1,060	66,231
1935	40,180	1,271	91,641
1940	47,897	1,257	116,305

3. *Committee on Instruction.* The primary purpose of this Laboratory is to encourage research by qualified investigators and to increase their number by preparing students to undertake original work. To determine whether the courses of instruction are fulfilling this purpose, the Committee on Instruction, under the able leadership of Dr. Allee, has made a careful study of the situation. They were guided in their deliberations by the following principles adopted by the Executive Committee.

1. That instructorships are to be regarded as aids to research.
2. That the duty of the instructors consists of research and teaching, and that they consequently are to remain in residence for a period longer than the duration of the course.
3. That instructorships should be distributed widely among American institutions of learning.
4. That long tenure of instructorships should be discouraged.
5. That it is better to have instructors who are specialists in the courses in which they teach.
6. That the Director be authorized to appoint a Standing Committee on Instruction to report to the Executive Committee each year.

The Committee met weekly during the summer, discussing these problems with the directors of the various courses and with many other interested investigators. Their general conclusion was that the work of instruction is on a fairly satisfactory basis. The instructors are actively engaged in productive research, many of them in the general subjects which they are called upon to teach. Not all, however, are in residence at Woods Hole for a substantial part of the summer. They are drawn from 23 colleges and universities, of which 12 are eastern, 5 are in the midwest, 4 in the south, and one each from the far west and Canada. The tenure of instructorships averages about 5 years; that of the directors, excluding their previous service as instructors, about 9 years. The Committee felt that a more frequent change of directors would be desirable.

The Committee also recommended that in place of Dr. Irving, who resigned after five successful years as head of the Physiology Course, Dr. A. K. Parpart be appointed. And furthermore, that Dr. Calkins' desire to be relieved of the course in Protozoölogy be granted, and that this course be discontinued. It was with regret that this action was taken. Under Dr. Calkins the Protozoölogy Course has for many years occupied an outstanding place in our summer work of instruction. A large proportion of his students, drawn from all parts of the world, have made significant contributions to biology, a lasting tribute to the training and inspiration which they received from him. The Committee were convinced that no one could replace him, and that since there are now many excellent courses in Protozoölogy offered in various parts of the country, the need for continuing such a course here is not imperative.

I wish to express the thanks of the Trustees and Corporation to the members of this Committee, and to the other Standing Committees who have during the year coped successfully with many difficult problems.

4. *Election of Officers and Trustees.* At the meeting of the Corporation held August 13, 1940 the following Trustees were elected Trustees Emeritus:

Caswell Grave, Washington University
Ross G. Harrison, Yale University
C. E. McClung, University of Pennsylvania

The new Trustees elected at that meeting were:

Dugald E. S. Brown, Class of 1942
C. W. Metz, Class of 1944
H. H. Plough, Class of 1944

5. There are appended as parts of this report:

1. Memorials of deceased Trustees.
2. The Staff, 1940.
3. Investigators and Students, 1940.
4. A Tabular View of Attendance, 1936–1940.
5. Subscribing and Coöperating Institutions, 1940.
6. Evening Lectures, 1940.
7. Shorter Scientific Papers, 1940.
8. The General Scientific Meeting, 1940.
9. Members of the Corporation, 1940.

Respectfully submitted,

CHARLES PACKARD,
Director.

1. MEMORIALS OF DECEASED TRUSTEES

MEMORIAL TO DR. M. M. METCALF

BY DR. R. A. BUDINGTON

It is altogether fitting that the Corporation of the Marine Biological Laboratory, at its annual meetings, should pause to pay such salutation and honor as it may to those recently removed by death, and who over many years supported the Laboratory by scientific work, wise counsel, and energetic endorsement.

Such a Corporation member was Maynard Mayo Metcalf, who died last April 19th after a very prolonged illness, which began suddenly while he was at work in this building. His age was seventy-two years.

Dr. Metcalf's chief biological mentors were Prof. Albert A. Wright at Oberlin (Wright was one of the very early workers at Woods Hole), and Prof. W. K. Brooks of the Hopkins, under whom he took the

doctorate in 1893. His academic appointments as teacher were as organizer and head of the Department of Zoology at Goucher College, 1893–1906; at Oberlin he reorganized the corresponding department and directed it from 1906 till 1914; from 1926 till 1933 he was research associate with rank of Professor at the Johns Hopkins University. During the year 1924–25 he was chairman of the Division of Biology and Agriculture of the National Research Council, Washington.

Among Metcalf's earliest published studies were some on morphological and embryological features of Amphineura and gastropods; but thereafter for several years his attention was given to the morphology, physiology, phylogeny, and taxonomy of the Tunicata with major emphasis on pelagic forms. He presented very comprehensive collections of these to the National Museum. His third and most arduous series of studies dealt with the morphology, taxonomy, and cytology of the Opalinidae; these led him to far-reaching analysis of specific host-parasite relations, with deductions therefrom as to the ancient distribution of Amphibia, as well as to evidences of former land connections between now-separated continents.

All his life an outstanding charaacteristic of Metcalf, which should be mentioned in any summary of his scientific work, was that of giving credit to collaborators. Especially in his later years was assistance necessary; and all such received appropriate acknowledgment in the publications involved.

Metcalf's publications include: papers exceeding 120 in number; a book, "Organic Evolution" (Macmillan); and three large monographic volumes on the opalinids. The most recent of these was issued by the Smithsonian Institution as a Bulletin of the National Museum last spring.

He was elected to membership in 28 American, 3 British, and 3 French learned societies, and was a member of the Authors Club, London. For 45 years he was a summer frequenter of the Woods Hole Laboratories, and a member of the Board of Trustees of the Marine Biological Laboratory from 1896 till his death—44 years. Few men indeed have been as deeply sincere in their solicitude for and belief in the functions of this Laboratory as was Maynard Metcalf. Directly or indirectly he assisted many a student, in financial or other ways, to come here for study and research; and mention should here be made of his gift of his large collection of reprints to our library.

As a man he was chronically of discriminating judgment, positive opinions, and uncompromising integrity. He was thoroughly human of the finest grade; an optimist; an idealist; a dispenser of cheer, with rare generosity of spirit, and capacity for friendship. He will not be forgotten.

Memorial to Dr. H. McE. Knower

By Dr. Ross G. Harrison

Henry McElderry Knower died in Baltimore on January 10, 1940, at the age of 71, after a long and distressing illness, which was borne with the courage, patience, and good humor that characterized his whole life.

He was born in Baltimore on August 5, 1868 and was educated in schools in that city and at the Johns Hopkins University, where he received the A.B. degree in 1890 and the Ph.D. in 1896. After graduation Knower held an instructorship for one year at Williams College and subsequently was for ten years on Doctor Mall's staff in the Department of Anatomy at the Johns Hopkins Medical School. From there he went to the University of Toronto as lecturer in 1909 and the following year to the University of Cincinnati as Professor and Head of the Department of Anatomy. After his resignation in 1924 he served as a visiting professor at the University of Georgia, later as Professor of Anatomy at the University of Alabama (1927–29) and as Associate Professor of Anatomy in the Albany Medical College. His last appointment was that of Research Associate in Biology in Yale University.

Knower spent the summer of 1896 at the Marine Biological Laboratory as an investigator and was elected to membership in the Corporation. The following year he was on the staff of the Invertebrate course. In 1908 he became one of the permanent members of the Woods Hole summer colony and a regular attendant at the Laboratory, until the failure of his health made that impossible. From 1909 till 1919 he served as librarian of the Marine Biological Laboratory, and it was during his administration that the library began its period of rapid growth. It was well arranged and catalogued, particularly after its removal to the Crane Building made that possible.

Knower first became interested in the embryology of termites when in Jamaica as a student. This was the subject of his doctoral dissertation, but on entering the Department of Anatomy at Johns Hopkins in 1899 his interest shifted to the development of the vascular system, the study of which became his life work. Much of his research in this field was done at Woods Hole. He developed very delicate methods of injection and accumulated a great collection of exquisitely injected embryos. Fortunately he was able to complete one of the major installments of this work shortly before his death.

His services to scientific publication in this country were unusual. Through his energy and enterprise the foundation of the *American Journal of Anatomy* was greatly hastened, and its establishment came

just at the time when most needed. Over twenty years of his life were devoted to this undertaking, which he served with devotion and skill as Secretary of the Editorial Board. In 1906 he initiated the publication of the *Anatomical Record*, first as a supplement to the *American Journal of Anatomy* but soon to become an independent journal with its own editorial board.

In all of his relations Knower was steadfast and sincere. He was good humored, sympathetic with youth and wise in his counsel. Through his death many of us here have lost a warm, devoted and genial friend. The Corporation of the Marine Biological Laboratory desire to record their sorrow at his death, their sense of personal loss, and their appreciation of his many contributions to biological science and his services to the institution.

Memorial to Dr. Charles Zeleny

By Dr. Fernandez Payne

Charles Zeleny, Professor of Zoölogy at the University of Illinois, died at his home in Urbana December 21, 1939. He was born at Hutchinson, Minnesota, September 17, 1878, and spent his early boyhood days there. Later his parents moved to Minneapolis where he entered the University of Minnesota and graduated in 1898. He remained as a graduate and received his M.S. in 1901. The next year he was a graduate student at Columbia University, working with T. H. Morgan and E. B. Wilson, and the following year he worked at the Naples Zoölogical Station. Returning to America in 1903, he entered Chicago University where he obtained the Ph.D. in 1904. He came to Indiana University as an instructor in the summer of 1904. Here he advanced rapidly and held the rank of Associate Professor at the time of call to the University of Illinois in 1909. Beginning at Illinois as an Assistant Professor, he was promoted the next year to the rank of Associate Professor and in 1915 to a Professorship. Upon the retirement of Professor H. B. Ward in 1933, he was made head of the Department of Zoölogy and chairman of the Division of Biological Sciences. Because of ill health, he had retired from his executive duties in 1938.

On May 29, 1911, he married Ida Benedicta Ellingson, of St. Morris, Wisconsin. Mrs. Zeleny and a son, Charles, Jr., survive.

Dr. Zeleny's family is unique in that three of his brothers are scientists of note. Anthony Zeleny, now retired, was Professor of Physics at the University of Minnesota; John Zeleny is Professor of Physics at Yale; and Frank Zeleny is an engineer with the Burlington Railway.

As is true with every great man, chronological facts such as those enumerated tell but little of the life of Charles Zeleny. They are cold, external. It was the writer's good fortune to have been a student in Dr. Zeleny's first class in embryology taught at the Biological Station in the summer of 1904. For the next three years, our associations were intimate. We worked together, ate at the same table, played together and tramped through the woods and fields together. The fact that one was teacher, the other student entered but little into our thinking. The friendship formed in those early years remained to the end. As a friend he was true, somewhat reserved, seldom talked of his own personal affairs, possessed a subtle, sometimes mischievous wit, appreciated by those who know him best. Seldom did he complain about anything. Bitterness, if present, was kept hidden.

As a teacher he was kind, helpful, encouraging, stimulating. As a zoölogist his papers in the fields of regeneration, experimental embryology and genetics speak for themselves. They rank among the best contributions of his time. Originality in thinking stands out prominently in all his work.

In recognition of his attainments, he was elected vice-president of section F of the A. A. A. S. in 1932, and president of the American Society of Zoölogists in 1933.

Dr. Zeleny's death at the early age of 61 years is not only a loss to his relatives and friends, but to science.

Memorial to Captain John J. Veeder

By Dr. F. R. Lillie

John J. Veeder, Captain of the fleet of the Marine Biological Laboratory from 1890 to 1933, was born on the island of Cuttyhunk January 27, 1859. Like all Cuttyhunkers, he was accustomed to the management of boats from early years, and acquired a most intimate knowledge of the shoals, tides, currents and weather conditions of Vineyard Sound and Buzzards Bay. He married and moved to Woods Hole in 1881.

The Marine Biological Laboratory was founded in 1888, and as Dr. Bumpus has written me, "The summer of 1890 found the steam launch "Sagitta" proudly added to the fleet of two old green dories that had been inherited from the Annisquam Laboratory." It became necessary to appoint a captain and John J. Veeder was called in for examination by Dr. Gardiner. He was asked to "box the compass." Dr. Bumpus relates, "The speed with which he went through the ritual settled the matter then and there. Captain Veeder was promptly commissioned."

For a year, until George M. Gray was appointed, Captain Veeder acted also as collector; and afterwards collaborated closely with the Supply Department, became thoroughly familiar with the collecting grounds, and located and set the fish traps of the Laboratory.

Captain Veeder was in charge of the class trips and picnics, and though many thousands were carried in the years of his service, no one was ever lost. He was a past master of the technique of the clambakes which added so greatly to the enjoyment of the picnics. He kept his eye on the weather and he always veteod a trip if his extraordinary weather sense and wisdom warned him that the trip would be dangerous. I cannot say how many times he came to the rescue of our amateur sailors in distress, when marooned by bad weather or ignorance of tidal currents; and very frequently he and the crew went to the aid of small craft grounded on shoals in the Hole or near the harbor.

He had the good old Cape Cod dignity and self-respect; he was a shrewd judge of men in all walks of life, and met all on an equal basis. He never regarded his position merely as a job; whatever was " for the good of the Laboratory," as he used to say, was always cheerfully and skilfully performed. He acted as interpreter of the Laboratory to the town folk or in town meetings, and was helpful in maintaining the good relations which we have always valued.

2. THE STAFF, 1940

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

ZOÖLOGY

I. Investigation

Gary N. Calkins, Professor of Protozoölogy, Emeritus, Columbia University.

E. G. Conklin, Professor of Zoölogy, Emeritus, Princeton University.

Caswell Grave, Professor of Zoölogy, Emeritus, Washington University.

H. S. Jennings, Professor of Zoölogy, University of California.

Frank R. Lillie, Professor of Embryology, Emeritus, The University of Chicago.

C. E. McClung, Professor of Zoölogy, Emeritus, University of Pennsylvania.

S. O. Mast, Professor of Zoölogy, Johns Hopkins University.

T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.

G. H. Parker, Professor of Zoölogy, Emeritus, Harvard University.

Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. Instruction

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

Junior Instructors

E. R. Jones, Jr., Professor of Biology, College of William and Mary.
N. T. Mattox, Instructor in Zoölogy, Miami University.

PROTOZOÖLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
Virginia Dewey, Assistant in Zoölogy, Vassar College.
G. W. Kidder, Assistant Professor of Biology, Brown University.

EMBRYOLOGY

I. Investigation

(*See Zoölogy*)

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 Zoölogy, University of North Carolina.
Viktor Hamburger, Assistant Professor of Zoölogy, 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

Laurence Irving, Professor of Biology, Swarthmore College.
Robert Chambers, Professor of Biology, New York University.
Kenneth C. Fisher, Assistant Professor of Experimental Biology, University of Toronto.
Rudolf Höber, Visiting Professor of Physiology, University of Pennsylvania.
C. Ladd Prosser, Assistant Professor of Zoölogy, University of Illinois.
James A. Shannon, Assistant Professor of Physiology, New York University Medical College.
F J. M. Sichel, Instructor in Physiology, University of Vermont, College of Medicine.

BOTANY

I. Investigation

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E. W. Sinnott, Professor of Botany, Barnard College.

II. Instruction

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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.
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LIBRARY

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Deborah Lawrence, Secretary.
Mary A. Rohan, S. Mabell Thomes, Assistants.

SUPPLY DEPARTMENT

James McInnis, Manager.
Milton B. Gray, Collector.
A. M. Hilton, Collector.
A. W. Leathers, Shipping Dept.
Geoffrey Lehy, Collector.
Walter Kahler, Collector.
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KRANTZ, MARION, Student, Bennington College.
LEE, RICHARD E., Harvard University.
LUDWIG, FRANCIS W., Villanova College.

McFarland, William, Student, Washington and Jefferson College.
Miller, Gerald, Student, Oberlin College.
Nichols, Myron McCall, Laboratory Assistant, DePauw University.
Pond, Sidney M., Wesleyan University.
Robinson, Edwin J., Jr., Teaching Fellow, Washington Square College, New York University.
Samorodin, Albert J., Graduate, University of Minnesota.
Sawyer, Charles H., Assistant in Biology, Yale University.
Sherman, Frederick G., Laboratory Assistant, Northwestern University.
Steele, Kenneth C., Dartmouth College.
Sweeny, Frank P., Amherst College.
Yancey, Maude J., Student, North Carolina College for Negroes.

PHYSIOLOGY

Baylor, Edward R., Student, University of Illinois.
Blanchard, Barbara D., Teacher, Placer Junior College, California.
Carleen, Mildred H., Graduate Assistant, Mount Holyoke College.
Chidsey, Jane L., Assistant Professor, Wheaton College.
Davies, Philip W., Johnson Foundation Scholar, University of Pennsylvania.
Edgerley, Robert H., Graduate Assistant, Ohio State University.
Edwards, George A., Graduate Assistant, Tufts College.
Everett, Guy M., Graduate Teaching Assistant, University of Maryland Medical School.
Fox, Ruth P., Assistant, Vassar College.
Henry, Richard J., University of Pennsylvania, School of Medicine.
Hohwieler, Harold J., Graduate Assistant, Washington University.
Holton, George W., Wesleyan University.
Jackson, Blanche E., Fellowship Student, Radcliffe College.
Jakus, Marie A., Graduate Assistant, Washington University.
Kasserman, Walter H., Washington and Jefferson College.
Norman, George R., Student, Wabash College.
Ormsbee, Richard A., Graduate Assistant, Brown University.
Rathbun, Edith N., 88 Fosdyke Street, Providence, Rhode Island.
Scholander, Per Fredrik, Research Associate, University of Oslo.
Stokes, Allen W., Harvard University.
Wolf, Mary H., Student, Duke University.
Woodward, Arthur, Jr., Graduate Assistant, Wesleyan University.

PROTOZOÖLOGY

Beam, Carl A., Student, Brown University.
Carroll, Kenneth M., Student, Franklin and Marshall College.
Cosgrove, William B., Student, Cornell University.
Dodge, Frances, Student, Gettysburg College.
Harrigan, Mary K., Special Instructor in Biology, Simmons College.
Macdonald, Katherine C., Graduate Student, McGill University.
Marchand, Doris, Teacher, St. Catherine's School, Richmond, Virginia.

INVERTEBRATE ZOÖLOGY

Adams, Esther F., Instructor in Biology, Moberly Junior College.
Allen, Jean, Miami University.
Beeman, Elizabeth A., Graduate Assistant in Zoölogy, Mount Holyoke College.
Bergstrom, William H., Student, Amherst College.

Böving, Bent G., Assistant, Swarthmore College.
Brush, Helen V., Assistant in Zoölogy, Vassar College.
Burns, John E., Graduate Laboratory Assistant, Wesleyan University.
Cairns, Malcolm G., New Jersey State Teachers College, Montclair, New Jersey.
Clark, Arnold M., Student, University of Pennsylvania.
Coe, Grace L., Student, New Jersey State Teachers College, Montclair, New Jersey.
Dent, James N., Graduate Assistant in Zoölogy, Johns Hopkins University.
Edwards, Gene C., Student, Wabash College.
Fitzgerald, Laurence R., State University of Iowa.
Gibbs, Elizabeth, Undergraduate, Wheaton College.
Goodrich, Mary W., Student, Wheaton College.
Gravett, Howard T., Associate Professor of Biology, Elon College.
Hale, Barbara, Student, Radcliffe College.
Hildebrandt, Wallace H., Undergraduate Instructor, Canisius College.
Holdsworth, Robert P., Jr., Austin Teaching Fellow, Harvard University.
Horwitz, Diana C., Teacher, Hyde Park High School, Hyde Park, Massachusetts.
Hoyt, Jane M., Barnard College.
James, Marian F., Graduate Fellow, University of Illinois.
Killough, John H., Graduate Student, Johns Hopkins University.
Kline, Irene T., Duke University.
Kreeger, Florence Brooks, Graduate Assistant in Biology, Newcomb College.
Lamoreux, Welford F., Assistant Professor, Cornell University.
Lerner, Eleanor, Brooklyn College.
Levitzky, Edward, Student, Rutgers University.
MacRae, Roberta M., Graduate Assistant, Wellesley College.
McKenzie, Helen E., Seton Hill College.
Marbarger, John P., Graduate Student, Johns Hopkins University.
Means, Oliver W., Jr., Yale University.
Micklewright, Helen L., Student, Wilson College.
Musser, Ruth E., Student, Goucher College.
Noce, Mildred W., Student, Southwestern College.
Powers, Samuel R., Jr., Swarthmore College.
Putnam, William S., Graduate Assistant, Amherst College.
Reeves, Walter P., Jr., Graduate Student, University of Alabama.
Royle, Jane G., Graduate Assistant in Anatomy and Invertebrate Zoölogy, Bryn Mawr College.
Samuels, Robert, University of Pennsylvania.
Saunders, Grace S., Hunter College.
Schnabel, Margaret J., Student, Oberlin College.
Scott, George T., Assistant, Harvard University.
Shank, Margaret L., Student, New Jersey State Teachers College, Montclair, New Jersey.
Smith, Fern W., Student, Smith College.
Smith, Frederick E., Massachusetts State College.
Smith, Julia P., Student, University of Rochester.
Stifler, Margaret C., Assistant, Goucher College.
Stone, Fred L., University of Rochester.
Syner, James C., Student, Springfield College.
Walker, Warren F., Jr., Student, Harvard University.
Wheeler, Bernice M., Instructor, Westbrook Junior College.
White, Francis M., Graduate Assistant in Biology, Purdue University.
Wolaver, John H., Jr., Student, DePauw University.
Wright, Margaret R., Student, Yale University.

4. TABULAR VIEW OF ATTENDANCE, 1936–1940

	1936	1937	1938	1939	1940
Investigators—Total	359	391	380	352	386
Independent	226	256	246	213	253
Under Instruction	76	74	53	60	62
Research Assistants	57	61	81	79	71
Students—Total	138	133	132	133	128
Zoölogy	55	57	54	55	55
Protozoölogy	17	16	10	12	7
Embryology	34	35	34	36	34
Physiology	22	16	22	21	22
Botany	10	9	12	9	10
Total Attendance	497	524	512	485	514
Less Persons registered as both students and investigators	24	13	16	14	7
	473	511	496	471	507
Institutions Represented—Total	158	165	151	162	148
By Investigators	120	134	125	132	112
By Students	77	79	67	72	79
Schools and Academies Represented					
By Investigators	2	3	4	2	1
By Students	3	2	1	2	2
Foreign Institutions Represented					
By Investigators	9	16	14	8	2
By Students	5	—	3	1	1

5. SUBSCRIBING AND COÖPERATING INSTITUTIONS

1940

Amherst College
Barnard College
Biological Institute, Philadelphia, Pennsylvania
Bowdoin College
Brooklyn College
Brown University
Bryn Mawr College
Canisius College
College of Physicians and Surgeons
Columbia University
Cornell University
Cornell University Medical College
DePauw University
Duke University
Fisk University
Goucher College
Harvard University
Harvard University Medical School
Hunter College
Industrial and Engineering Chemistry, of the American Chemical Society
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly and Company
Long Island University
Massachusetts State College
Memorial Hospital, New York City
Mount Holyoke College
Mount Sinai Hospital, New York City
Newcomb College
New York State Department of Health
New York University
New York University College of Medicine
New York University Washington Square College
North Carolina College for Negroes
Northwestern University
Oberlin College
Ohio State University
Princeton University
Purdue University
Radcliffe College
Rockefeller Foundation

Rockefeller Institute for Medical Research
Russell Sage College
Rutgers University
Smith College
Springfield College
Stanford University
State University of Iowa
Syracuse University
Syracuse University College of Medicine
Tufts College
Union College
University of Chicago
University of Cincinnati
University of Illinois
University of Kansas
University of Missouri
University of Pennsylvania
University of Pennsylvania School of Medicine
University of Pittsburgh
University of Rochester
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
Wilson College
Yale University
Yale University Medical School

6. EVENING LECTURES, 1940

Friday, July 5
Dr. Leonor Michaelis ..... "Oxidation and Reduction in Organic and Biological Chemistry."

Friday, July 12
Dr. Kenneth V. Thimann ..... "Hormones and the Physiology of Growth in Plants."

Friday, July 19
Dr. K. S. Cole ..... "Electrical Properties of the Cell Membrane."

Friday, July 26
Dr. D. H. Wenrich ..... "Chromosomes in Protozoa."

Wednesday, July 31
Mr. George C. Lower ..... "Local Marine Life in Color."

Friday, August 2
Dr. Eric G. Ball ..... "Catalysts of Biological Oxidations, their Composition and Mode of Action."

Thursday, August 8
Dr. L. J. Milne ..... "Animated Diagrams of Biological Processes."

Friday, August 9
Prof. F. O. Schmitt ..... "Modern Concepts of Protoplasmic Organization."

Friday, August 16
Dr. Alfred S. Romer ..... "The Phylogeny and Structure of the Lower Vertebrates."

Friday, August 23
Dr. Dugald Brown ..... "The Regulation of Metabolism in Contracting Muscle."

Friday, August 30

Dr. Curt Stern ..... "On Dependent Growth and Form of the Testes in Various Species of Drosophila."

Saturday, August 31

Dr. Per Höst ..... "Arctic Seal Hunting in the White Sea and in Greenland Waters."

7. SHORTER SCIENTIFIC PAPERS, 1940

Tuesday, July 9

Mr. Edward L. Chambers ..... "Inter-relations between Egg-Nucleus, Sperm-Nucleus and Cytoplasm of the Asterias Egg."

Dr. Daniel Mazia ..... "Digestion Studies on Salivary Chromosomes."

Dr. M. J. Kopac ..... "Some Properties of the Residue from Rapidly Disintegrated Arbacia Egg Cytoplasm."

Tuesday, July 16

Dr. S. C. Brooks ..... "Ion Intake by Living Cells."

Dr. L. I. Katzin ..... "The Use of Radioactive Tracers in the Determination of Irreciprocal Permeability of Biological Membranes."

Dr. K. C. Fisher ..... "Urethane and the Respiration of Yeast Cells."

Dr. M. M. Brooks ..... "Spectrophotometric Determinations on Hemoglobin and its Derivatives."

Tuesday, July 23

Dr. Nelson J. Spratt, Jr. ..... "An in vitro Analysis of the Organization of the Eye-forming Area in the Early Chick Blastoderm."

Dr. Ernest Scharrer ..... "On the Determination of the Vascular Pattern of the Brain of the Opposum."

Dr. Paul Weiss ..... "Functional Properties of Transplanted and Deranged Parts of the Central Nervous System of Amphibians."

Tuesday, July 30

Dr. B. H. Willier ..... "A Study of Feather Color Patterns Produced by Grafting Melanophores During Embryonic Development."

Dr. H. B. Goodrich ..... "The Cellular Basis of the Color Pattern in some Bermuda Coral Reef Fish."

Tuesday, August 6

Dr. Albert E. Oxford ..... "Production of a Complex Nitrogenous Compound Related to Tyrosine by a Species of Penicillium."

Dr. Kurt Salomon ..... "Studies on Erythrocruorin (Invertebrate Hemoglobin)."

Dr. Kurt G. Stern,
Dr. Joseph L. Melnick and
Dr. Delafield DuBois ..... "Photochemical Spectrum of the Pasteur Enzyme."

Tuesday, August 13

Dr. A. C. Giese ..... "Effects of Ultra-violet Light on Respiration of the Luminous Bacteria."

Dr. Ivor Cornman ..... "Effects of Ether upon the Development of Drosophila melanogaster."

Dr. Berta Scharrer ..... "Neurosecretory Cells in Cockroaches."

Dr. G. Haugaard ..... "The Mechanism of the Glass Electrode."

Tuesday, August 20

Dr. W. Gordon Whaley ..... "Developmental Changes in Apical Meristems."

Dr. Harry G. Albaum and
Dr. Barry Commoner ..... "The Relation between the Four-Carbon Acid Respiratory System and the Growth of Oat Seedlings."

Dr. R. K. Skow and
L. R. Blinks ..... "Respiratory Changes following Stimulation in Nitella."

Dr. L. R. Blinks ..... "The Relation of Potassium to the Bio-electric Effects of Temperature and Light in Valonia."

8. GENERAL SCIENTIFIC MEETINGS, 1940

Tuesday, August 27

Dr. S. O. Mast and
Dr. W. J. Bowen ..... "The Hydrogen Ion and the Osmotic Concentrations of the Cytoplasm in Vorticella Similis Stokes, as Indicated by Observations on the Food Vacuoles."

Dr. M. H. Jacobs and
Mr. W. D. Jones ..... "The Reversibility of Certain Artificially Induced Changes in the Permeability of the Erythrocyte."

Dr. E. J. Boell,
Dr. R. Chambers,
Miss E. A. Glancy,
Dr. K. G. Stern and
Miss B. Meyerhof ..... "Oxidase Activity and Respiration of Cells and Cell Fragments."

Dr. E. J. Boell and
Dr. L. L. Woodruff ..... "Respiratory Metabolism of Mating Types in Paramecium Calkinsi."

Dr. Eric G. Ball and
Miss Pauline A. Ramsdell ..... "Squid Ink, a Study of its Composition and Enzymatic Production."

Dr. A. E. Oxford ..... "Observations on the Occurrence of Simple Ethereal Sulphates in Marine Algae."

Dr. E. J. W. Barrington ..... "Blood-sugar and the Problem of the Pancreas in Lampreys."

Dr. A. E. Navez and
Mr. A. DuBois ..... "Fatty Acid Compounds in the Unfertilized Egg of Arbacia punctulata."

Mr. C. B. Giddings ..... "Distribution of Plasmalogen in Certain Invertebrate Forms."

Dr. G. H. Parker ..... "Lipoids and their probable Relation to Melanophore Activity."

Mr. Samuel Belfer,
Mr. B. Bailey,
Dr. H. C. Bradley, and
Mr. Howard Eder ..... "Studies of the Distribution of the Autolytic Mechanism."

Dr. Carl C. Smith ..... "The Effect of Various Cholinergic Drugs on the Radula Protractor Muscle of Busycon canaliculatum."

Dr. E. J. Boell and
Dr. D. Nachmansohn ..... "Choline Esterase in Nerve Fibers."

Dr. R. G. Abell and
Dr. Irvine H. Page ..... "Vascular Reactions to Renin and Angiotonin."

Mr. J. Crawford,
Miss D. Benedict, and
Dr. A. E. Navez ..... "Factors Affecting the Frequency of Contraction of the Heart of Venus mercenaria."

Mr. Charles E. Wilde, Jr. ..... "Determining Factors in the Regeneration of Hydractinia."

Dr. Edgar Zwilling ..... "Time of Determination and Dominance in Tubularian Reconstitution."

DR. S. MERYL ROSE ..... "A Regeneration-Inhibiting Substance Released by Tubularia Tissue."
DR. L. G. BARTH ..... "The Rôle of Oxygen in Regeneration of Tubularia."
DR. HARRY G. ALBAUM ..... "The Growth of Oat Coleoptiles after Seed Exposure to Different Oxygen Concentrations."
DR. W. GARDNER LYNN ..... "Results of Transplantation of the Pituitary Anlage to the Thyroid Region in Amblystoma."

Wednesday, August 28

DR. T. C. EVANS ..... "Oxygen Consumption of Arbacia Eggs Following Exposure to Roentgen Radiation."
DR. T. C. EVANS ..... "Effects of Roentgen Radiation on the Jelly of Arbacia Egg. I. Disintegration of the Jelly."
DR. M. E. SMITH AND DR. T. C. EVANS ..... "Effects of Roentgen Radiation on the Jelly of the Arbacia Egg. II. Changes in pH of Egg Media."
MR. E. P. LITTLE AND DR. T. C. EVANS ..... "Delay in First Cleavage of Arbacia Eggs Following Roentgen Irradiation of Zygotes."
DR. GRACE TOWNSEND ..... "Concerning Sensitivity of Cells to X-Ray."
DR. GRACE TOWNSEND ..... "Laboratory Ripening of Arbacia in Winter."
DR. ETHEL BROWNE HARVEY .I. "A Note on Determining the Sex of Arbacia punctulata."
DR. ETHEL BROWNE HARVEY .II. "Centrifugal Speed and the Arbacia Egg."
DR. ETHEL BROWNE HARVEY III. "Colored Photographs of Stratified Arbacia punctulata Eggs Stained with Vital Dyes."
DR. HERBERT SHAPIRO ..... "Elongation and Return in Spherical Cells."
MR. IVOR CORNMAN ..... "Echinochrome as the Sperm-activating Agent in Egg-water."
MR. TERU HAYASHI ..... "A Relation between the Dilution Medium and the Survival of Spermatozoa of Arbacia punctulata."
DR. WM. H. F. ADDISON ..... "The Occurrence of Cartilage at the Bifurcation of the Common Carotid Artery in an Adult Dog."
DR. HOPE HIBBARD ..... "Cytoplasmic Morphology in the Gizzard of Gallus domesticus."

PAPERS READ BY TITLE

Mr. Fred W. Alsup ..... "Further Studies of Photodynamic Action in the Eggs of Nereis limbata."

Mr. C. W. J. Armstrong and
Dr. Kenneth C. Fisher ..... "A Quantitative Study of the Effect of Cyanide and Azide on Carbonic Anhydrase."

Dr. Frank A. Brown, Jr., and
Dr. Alison Meglitsch ..... "Upon the Sources in the Insect Head of Substances which Influence Crustacean Chromatophores."

Dr. Ralph H. Cheney ..... "Myofibrillar Modifications in the Caffeinized Frog Heart."

Dr. Leonard B. Clark ..... "Effects of Visible Radiation on Arbacia Eggs Sensitized with Rhodamine B."

Dr. A. C. Clement ..... "Effects of Cyanide on Cleavage in Eggs of Ilyanassa and Crepidula."

Dr. D. P. Costello ..... "The Cell Origin of the Prototroch of Nereis limbata."

Dr. James Donnellon ..... "Blood Clotting in Callinectes sapidus."

Dr. Llewellyn T. Evans ..... "Effects of Light and Hormones upon the Activity of Young Turtles, Chrysemys picta."

Dr. Llewellyn T. Evans ..... "Effects of Testosterone Propionate upon Social Dominance in Young Turtles, Chrysemys picta."

Dr. Kenneth C. Fisher and
Mr. Richard J. Henry ..... "The Use of Urethane as an Indicator of "Activity" Metabolism in the Sea Urchin Egg."

Mr. Mordecai L. Gabriel ..... "The Inflation Mechanism of Spheroides maculatus."

Miss E. A. Glancy ..... "Micromanipulative Studies on the Nuclear Matrix of Chironomus Salivary Glands."

Dr. John E. Harris ..... "The Reversible Nature of the Potassium Loss from Erythrocytes during Storage of Blood at 2–5° C."

Dr. Arne V. Hunninen and
Dr. Raymond M. Cable ..... "Studies on the Life History of Anisoporus Manteri sp. nov. (Trematoda: Allocreadiidae)."

Dr. Cornelius T. Kaylor ..... "Histological Studies on the Problem of Edema in Haploid Triturus pyrrhogaster Larvae."

Dr. Baldwin Lucké,
Dr. Arthur K. Parpart, and
Mr. R. A. Ricca ..... "Do Carcinogenic Compounds affect Cell Permeability?"

Dr. W. G. Lynn ..... "The Development of the Skull in the Non-aquatic Larva of the Tree-toad, Eleutherodactylus nubicola."

Dr. W. G. Lynn ..... "The Embryonic Origin and Development of the Pharyngeal Derivatives in Eleutherodactylus nubicola."

Sister Maria Laurence Maher . "Preliminary Report on Effect of Indole Acetic Acid on Growth of Chlamydomonas."

Dr. H. Shapiro ..... "Further Studies on the Metabolism of Cell Fragments."

Dr. Carl C. Smith,
Miss Blanche Jackson and
Dr. C. Ladd Prosser ..... "Responses to Acetylcholine and Cholinesterase Content of Cerebratulus."

Dr. A. J. Waterman ..... "Response of the Heart of the Compound Ascidian, Perophora Viridis, to Pilocarpine, Atropine and Nicotine."

DEMONSTRATIONS

Wednesday, August 28

Dr. W. H. F. Addison ..... "Corrosion Preparations of the Branchial Circulation in the Dogfish."

Dr. E. Scharrer ..... "Vascularization of the Extramedullary Nerve Cells of the Puffer, Spheroides Maculatus."

Dr. E. R. Clark and
Mrs. Eleanor Linton Clark .... "The Microscopic Study of Living Tissues in Transparent Chambers Installed in Rabbits' Ears."

Mr. E. P. Little ..... "Color and Luminescence Produced by Roentgen Rays in Glass and Chemicals."

Dr. E. J. Boell ..... "The Cartesian Diver Ultramicro-Respirometer."

Per F. Scholander,
Dr. S. W. Grinnell and
Dr. L. Irving ..... "Apparatus for Measurement of Respiratory Metabolism and Circulation Changes."

9. MEMBERS OF THE CORPORATION, 1940

1. Life Members

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Maryland.
Billings, Mr. R. C., 66 Franklin Street, Boston, Massachusetts.
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania.
Conklin, Prof. Edwin G., Princeton University, Princeton, New Jersey.
Evans, Mrs. Glendower, 12 Otis Place, Boston, Massachusetts.
Foot, Miss Katherine, Care of Morgan Harjes Cie, Paris, France.
Gardiner, Mrs. E. G., Woods Hole, Massachusetts.
Jackson, Miss M. C., 88 Marlboro Street, Boston, Massachusetts.
Jackson, Mr. Chas. C., 24 Congress Street, Boston, Massachusetts.
King, Mr. Chas. A.
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Maryland.
Lowell, Mr. A. L., 17 Quincy Street, Cambridge, Massachusetts.
Means, Dr. J. H., 15 Chestnut Street, Boston, Massachusetts.
Moore, Dr. George T., Missouri Botanical Gardens, St. Louis, Missouri.
Morgan, Mr. J. Pierpont, Jr., Wall and Broad Streets, New York City, New York.
Morgan, Prof. T. H., Director of Biological Laboratory, California Institute of Technology, Pasadena, California.
Morgan, Mrs. T. H., Pasadena, California.
Morrill, Dr. A. D., Hamilton College, Clinton, New York.
Noyes, Miss Eva J.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
Sears, Dr. Henry F., 86 Beacon Street, Boston, Massachusetts.
Shedd, Mr. E. A.
Thorndike, Dr. Edward L., Teachers College, Columbia University, New York City, New York.
Treadwell, Prof. A. L., Vassar College, Poughkeepsie, New York.
Trelease, Prof. William, University of Illinois, Urbana, Illinois.
Wallace, Louise B., 359 Lytton Avenue, Palo Alto, California.

2. Regular Members

Abramowitz, Dr. Alexander A., Biological Laboratories, Harvard University, Cambridge, Massachusetts.
Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts.

Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.

Adolph, Dr. Edward F., University of Rochester Medical School, Rochester, New York.

Albaum, Dr. Harry G., 3115 Avenue I, Brooklyn, New York.

Allee, Dr. W. C., The University of Chicago, Chicago, Illinois.

Amberson, Dr. William R., Department of Physiology, University of Maryland, School of Medicine, Lombard and Greene Streets, Baltimore, Maryland.

Anderson, Dr. Rubert S., Memorial Hospital, 444 East 58th Street, New York City, New York.

Angerer, Dr. Clifford A., Department of Physiology, Ohio State University, Cleveland, Ohio.

Armstrong, Dr. Philip B., College of Medicine, Syracuse University, Syracuse, New York.

Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts.

Baitsell, Dr. George A., Yale University, New Haven, Connecticut.

Baker, Dr. H. B., Zoölogical Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.

Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire.

Ball, Dr. Eric G., Department of Biological Chemistry, Harvard University Medical School, Boston, Massachusetts.

Bard, Prof. Philip, Johns Hopkins Medical School, Baltimore, Maryland.

Barron, Dr. E. S. Guzman, Department of Medicine, The University of Chicago, Chicago, Illinois.

Bartii, Dr. L. G., Department of Zoölogy, Columbia University, New York City, New York.

Beadle, Dr. G. W., School of Biological Sciences, Stanford University, California.

Beams, Dr. Harold W., Department of Zoölogy, State University of Iowa, Iowa City, Iowa.

Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York.

Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, Louisiana.

Bigelow, Dr. H. B., Museum of Comparative Zoölogy, Cambridge, Massachusetts.

Bigelow, Prof. R. P., Massachusetts Institute of Technology, Cambridge, Massachusetts.

Binford, Prof. Raymond, Guilford College, Guilford, North Carolina.

Bissonnette, Dr. T. Hume, Trinity College, Hartford, Connecticut.

Blanchard, Prof. Kenneth C., Washington Square College, New York University, New York City, New York.

Bodine, Dr. J. H., Department of Zoölogy, State University of Iowa, Iowa City, Iowa.

Boring, Dr. Alice M., Yenching University, Peking, China.

Bradley, Prof. Harold C., University of Wisconsin, Madison, Wisconsin.

Bronfenbrenner, Dr. Jacques J., Department of Bacteriology, Washington University Medical School, St. Louis, Missouri.

Brooks, Dr. S. C., University of California, Berkeley, California.

Brown, Dr. Dugald E. S., New York University, College of Medicine, New York City, New York.

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

Buckingham, Miss Edith N., Sudbury, Massachusetts.

Buck, Dr. John B., Department of Zoölogy, University of Rochester, Rochester, New York.

Budington, Prof. R. A., Winter Park, Florida.

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

Bumpus, Prof. H. C., Duxbury, Massachusetts.

Byrnes, Dr. Esther F., 1803 North Camac Street, Philadelphia, Pennsylvania.

Calkins, Prof. Gary N., Columbia University, New York City, New York.

Cannan, Prof. R. K., New York University College of Medicine, 477 First Avenue, New York City, New York.

Carlson, Prof. A. J., Department of Physiology, The University of Chicago, Chicago, Illinois.

Carothers, Dr. E. Eleanor, Department of Zoölogy, State University of Iowa, Iowa City, Iowa.

Carpenter, Dr. Russell L., Tufts College, Tufts College, Massachusetts.

Carroll, Prof. Mitchel, Franklin and Marshall College, Lancaster, Pennsylvania.

Carver, Prof. Gail L., Mercer University, Macon, Georgia.

Cattell, Dr. McKeen, Cornell University Medical College, 1300 York Avenue, New York City, New York.

Cattell, Prof. J. McKeen, Garrison-on-Hudson, New York.

Cattell, Mr. Ware, Garrison-on-Hudson, New York.

Chambers, Dr. Robert, Washington Square College, New York University, Washington Square, New York City, New York.

CHENEY, DR. RALPH H., Biology Department, Long Island University, Brooklyn, New York.

CHIDESTER, PROF. F. E., Auburndale, Massachusetts.

CHILD, PROF. C. M., Jordan Hall, Stanford University, California.

CHURNEY, DR. LEON, Zoölogical Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.

CLAFF, MR. C. LLOYD, Department of Biology, Brown University, Providence, Rhode Island.

CLARK, PROF. E. R., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.

CLARK, DR. LEONARD B., Department of Biology, Union College, Schenectady, New York.

CLELAND, PROF. RALPH E., Indiana University, Bloomington, Indiana.

CLOWES, DR. G. H. A., Eli Lilly and Company, Indianapolis, Indiana.

COE, PROF. W. R., Yale University, New Haven, Connecticut.

COHN, DR. EDWIN J., 183 Brattle Street, Cambridge, Massachusetts.

COLE, DR. ELBERT C., Department of Biology, Williams College, Williamstown, Massachusetts.

COLE, DR. KENNETH S., College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York City, New York.

COLE, DR. LEON J., College of Agriculture, Madison, Wisconsin.

COLLETT, DR. MARY E., Western Reserve University, Cleveland, Ohio.

COLTON, PROF. N. S., Box, 601, Flagstaff, Arizona.

COONFIELD, DR. B. R., Brooklyn College, Bedford Avenue and Avenue H, Brooklyn, New York.

COPELAND, PROF. MANTON, Bowdoin College, Brunswick, Maine.

COSTELLO, DR. DONALD P., Department of Zoölogy, University of North Carolina, Chapel Hill, North Carolina.

COSTELLO, DR. HELEN MILLER, Department of Zoölogy, University of North Carolina, Chapel Hill, North Carolina.

COWDRY, DR. E. V., Washington University, St. Louis, Missouri.

CRAMPTON, PROF. H. E., Barnard College, Columbia University, New York City, New York.

CRANE, MRS. C. R., Woods Hole, Massachusetts.

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DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER

IV. Interrelationship of Various Stages; Influence of Concentration and Nature of Particulate Matter [1]

THEODOR VON BRAND AND NORRIS W. RAKESTRAW

(From the Woods Hole Oceanographic Institution, Woods Hole, Mass.)

In previous investigations (1937, 1939, 1940) it has been demonstrated that the decomposition of particulate organic matter in sea water proceeds in well-defined steps, the main stages being the formation of ammonia, nitrite and nitrate. The appearance of these substances is due to the successive development of different bacterial floras acting upon the original substratum of organic matter. In nature, however, a mixture of the various floras will probably occur, with frequent or continuous addition of new decomposing material. It seemed desirable, therefore, to study the interrelationship of the different stages of the cycle and the results obtained when two or more stages occur simultaneously.

For this purpose a 20-liter carboy of filtered sea water from Woods Hole Harbor, to which washed diatoms (*Nitzschia Closterium*) were added, was kept at room temperature in the dark. In order to determine what effect the bacterial flora present at various stages would have on the decomposition of fresh organic matter, portions of the culture were withdrawn at various times during the decomposition cycle. New particulate organic matter was added to these subcultures, as indicated below, after which they were put in fresh containers in the dark. The parent culture (No. 42) and the various subcultures (42A to 42H) were analyzed regularly for the different forms of nitrogen and the changes found are shown in Fig. 1.

The first subculture, No. 42A, was separated from the parent culture when the ammonia in the latter had reached its maximum and when the first trace of nitrite had appeared; the second subculture (42B) a few days later, when the nitrite formation was well under way; and the third (42C), when the nitrite had about reached its maximum. Later, when the nitrite began to decline in subculture No. 42B and when nitrate formation had begun, a portion of it was used in preparing a new subculture (42G). To all these subcultures new particulate matter was added in the form of living, washed diatoms.

[1] Contribution No. 292 from the Woods Hole Oceanographic Institution.

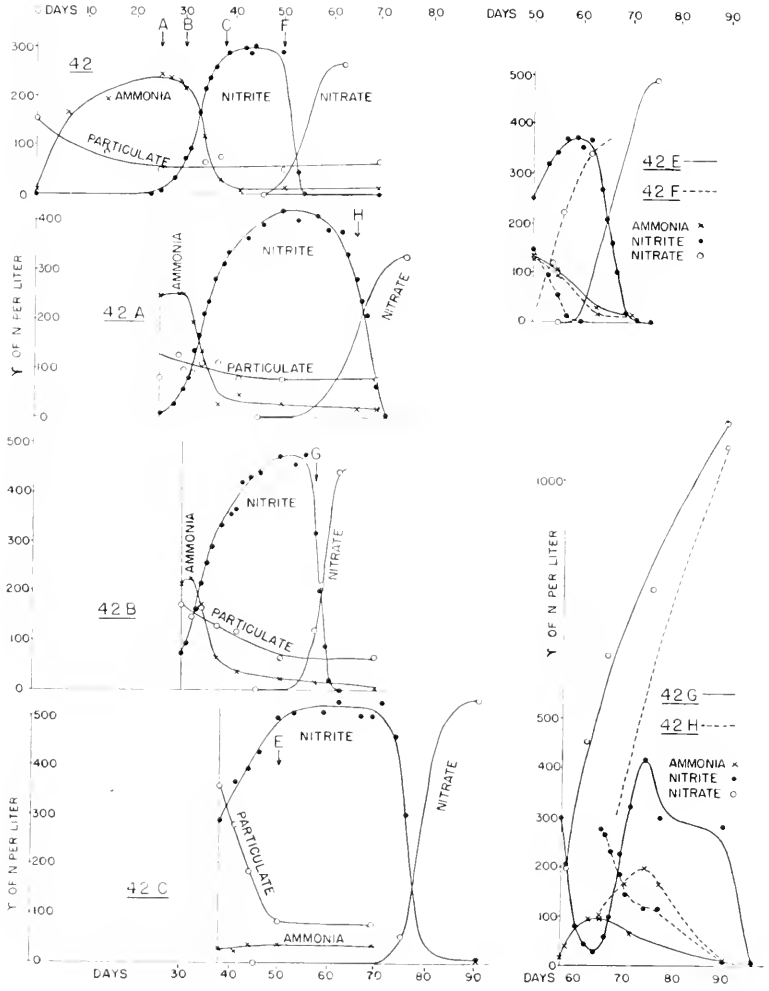

FIG. 1. Interrelationship of different stages of the decomposition cycle. Time in days. Different forms of nitrogen in micrograms (gamma) per liter. The original culture, No. 42, consisted of filtered sea water with washed diatoms (*Nitzschia Closterium*) added. Decomposition in the dark. Subcultures *A, B,* etc., separated at times indicated by arrows and with new particulate organic matter added.

Figure 1 shows that nitrite formation in the first three subcultures (42A, B and C) was in no way interfered with by the addition of new diatoms, but proceeded at normal speed without interruption. In each case ammonia rose only slightly higher than in the original culture, No.

42, indicating that the ammonia formed from the new decomposing diatoms was at once oxidized to nitrite. Finally, the nitrite disappeared from all cultures in the usual way, appearing quantitatively as nitrate.

A somewhat different result was obtained in subculture 42G, which was prepared by adding new organic matter to a portion of 42B when the latter was approaching the end of the nitrite stage. As before, ammonia remained low throughout the whole time. During the first week nitrite disappeared exactly as in the mother-culture from which it had been prepared (42B), but after this it increased again, reaching a new maximum ten days later. Apparently the nitrite-forming flora was on the decline when this subculture was begun but was able to recover under the influence of the newly-formed products of decomposition. Nitrate formation seems to have occurred throughout this subseries. It is probable that we have here a case in which ammonia-, nitrite- and nitrate-forming floras were active at the same time.

In the cultures so far described living diatoms were used as a source of new organic matter; consequently, vigorous ammonia formation was not actually under way at the start of each subculture. In the next two cultures organic matter was introduced which was already in the ammonia-formation stage. Fresh diatoms were added to a fresh quantity of harbor water (No. 42D). After 12 days in the dark, when ammonia was being formed rapidly, portions of this culture were withdrawn and mixed with equal amounts of older cultures in various stages of the decomposition cycle. Thus, subculture 42E consisted of an equal mixture of 42D and 42C, the latter taken when the nitrite had reached its maximum. In this case the ammonia introduced with culture 42D disappeared rapidly, with a corresponding rise in nitrite. Evidently the nitrite-forming flora of culture 42C was still active when the new, partially-decomposed organic matter was added.

Subculture 42F was prepared by separating a portion of the original culture, No. 42, when nitrite had begun to diminish, and adding an equal amount of 42D containing organic matter in the stage of ammonia formation. Both ammonia and nitrite disappeared rapidly, in contrast to the last preceding subculture, 42E, probably due to the fact that the nitrate-forming flora in the parent culture was at that time the most potent one.

The last culture, 42H, behaved in a somewhat similar manner. This consisted of a portion of culture 42A, separated at a time when the nitrite was about half converted to nitrate. To this was added a large number of partially decomposed diatoms, centrifuged from a culture which had stood for six days in the dark. A relatively small increase in ammonia was observed during the first days, with a subsequent rapid decrease.

Nitrite was present somewhat longer than in 42A, but never reached a very high level. It is likely that during this whole time nitrate formation proceeded rapidly.

The following conclusions may be drawn from the study of culture 42 and its subcultures: Ammonia formation does not interfere with the formation of either nitrite or nitrate, in such concentrations as we observed. The strict sequence of processes in our normal decomposition experiments can therefore hardly be due to any inhibiting action of ammonia or other initial products of decomposition upon nitrite or nitrate formation. More likely is it connected with a very slow development of the oxidizing floras. Doubtless, however, some other, hitherto unrecognized factor must also be involved. A slow increase in the nitrate-forming population, for example, is alone insufficient to explain why it should require weeks, or even months, for the first traces of nitrate to appear, whereas once the process has started the nitrate maximum may be reached in five days.

These observations are not necessarily inconsistent with experiments we have described previously, involving deep sea water, in which there was evidence of a retarding influence on the development of the oxidizing floras. This influence has not yet been explained, but seems to be connected with some unknown special property of the deep sea water used.

The course which the decomposition will take, when new organic material is added, will depend upon the flora which predominates. In general, a shortening of the cycle will occur, as far as the newly added material is concerned. The original culture, No. 42, took 55 days to complete its cycle. In the various subcultures the mean time from the addition of new organic matter to the end of the cycle was 36 days, or 41 days if one includes the initial period of decomposition of the added organic matter before its addition to Series 42E, F and H.

Duration of the Cycle

As pointed out in previous papers, the time required for the decomposition cycle varies considerably in different series. It seemed possible that the initial concentration of organic matter might be a determining factor in this connection and Series 47 to 50 were set up to investigate this point. The four cultures contained amounts of particulate nitrogen varying from 185 γ to 768 γ per liter. As shown in Fig. 2, this factor seems to be of some, though not of very great importance. The rate of disappearance of particulate nitrogen was nearly the same in each case. In the higher concentrations the ammonia maxi-

mum was reached a little earlier and nitrite appeared and disappeared more rapidly. The total time for the cycle varied from 61 days in the highest concentration to 88 days in the lowest.

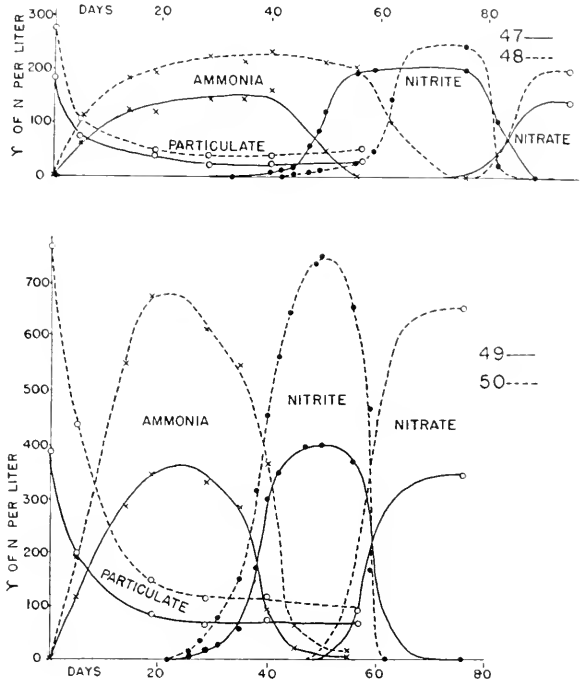

FIG. 2. Series 47 to 50. Influence of varying concentration of particulate matter. Filtered sea water with different amounts of *Nitzschia Closterium* added. Decomposition in the dark. Time in days. Different forms of nitrogen in micrograms (gamma) per liter.

The nature of the suspended organic matter also determines the duration of the cycle, as the next series show. Series 43 and 45 (Figs. 3 and 4) were set up with the same harbor water; in 43 was suspended a small amount of mixed plankton, in 45 a large amount of yeast. In No. 43 the nitrogen cycle proceeded in the normal way, but in No. 45 nitrite appeared only very slowly, with no formation of nitrate when the experiment was terminated after 5½ months.

It has been shown that the length of the decomposition cycle depends upon the source of the water and it has been suggested that this might involve the action of growth-promoting substances upon the bacterial flora. Series 44 and 46 (Figs. 3 and 4) were planned as an approach to this question. Two samples of harbor water, the same as in Nos. 43

and 45, were evaporated to dryness and the salt residues ignited at 600–700° C. for 5 hours, to destroy organic matter. The salts were dissolved in the original volume of distilled water, with a little HCl, and the pH brought back to between 7.5 and 8.2 with NaOH. To the two (Nos. 44 and 46) were added amounts of mixed plankton and yeast, respectively, corresponding to the quantities in Series 43 and 45. In

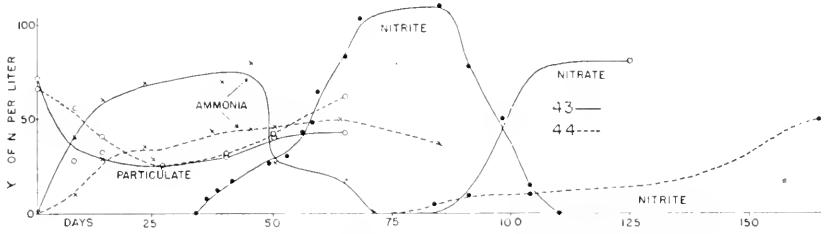

FIG. 3. Series 43 and 44. Mixed plankton added to filtered sea water (No. 43) and to a "synthetic" water made by redissolving the ignited salt residue of evaporated sea water (No. 44). Time in days. Different forms of nitrogen in micrograms (gamma) per liter.

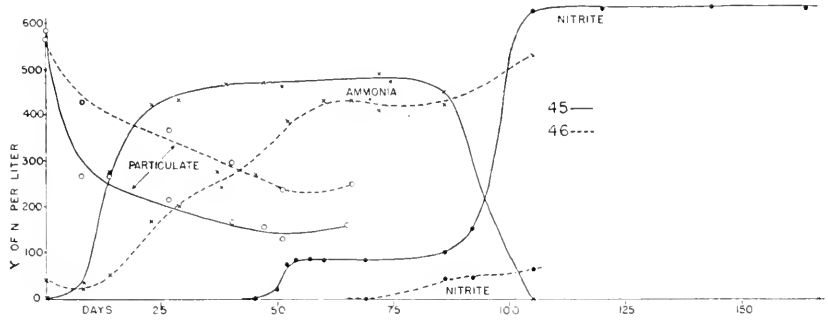

FIG. 4. Series 45 and 46. Yeast added to filtered sea water (No. 45) and to a "synthetic" water made by redissolving the ignited salt residue of evaporated sea water (No. 46). Time in days. Different forms of nitrogen in micrograms (gamma) per liter.

both 44 and 46 the formation of ammonia was much slower than in the untreated water of Series 43 and 45, an effect even more pronounced on the formation of nitrite, which had not reached its maximum at the termination of the experiments. This apparently indicates that some "growth-promoting factor" had been eliminated from the water by the process of ignition, but further investigation will be necessary before a definite conclusion can be reached.

SUMMARY

1. With a recurrent supply of particulate organic matter, the formation of ammonia, nitrite and nitrate may take place simultaneously. The process which predominates will depend upon the stage at which the new organic matter is introduced.

2. The nature of the suspended particulate matter is of considerable importance in determining the total duration of the decomposition cycle, but the level of its original concentration is only a minor determining factor.

3. There is some evidence of a "growth-promoting" factor, normally effective in the decomposition cycle, but which can be destroyed by high temperature.

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THE REPRODUCTIVE CYCLE OF THE VIVIPAROUS TELEOST, NEOTOCA BILINEATA, A MEMBER OF THE FAMILY GOODEIDAE

III. The Germ Cell Cycle

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Introduction

In previous articles on the reproductive cycle of *Neotoca bilineata*, the writer has described the breeding cycle and the somatic cycle of the ovary (Mendoza, 1939, 1940). It was shown that, in *Neotoca*, as is true for many viviparous teleosts, the height of the breeding season occurs during the spring and early summer; broods are spaced approximately 44 days apart. During the breeding cycle, the soma of the ovary was shown to undergo very marked cyclic changes. These changes include (*a*) an increased tumescence of the ovarian stroma, (*b*) a secretory activation of the internal ovarian epithelium, and (*c*) marked changes in the free cellular elements. These conditions are particularly prominent during the middle stages of gestation and later recede to the more normal resting condition. In order to complete the analysis of the reproductive cycle, a study was made of the germ cells and their cyclic variation during gestation. The present account, therefore, presents such an analysis of the germ cells. The detailed description of the germ cells, follicles, their changes during growth, and the fate of the atretic and evacuated follicles, etc., will be considered in a later report.

Previous accounts of the reproductive cycle of viviparous teleosts have made little or no reference to the germ cell cycle, an omission that is difficult to justify since, obviously, the phenomenon is an important phase of the reproductive cycle. It is true, of course, that in many investigations of viviparous teleosts the paucity of material prevented a detailed analysis of the complete gestation cycle. Among recent accounts, the ones on *Jenynsia* (*Fitzroyia*) *lineata*[1] (Scott, 1928) and *Xiphophorus helleri* (Bailey, 1933) contain no reference to the germ cell cycle whereas

[1] E. J. MacDonagh of the Museo de La Plata has kindly informed me that the name *Jenynsia lineata* is considered preferable over the name *Fitzroyia lineata*. In two publications (1934, 1938), MacDonagh uses the name *Jenynsia;* in the latter, he quotes correspondence with C. L. Hubbs of the University of Michigan in which the latter supports the use of the name *Jenynsia lineata*.

Turner (1938*a*) mentions it but briefly in his description of the reproductive cycle of *Cymatogaster aggregatus*. However, Turner does devote considerable attention to the germ cells in his general study of poeciliid fishes (Turner, 1937) and in a special article on the poeciliid, *Brachyrhaphis episcopi* (Turner, 1938*b*). In general, nevertheless, a complete count and measurement of the germ cells during gestation has not been made in a viviparous teleost. Hence it is the purpose of the present account to supplement a preliminary description (Mendoza, 1938) by considering in greater detail the cyclic variation of the germ cells during gestation.

Materials

The present analysis of the germ cell cycle is based on a study of the gonads used in the preceding study on the somatic cycle. Fourteen of these ovaries were chosen at well-spaced intervals during the resting and gestation periods. Detailed cell counts were made in one lateral half of each of the above ovaries; these cell counts, involving a total of 4686 oöcytes, form the basis of the present analysis. The brief description of the germ cells and follicles is based on gonads fixed in Bouin's fluid and stained with iron hematoxylin. In a considerable number of sections, Mallory's triple connective tissue stain was used. A detailed account of the ovaries used and the method of treating the data is given later.

Description of the Germ Cells

As is true for all the Goodeidae, the germinal tissue is confined to the lobulated, ovigerous folds in the ovary. In *Neotoca* there are two such folds, one on either side of the median sagittal septum. The germ cells are confined solely to the ovigerous folds and are more or less evenly distributed throughout the gonad except at the extreme anterior and posterior ends.

In general, the oöcytes of *Neotoca* are essentially similar to those of other viviparous teleosts. The eggs are spherical, attain a maximal size of 180–200 micra in diameter and are characterized by the absence of large masses of yolk. The cytoplasm is coarsely granular and contains the scattered, flocculent, albumen-like yolk. The nucleus of the fully-grown oöcyte is granular, oxyphylic in reaction, and contains typical "lampbrush" chromosomes. Numerous vacuolated nucleoli may appear within the nucleus. Surounding the oöcyte is a follicle composed of a single row of tall columnar cells tightly pressed together. External to the follicle is a thin sheath of connective tissue fibers in which appears a plexus of capillaries.

Germ Cell Cycle

A detailed account of the germ cell cycle during gestation was obtained by using fourteen females (see Table I), chosen at well-spaced intervals before and during gestation. In each female a count was made of every oöcyte, germ cell nest, and atretic follicle in one of the two ovigerous folds. Each oöcyte counted was measured and placed in one of six groups depending upon its diameter (see Table II). The groups

Table I

Females used in the analysis of the germ cell cycle.

Number of Female	Stage of Gestation	Number of Female	Stage of Gestation
6	Non-gravid ovary	12	Embryos 4.5 mm. in length
20	Non-gravid ovary	8	" 4.5 " " "
22	Early segmentation	23	" 6.0 " " "
19	Late segmentation	27	" 6.0 " " "
47	Embryos 1.5 mm. in length	9	" 7.0 " " "
16	" 2.7 " " "	11	" 7.2 " " "
44	" 3.5 " " "	1	" 9.0 " " "

were chosen arbitrarily to facilitate counting and measuring the cells; the actual limits of each group were determined largely by the ocular micrometer units at that particular magnification. Since the total number of oöcytes in each group would vary with the size of the gonad, the average number of cells per half-section of ovary was obtained. Thus, an average figure was obtained that could be compared with those of other gonads regardless of size differences. In order to further reduce the possibilities of individual vaiations of different ovaries, counts were made, wherever possible, of two ovaries for each stage of gestation. Thus six of the eight representative stages chosen are based on the average figures between two different ovaries; only in two stages, IV (3.5 mm.) and VIII (9.0 mm.) are the figures based on a single ovary. All in all, fourteen ovaries were examined histologically, 5633 sections were checked for oöcytes, and 4686 germ cells were counted and measured (see Table II). Graph I is based on these figures. From a careful study of these graphs and figures certain definite and interesting conclusions were obtained.

Conclusions on the Germ Cell Cycle

Continuous Production of Oöcytes

There is no evidence of a complete cessation of egg production during gestation. Nests occur abundantly at all times although, with the excep-

Table II

The germ cell count during gestation.

In order to facilitate the analysis of the germ cells an arbitrary segregation was made of the oöcytes into germ cell nests and six other groups on the basis of size. The six groups and the diameter in micra of the oöcytes involved are indicated below. Similarly, gestation and the resting period were divided into eight arbitrary stages. The pre-fertilization or resting period forms Stage I; thereafter the different stages are distinguished by the stage of development or size of the contained young. Birth normally follows immediately after Stage VIII. In all Stages except IV and VIII, two ovaries were used for the analysis of the germ cells. By using two ovaries it was hoped to get a more typical picture. In the two exceptional cases only one ovary was available for each, but since both ovaries were normal in every respect it is assumed that the cell counts also are typical. For each cell group there are two figures: the whole numbers are the total numbers of cells of each particular group in the different stages of gestation; the numbers in decimals represent the average number of cells of each group per half-section of the ovary.

Number of Ovary	Stage of Gestation	Number of Sections	Number of Nests	Groups of Oöcytes					
				I 10–36μ	II 40–72μ	III 76–108μ	IV 112–144μ	V 148–180μ	VI 184–216μ
6, 20	I Resting	651	136 .2149	560 .8889	121 .1993	54 .0861	29 .0461	41 .0635	4 .0061
19, 22	II Segmentation	640	169 .2641	496 .7750	178 .2781	57 .0891	32 .0500	26 .0406	4 .0063
16, 47	III 2.0 mm.	437	83 .1899	253 .5789	79 .1878	39 .0892	26 .0595	14 .0320	
44	IV 3.5 mm.	154	29 .1883	86 .5584	29 .1883	14 .0909	8 .0521	6 .0389	
8, 12	V 4.5 mm.	507	129 .2544	245 .4832	92 .1815	33 .0651	23 .0454	12 .0237	
23, 27	VI 6.0 mm.	977	106 .1085	681 .6970	162 .1658	54 .0553	65 .0665	14 .0144	
9, 11	VII 7.2 mm.	1417	153 .1079	304 .2145	143 .1009	50 .0353	25 .0176	12 .0085	1 .0007
1	VIII 9.0 mm.	850	128 .1506	493 .5800	72 .0847	22 .0259	14 .0165	13 .0153	
	Totals	5633	933	3118	876	323	222	138	9

tion of the rise in number of nests in Stage V, it can be stated that nests of germ cells decrease in number until the latter part of gestation; at that time they are approximately half as abundant as in pre-fertilization stages.

Time of Onset of Egg-production

The onset of the wave of egg-production for the following brood is not a prominent one as is true for some of the poeciliids described by Turner (1937). Following the general decrease in number of nests and small oöcytes during gestation, there is a rather sharp rise again at the end of gestation when the current brood is about ready for birth. It is unlikely that these minute oöcytes are the ones destined for fertilization and the formation of the following brood. Rather it is more probable that the rise in number of small oöcytes is the first indication of a general activation which results in an increase in the number of eggs of all groups before the following fertilization period. Hence, it is more likely that eggs of Groups III and IV will grow sufficiently during the resting period to form the bulk of eggs to be fertilized for the succeeding brood. Growth of these larger eggs, however, must of necessity occur in the interval between the expulsion of the current brood and the fertilization of the next group of eggs since there is no indication of an increase in number among these larger groups (III and IV) before the end of gestation. The only other noticeable increase in the number of eggs before the end of gestation apparently occurs in Group V eggs, and that increase is but a slight one.

Variation in Number of Eggs with Size

Regardless of the stage of gestation, oöcytes of the 10–36 μ group are the most numerous. Following that there is a regular and almost perfect drop in the number of cells in each succeedingly larger group so that, actually, cells of maximal size, 184–216 μ, are the smallest in number. Only two exceptions occur to this generalization. The first, in Stage VI of gestation, is caused by an abnormally large count of cells of Group 112–144 μ in ovary number 27; this may be purely an individual variation. The second exception is one that is readily understood. In Stage I, preceding gestation, cells of Group 148–180 μ include the bulk of the cells that are to be fertilized and hence appear in large numbers. The number is so large, in fact, that it exceeds that of the next smaller group of cells, Group IV. Furthermore, it is interesting to note that, during the stage of segmentation, since a large percentage of the cells of Group V were fertilized, the count once more drops below that of Group IV eggs and remains so during the rest of gestation.

Decrease in Number of Eggs During Gestation

Not only do eggs decrease in number with increase in size in any one ovary but eggs of all sizes also decrease in number during gestation.

The cell count for each size group is maintained fairly well until the middle period of gestation; from that time on there is a noticeable drop in the number of cells to one-third or one-quarter of their number in the middle stages of gestation. In keeping with this observation, there is a noticeable increase in atretic follicles during the latter half of gestation.

Maximal Size of Eggs

Normally, oöcytes attain maximal size during the resting period of the ovary and remain until stages of segmentation. Following that there is a noticeable absence of large eggs. This condition is verified further by the large number of degenerating follicles of maximal size which are found in pre-gestation stages. Absence of large degenerating follicles and large eggs in the later stages of gestation indicates failure of eggs normally to attain maximal size during that period.

Superfetation

It has been determined before (Turner, 1933; Mendoza, 1939) that superfetation normally does not occur among the growing embryos. It is interesting that, in keeping with this fact, there is no evidence among growing oöcytes of a phenomenon similar to superfetation; rather there is a continuous gradation in the size of oöcytes in all ovaries.

Variation in Number of Oöcytes

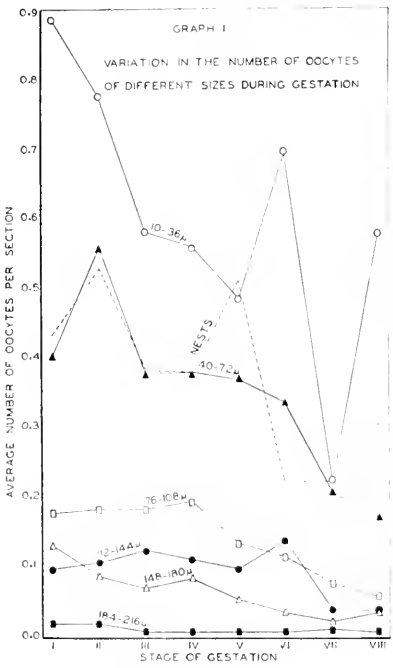
The fluctuation in number of oöcytes of different sizes during gestation varies with the size of the cells. Larger eggs vary but little in number during gestation; smaller oöcytes, however, fluctuate widely in number. The almost perfect inverse relation between the size of oöcytes and fluctuation in number is clearly evident in Table III.

Percentage of Eggs Fertilized

Failure to find fertilization occurring before the expulsion of the previous brood is explainable, in part at least, by the presence of only 20–25 per cent as many large eggs at the end of gestation as there are in the resting ovary immediately preceding the time for fertilization.

An indication of the large number of eggs prepared for fertilization is obtained from the fact that despite the large number of atretic follicles in the gonad prior to fertilization, there still is a greater number of large eggs present at that time than at any other stage of gestation. It appears likely that fully 40–50 per cent of the large eggs available at time of

fertilization actually are activated to start development. This is verified by the knowledge that a normal brood averages from fifteen to twenty young, a number which is approximately half of the total number of



Explanation of Graph I

The graph represents in diagrammatic form the variation of germ cells during gestation. Each curve represents the number of cells of a limited size range during each of the stages of gestation. The different curves are identified by the diameters of the eggs involved. The group of the smallest oöcytes is called Group I; groups of successively larger eggs are called respectively Groups II, III, etc. The stages of gestation are plotted on the horizontal axis (see Table II for the different stages); the numbers of oöcytes of each size group are plotted on the vertical axis. The vertical scale is plotted equally for all curves with exception of the one for Group I (10–36 μ). The values for Group I were so great that, in order to facilitate plotting all curves on the same graph, only the number of cells per half-section are plotted. All other curves are plotted on the basis of number of cells per whole section. The latter figure is hypothetical and was derived by multiplying by two the actual count of cells per half-section obtained during the investigation.

eggs available at time of fertilization (see Table II). The marked drop in the number of eggs of Group 148–180 μ (see Graph I) following fertilization (between Stages I and II) is a further indication of the large number of eggs fertilized. The fact that eggs over 184 μ remain

constant in number until after stages of segmentation probably indicates that growth of the next smaller group of eggs continues until after fertilization and thus maintains the same level, replacing those which had been fertilized. Normally, all eggs fertilized develop completely for degeneration of developing embryos is very scarce.

Atretic Follicles

With reference to the atretic follicles, only two generalizations are justified: (1) large degenerating follicles may occur throughout gestation but are more numerous in early and middle stages of gestation; (2) there is a noticeable increase in the number of small atretic follicles during the

TABLE III

Size of Oöcytes	Variation in Number of Oöcytes per Half Section	Extent of Fluctuation per Half Section
10– 36 μ	.2145–.8889	.6744
40– 72 μ	.0847–.2781	.1934
76–108 μ	.0259–.0909	.0650
112–144 μ	.0165–.0665	.0500
148–180 μ	.0085–.0635	.0550
184–216 μ	.0007–.0063	.0056

latter half of gestation, an observation that agrees with the general decrease of oöcytes of all sizes. On the whole, atresia of the eggs and their follicles does not undergo a cyclic behavior as evident as that found in the development of the oöcytes.

DISCUSSION

The only serious discussion of the variation of germ cells during gestation in viviparous teleosts is that of Turner (1937) on the ovoviviparous poeciliid fishes. In that article he compares a large number of poeciliids and makes several classifications on the basis of (1) number of broods of young in the ovary and (2) the relationship of the growing oöcytes to the stage of gestation. It is evident that *Neotoca,* a true viviparous fish, cannot be classified with any of the poeciliids reviewed by Turner in his interesting article.

In the *Gambusia affinis* type the growing oöcytes are held back until the birth of the current brood. Following that, they grow very rapidly from 0.5 mm. to as much as 1.5 mm. in order to attain maximal size before the next fertilization period.

In the *Lebistes reticulatus* type, the oöcytes are more or less grouped into different sizes, the largest eggs being nearly of maximal size at the end of gestation.

In the *Quintana atrizona* type, fertilization follows immediately upon birth of the previous brood, necessitating that eggs be fully grown and matured at time of birth of the preceding brood.

Two further types are those of *Poecilistes pleurospilus* and *Heterandria formosa* in which superfetation occurs, requiring that different sets of oöcytes grow, mature, and be fertilized while one or more broods of young are still growing within the ovary. Even in *Heterandria* where there are six levels of embryos, Turner recognizes one or two definite waves of eggs.

Neotoca, on the other hand, is unique in that there are no such waves or groups of embryos or oöcytes. The present analysis shows a condition different from any described by Turner for the poeciliids. Here the oöcytes show a continuous gradation in size throughout gestation. Unlike the *Gambusia* type, growth of oöcytes is not inhibited completely, merely somewhat retarded; throughout gestation there is a variable number of eggs of maximal size. Because of this, there is no necessity for a period of marked growth in the interval between birth of young and the following fertilization period. The different sizes of oöcytes merely continue their growth over the non-gestation period. It is true, however, that in *Neotoca* there is a general activation of oöcytes of all sizes in the resting interval because each size group shows a doubling or tripling in the number of cells before the following fertilization period.

Furthermore, since there are eggs of maximal size at the end of gestation in *Neotoca,* it cannot be compared to *Lebistes.* A second point of difference is that in *Lebistes* definite groups of oöcytes are recognized; in *Neotoca* the gradation seems to be complete.

Neotoca resembles *Quintana atrizona* more than the others since in both forms there are fully grown eggs at the end of gestation; however, *Neotoca* differs in that fertilization does not occur until seven days later whereas in the poeciliid, fertilization follows in a few hours. Furthermore, in *Quintana,* apparently the full complement of eggs is present at the end of gestation; in *Neotoca,* however, only between 20–25 per cent of the large eggs are present.

Finally, the condition of superfetation as in *Poecilistes* and *Heterandria* has been made possible in part by the maturing of eggs before the expulsion of the growing young from the ovary and in part by the removal of a physiological block that prevents such growth and fertilization. In view of this, superfetation technically could occur in *Neotoca* since there are eggs fully grown throughout gestation but, in addition,

there still is some physiological obstacle that normally prevents copulation and fertilization. Despite these normal conditions, Turner has reported (1940) occasional examples of superfetation in *Neotoca*. Furthermore, there was one ovary known to the writer in which several eggs had been fertilized within a few hours after the release of a previous brood but the eggs were retained most abnormally within the follicles and not evacuated as is normally true. This observation, in addition to Turner's finding of occasional cases of superfetation, shows that the phenomenon normally does not occur but may, in exceptional cases, get started. These exceptional cases in *Neotoca* apparently are always abortive.

If the germ cell cycle as it occurs in *Neotoca* is true of most or all Goodeidae, certainly an interesting difference occurs between two large and important families of viviparous teleosts.

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UPON THE PRESENCE AND DISTRIBUTION OF A CHROMATOPHOROTROPIC PRINCIPLE IN THE CENTRAL NERVOUS SYSTEM OF LIMULUS[1]

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Certain definite glandular bodies in arthropods have been shown to produce hormone substances. The more important of these are the crustacean sinus gland, located in the eyestalks of a majority of decapod crustaceans, and the corpora allata and the corpora cardiaca located in the vicinity of the esophagus in insects. These glands appear to be concerned with chromatic adaptations, growth, molt, reproduction, metamorphosis, and certain other functions.

Recently it has been pointed out that certain portions of the nervous system act in an endocrine capacity. This has been demonstrated by the work of Kopeć (1922), Brown (1933), Fraenkel (1935), Hosoi (1934), Brown and Ederstrom (1940), Wigglesworth (1940). Furthermore, histological studies of the nervous system of invertebrates as well as vertebrates have shown certain cells and cell clusters whose cytoplasm is definitely filled with granules or colloid, very strongly suggesting glandular activity (See Scharrer and Scharrer, 1940). This latter paper also describes the presence of such neurosecretory cells in *Limulus*.

With these facts in mind, we attempted to discover and measure an endocrine activity of certain tissues in the arachnid *Limulus*. The only work which had been done previously was that of Snyder-Cooper (1938). She was unable to discover any endocrine activity of the eyes, optic nerves, or central nervous system of *Limulus*, using the chromatophore system of *Palaemonetes vulgaris* as a test object. Since there are many chromatophore types in the crustaceans and recent work has demonstrated that the chromatophores show fundamental differences in their responses to known endocrine materials, it appeared worthwhile to reinvestigate the problem using *Limulus* with a number of chromatophore types other than those of *Palaemonetes* as an index of the presence of an active chromatophoric substance.

[1] This investigation was supported by a research grant from the graduate school of Northwestern University.

The experiments reported here are restricted to a consideration of the activity of the central nervous system, because it appeared to be the most likely place of origin of an endocrine substance should any occur within the group, especially so since a portion of the nervous system has been shown to be active in both the other two classes of arthropods investigated. In this report a chromatophorotropic activity of the nervous system of *Limulus* will be described and it will be demonstrated conclusively that the active principle found within the nervous system is not uniformly distributed throughout the nervous tissue but shows a definite differential distribution. It may be seen in the paper following upon this one (Scharrer, 1941) that this differential distribution can be correlated with the distribution of neurosecretory cells within the central nervous system of the same species.

Materials and Methods

The experiments were commenced at the Marine Biological Laboratory at Woods Hole, Massachusetts, where freshly-caught *Limulus* and *Uca* were available, and completed at Evanston, Illinois, using *Limulus* and *Uca* which had been shipped from Woods Hole.

For the preparation of extracts of the nervous system, the live *Limulus* was quickly opened up, the nervous system removed and placed in sea water in a shallow container. The lateral nerves were trimmed away, leaving only their short stubs attached to the large nerve ring and the longitudinal chain of abdominal ganglia. In order to determine the effectiveness of various regions of the central nervous system, the system was cut with a scalpel into seven portions: section 1 included the anterior portion of the nerve ring; section 2, the lateral portions; section 3, the posterior portion of the nerve ring; sections 4, 5, 6 and 7 included respectively the first, second, third, and terminal ganglionic masses of the longitudinal cord. The relative positions of these cuts through the nervous system can be seen in Fig. 1. Each of the seven portions of the central nervous system was placed in a separate mortar and permitted to dry briefly; the nerve masses were then triturated thoroughly with pestles, in 2 cc. of sea water. It is appreciated that the total volume of extract obtained for the various nerve sections was somewhat different, due to the different sizes of the nerve masses. However, since the largest portion of the nervous system used in our experiments weighed less than .04 gram (except in one animal), this error was not considered an appreciable one. The extracts were then brought to a boil in order to precipitate out protein materials from the solution. The clear supernatant fluid was then used for assay purposes.

With each experimental series a control solution was prepared, consisting of a piece of muscle or digestive tract wall or gonad of approximately the same size as the largest nerve portion, extracted and treated in the same manner as the experimental solutions.

A sample of each extract, including the control, was injected into three blinded *Uca pugnax,* each *Uca* receiving an injection of approximately .05 cc. The injection was made into the basal segment of the third or fourth thoracic appendage.

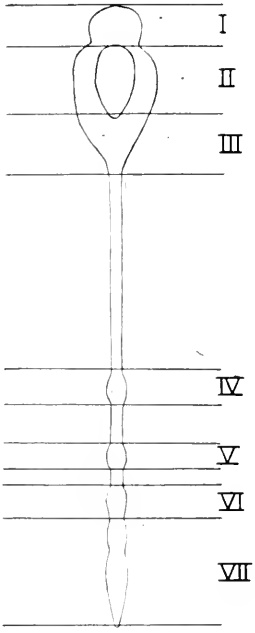

FIG. 1. Diagram of *Limulus* central nervous organs showing the sections of the system which were separately assayed for the chromatophorotropic principle.

Five experimental series were run. The chromatophore index for both black and white chromatophores was recorded at the beginning of each experiment and readings were taken at 15, 30, 45, 60 and 90 minutes. In the first experiment a large *Limulus,* approximately 30 cm. in length from the anterior end of the cephalothorax to the base of the telson, served as the source of nervous tissue. In the remaining four experiments smaller specimens of *Limulus* (about 12 cm. from anterior tip to base of telson) provided the nervous tissue.

Results

The results of these five experiments are shown in tabular form in Tables Ia and Ib. These tables give only the average chromatophore index for the three animals injected with each of the extracts, with the indices for the black and white chromatophores of course averaged separately. In these tables is shown also what has been called the coefficient of effectiveness of the various extracts. This coefficient of effectiveness we realize has only relative significance. It was calculated in the following manner: the sum of the averaged chromatophore indices for each of the two pigments at 15, 30, 45, 60 and 90-minute intervals was obtained. Since an extract having no effect upon the black chromatophores would leave these chromatophores with a chromatophore index of 1 (complete contraction) at each interval—hence a sum of 5—it was considered reasonable to subtract the constant 5 from the sum obtained following injections of active extracts. Similarly, since an extract which would leave the white chromatophores in an initially full dispersed condition (5) would yield a sum of 25, a true index of the effect of an active extract upon the white chromatophores would be the difference between the sum of the average indices and 25.[2] In brief, the coefficient of effectiveness for the black pigment is taken to be $x - 5$ and the coefficient of effectiveness of an extract in concentrating the white pigment is taken to be $25 - x$. In both of these instances x is equal to the sum of the averaged indices. This we believe to be a fair indication of the effectiveness of the extract since it takes into consideration both the magnitude and rate of the response, and, in many cases, duration of the response as well.

Another step was taken to make all the data of the five experiments comparable by obviating the differences which might exist as a result of the different sizes of *Limulus* used for the experiments. This was done by stating the effectiveness of the various portions of the nervous system in terms of the percentage of the effectiveness of Part III (the posterior portion of the ring), which was found in the earliest experiments to be obviously far more effective than any other portion of the nervous system. Thus, in Table I in the column "relative effectiveness" Part III has been arbitrarily assigned an activity of 100 and percentages have been calculated, on the basis of their coefficients of effectiveness, representing the effectiveness of the remaining portions of the nervous system in proportion to Part III.

[2] One difficulty arose which appeared to have no simple solution, namely, that the white chromatophores sometimes initially had their white pigment partially concentrated. It may readily be understood that to the extent to which this is true, the demonstrated differences in concentration of the substance in the nervous system assayed will be minimized.

The results of these experiments are summarized in Table II, in which the relative effectiveness of all the parts of the nervous system and of the control solution have been assembled and averaged. Inspection of these data indicates clearly that Part III is the most active, then,

TABLE Ia

Effect of Extracts upon Uca White

Exp.		0	15	30	45	60	90	Sum	Coefficient of Effectiveness (25−x)	Relative Effect
I	I	5.0	2.3	1.5	1.2	1.3	1.5	7.7	17.2	100.0
	II	5.0	2.5	2.5	1.75	1.25	1.0	9.0	16.0	93.0
	III	5.0	2.8	1.5	1.5	1.0	1.0	7.7	17.2	100.0*
	IV	5.0	2.0	3.0	1.8	2.0	2.0	10.7	14.2	82.5
	V	5.0	1.8	2.5	2.5	2.5	2.5	11.7	13.2	76.6
	C	5.0	5.0	5.0	5.0	5.0	5.0	25.0	0.0	
II	I	5.0	1.5	1.4	1.4	1.3	2.2	7.7	17.3	102.2
	II	4.8	2.2	1.9	1.5	1.2	1.1	7.9	17.1	101.2
	III	5.0	2.0	1.7	1.4	1.2	1.8	8.1	16.9	100.0*
	IV	5.0	3.2	3.1	2.8	2.7	3.5	15.3	9.7	57.4
	V	4.5	2.5	2.2	2.0	1.8	2.2	10.7	14.3	84.5
	C	4.0	4.2	4.2	4.1	4.0	4.0	20.5	4.5	26.6
III	I	4.7	2.7	2.7	2.7	2.7	3.0	13.8	11.2	66.2
	II	4.7	2.0	1.8	1.5	1.3	1.7	8.3	16.7	98.7
	III	5.0	2.3	1.8	1.6	1.3	1.1	8.1	16.9	100.0*
	IV	5.0	2.8	2.3	2.5	2.7	2.8	13.1	11.9	70.4
	V	5.0	3.0	3.0	3.4	3.8	4.5	17.7	7.3	43.2
	VI	4.7	3.3	3.3	3.1	3.0	3.4	16.1	8.9	52.6
	VII	3.4	2.0	1.8	1.8	1.8	2.3	9.7	15.3	90.5
	C	4.5	4.5	5.0	4.7	4.5	4.5	23.2	1.8	10.7
IV	I	3.7	1.8	1.8	1.7	1.5	1.5	8.3	16.7	91.3
	II	2.8	1.5	1.5	1.3	1.1	1.1	6.5	18.5	101.0
	III	2.3	1.7	1.5	1.3	1.1	1.1	6.7	18.3	100.0*
	IV	3.5	2.8	2.8	2.7	2.5	2.8	13.6	11.4	62.0
	V	3.8	2.8	2.7	2.7	2.7	3.1	14.0	11.0	60.1
	VI	3.0	2.8	2.7	2.7	2.8	2.8	13.8	11.2	61.1
	VII	3.2	2.7	2.7	2.8	3.0	3.0	14.2	10.8	59.0
	C	2.8	3.7	3.7	3.7	3.7	3.7	18.5	6.5	35.5
V	I	2.8	1.5	1.3	1.4	1.5	1.3	7.0	18.0	102.2
	II	3.2	2.0	1.7	1.4	1.3	1.5	7.9	17.1	97.2
	III	3.2	1.7	1.5	1.4	1.3	1.5	7.4	17.6	100.0*
	IV	2.7	2.7	2.5	2.4	2.3	2.6	12.5	12.5	71.0
	V	3.3	3.2	3.2	3.1	3.0	3.0	15.5	9.5	54.0
	VI	4.3	3.5	3.0	3.0	3.0	3.2	15.7	9.3	52.9
	VII	3.0	2.3	2.2	2.4	2.7	2.7	12.3	12.7	72.3
	C	3.3	3.2	3.3	3.7	4.0	3.5	17.7	7.3	41.5

TABLE Ib

Effect of Extracts upon Uca Black

Exp.		0	15	30	45	60	90	Sum	Coefficient of Effectiveness (x−5)	Relative Effect
I	I	1.0	2.5	2.5	3.3	2.7	3.0	14.0	9.0	81.8
	II	1.0	2.0	1.8	2.2	2.7	3.3	12.0	7.0	63.6
	III	1.0	2.3	2.5	2.7	4.3	4.2	16.0	11.0	100.0*
	IV	1.0	2.0	2.5	2.7	2.5	2.8	12.7	7.7	70.0
	V	1.0	3.0	2.5	3.0	3.2	2.3	14.0	9.0	81.8
	C	1.0	1.0	1.0	1.0	1.0	1.0	5.0	0.0	0.0
II	I	1.0	1.7	1.8	1.8	1.8	1.7	8.8	3.8	38.8
	II	1.0	1.5	2.6	3.5	4.5	3.0	15.1	10.1	103.0
	III	1.0	1.8	2.5	3.7	4.4	2.4	14.8	9.8	100.0*
	IV	1.0	1.8	1.6	1.3	1.1	1.0	6.8	1.8	18.3
	V	1.0	1.3	1.3	1.3	1.3	1.0	6.2	1.2	12.2
	C	1.0	1.0	1.0	1.0	1.0	1.0	5.0	0.0	0.0
III	I	1.0	2.2	2.3	2.3	2.2	2.7	11.7	6.7	58.8
	II	1.0	1.5	2.5	3.0	3.3	2.7	13.0	8.0	70.2
	III	1.0	1.5	3.2	3.7	4.2	3.8	16.4	11.4	100.0*
	IV	1.0	1.5	1.7	1.4	1.2	1.0	6.8	1.8	15.8
	V	1.0	1.5	1.3	1.1	1.0	1.0	5.9	0.9	7.9
	VI	1.0	1.3	1.2	1.0	1.0	1.0	5.5	0.5	4.4
	VII	1.0	1.5	1.5	1.3	1.0	1.0	6.3	1.3	11.4
	C	1.0	1.0	1.0	1.0	1.0	1.0	5.0	0.0	0.0
IV	I	1.3	2.3	3.5	3.2	3.0	3.2	15.2	10.2	104.1
	II	1.0	1.3	1.8	2.7	3.7	4.3	13.8	8.8	88.6
	III	1.0	1.8	2.3	3.2	4.2	3.3	14.8	9.8	100.0*
	IV	1.0	1.7	1.7	1.7	1.7	2.0	8.8	3.8	38.8
	V	1.0	1.8	1.8	1.7	1.7	1.1	8.1	3.1	31.6
	VI	1.0	1.1	1.7	1.4	1.1	1.0	6.3	1.3	13.3
	VII	1.0	1.5	1.3	1.2	1.0	1.0	6.0	1.0	10.2
	C	1.0	1.2	1.0	1.0	1.0	1.0	5.2	0.2	2.0
V	I	1.0	2.0	2.8	2.8	2.8	3.2	13.6	8.6	90.5
	II	1.0	2.2	2.8	3.2	3.8	2.6	14.6	9.6	101.0
	III	1.0	1.8	3.0	3.2	3.5	3.0	14.5	9.5	100.0*
	IV	1.0	1.3	1.5	1.3	1.1	1.0	6.2	1.2	12.6
	V	1.0	1.0	1.0	1.0	1.2	1.0	5.2	0.2	2.1
	VI	1.0	1.2	1.2	1.2	1.0	1.0	5.6	0.6	6.3
	VII	1.0	1.5	1.5	1.5	1.5	1.3	7.3	2.3	24.2
	C	1.0	1.0	1.0	1.0	1.0	1.0	5.0	0.0	0.0

in order, Part II and Part I, and finally the nerve tissue of the ganglia of the longitudinal cord. With these data only, it is obviously impossible to determine the concentration of active principle within the various parts of the nervous system, since the portions varied considerably in size.

TABLE II

Relative Effects of Parts of Central Nervous System

Exp.	On White							
	I	II	III	IV	V	VI	VII	Control
I	100.0	93.0	100.0	82.5	76.6	—	—	0.0
II	102.2	101.2	100.0	57.4	84.5	—	—	26.6
III	66.2	98.7	100.0	70.4	43.2	52.6	90.5	10.7
IV	91.3	101.0	100.0	62.0	60.1	61.1	59.0	35.5
V	102.2	97.2	100.0	71.0	54.0	52.9	72.3	41.5
Av.	92.4	98.2	100.0	68.7	63.7	55.5	73.9	22.8
Exp.	On Black							
	I	II	III	IV	V	VI	VII	Control
I	81.8	63.6	100.0	70.0	81.8	—	—	0.0
II	38.8	103.0	100.0	18.3	12.2	—	—	0.0
III	58.8	70.2	100.0	15.8	7.9	4.4	11.4	0.0
IV	104.1	83.6	100.0	38.8	31.6	13.3	10.2	1.0
V	90.5	101.0	100.0	12.6	2.1	6.3	24.2	0.0
Av.	74.8	84.3	100.0	31.1	27.1	8.0	15.3	0.2

To make this calculation, it was necessary to know two more facts: first, the volume of the various parts of the nervous system extracted, and second, the relation between the concentration of active principle within an extract and the calculated coefficients of effectiveness.

In order to answer the first problem, the various parts of the nervous system used were individually weighed prior to their extraction. The results of these weighings are found in Table III.

TABLE III

Weights of Parts

No.	Part	Exp. IV Wgt. Gms.	Exp. V Wgt. Gms.	Average
I	Anterior nerve ring	.0291	.0350	.032
II	Lateral nerve ring	.0310	.0347	.033
III	Posterior nerve ring	.0219	.0306	.026
IV	First ganglion	.0047	.0044	.0046
V	Second ganglion	.0041	.0030	.0036
VI	Third ganglion	.0032	.0061	.0047
VII	Fourth ganglion	.0021*	.0073	.0047
Control	Muscle	.0186	.0246	.022

* Part of ganglion was lost in the preparation of the ganglion for weighing, therefore average should be higher.

An experiment was then designed to determine the relationship between the coefficients of effectiveness and the concentration of the active principle in the extracts. In two of the preceding five experiments, a portion of the extract prepared from Part III was set aside in order to determine the effects of known dilutions upon the two chromatophoric types of *Uca*. In this experiment the extract of Part III was diluted to half its original concentration, then one-fourth, one-eighth, one-sixteenth, one-thirty-second, and one-sixty-fourth. Each dilution stage was injected into three *Uca* just as in the original assay experiments and the coefficient of effectiveness of each dilution was calculated in the same way. The results were expressed as percentages, keeping the original concentration of Part III of the nervous system as 100 per cent with the various dilution stages decreasing according to their coefficients. The results of this experiment with respect to the white chromatophores are seen in Table IVa. and for the black chromatophores in Table IVb.

These data were used for calculating the relative concentration of active principle throughout the nervous system of *Limulus,* as follows: a graph was constructed, the abscissa of which represented the logarithm of the relative concentration and the ordinate the effectiveness in terms of percentage of the original concentration. The results are plotted in Fig. 2. The best smooth curves possible have been drawn through the two series of eight points. With the aid of these plots, it was possible to determine the relative concentration of active chromatophorotropic principle by locating the percentage response on the graph and reading the log of the relative concentration on the abscissa. Using this technique, the relative concentration of active principle for the various portions of the nervous system used in this experiment were calculated.

In Fig. 3 we have plotted together, upon the same abscissa (the segments of the nervous system) but on different ordinates, the weights of the various experimental sections of the nervous system and the apparent relative total quantity of active principle in each part of the nervous system. We have assumed that the specific gravity of all portions of the nervous system is roughly constant, which seems reasonable.

Now the apparent relative quantity of active principle in each part of the nervous system was divided by the weight in grams of that particular portion, and a figure was obtained which indicates the relative concentration of the active principle in these portions. These calculations are summarized in Table V. Inspection of this table indicates that Part III of the nervous system has double the concentration of Part II and nearly four times the concentration of Parts I, IV, V and VII, and nearly ten times the concentration of Part VI.

TABLE IVa

Effect of Dilution on White

	15	30	45	60	90	Sum	Coeff.	Percentage Relative Effect	
Exp. IV									
1	1.7	1.5	1.3	1.1	1.1	6.7	18.3	100.0*	
1/2	2.0	1.7	1.5	1.3	1.5	8.0	17.0	93.0	
1/4	2.0	1.5	1.6	1.7	2.2	9.0	16.0	87.5	
1/8	1.7	1.8	1.8	1.8	1.7	8.8	16.2	88.5	
1/16	2.2	2.5	2.6	2.7	2.3	12.3	12.7	69.4	
1/32	2.3	2.7	2.5	2.3	2.5	12.3	12.7	69.4	
1/64	3.7	3.3	3.7	4.0	4.0	18.7	6.3	34.4	
0	3.7	3.7	3.7	3.7	3.7	18.5	6.5	35.5	
Exp. V									Average
1	1.7	1.5	1.4	1.3	1.5	7.4	17.6	100.0*	100.0
1/2	1.8	1.5	1.4	1.3	1.3	7.3	17.7	100.8	96.9
1/4	2.2	2.3	2.0	1.7	1.5	9.7	15.3	87.0	87.3
1/8	2.2	2.0	1.9	1.8	2.2	10.1	14.9	84.7	86.6
1/16	2.2	2.2	2.0	1.8	1.8	10.0	15.0	85.3	77.4
1/32	3.5	3.2	3.2	3.2	3.2	16.3	8.7	49.5	59.5
1/64	3.0	2.5	2.6	2.6	2.6	13.3	11.7	66.5	50.5
0	3.2	3.3	3.7	4.0	3.5	17.7	7.3	41.5	38.5

TABLE IVb

Effect of Dilution on Black

	15	30	45	60	90	Sum	Coeff.	Percentage Relative Effect	
Exp. IV									
1	1.8	2.3	3.2	4.2	3.3	14.8	9.8	100.0*	
1/2	2.2	2.3	2.7	3.2	3.2	13.6	8.6	87.7	
1/4	2.8	3.8	3.5	3.2	3.0	16.3	11.3	113.0	
1/8	2.3	2.3	2.4	2.5	2.3	11.8	6.8	69.4	
1/16	1.8	1.7	1.6	1.5	1.3	7.9	2.9	29.6	
1/32	1.5	1.3	1.3	1.3	1.2	6.7	1.7	17.3	
1/64	1.2	1.3	1.3	1.3	1.2	6.3	1.3	13.3	
0	1.0	1.2	1.0	1.0	1.0	5.2	0.2	2.0	
Exp. V									Average
1	1.8	3.0	3.3	3.5	3.0	14.3	9.3	100.0*	100.0
1/2	2.0	2.3	2.3	2.3	2.6	11.5	6.5	69.9	78.8
1/4	1.5	1.5	1.6	1.7	1.7	8.0	3.0	32.2	72.6
1/8	1.7	1.7	1.9	2.2	1.7	9.2	4.2	45.1	57.2
1/16	1.5	1.5	1.6	1.8	1.7	8.1	3.1	33.3	31.5
1/32	1.3	1.3	1.5	1.7	1.5	7.3	2.3	24.7	21.0
1/64	1.0	1.0	1.0	1.3	1.0	5.3	0.3	3.2	8.2
0	1.0	1.0	1.0	1.0	1.0	5.0	0.0	0.0	1.0

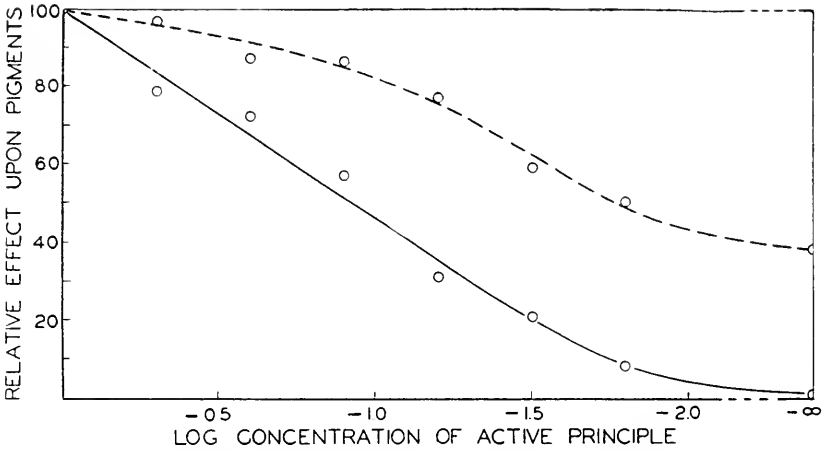

FIG. 2. The relationship between the log concentration of the active principle and the relative effectiveness upon the *Uca* white (dashed line) and black (solid line) pigments.

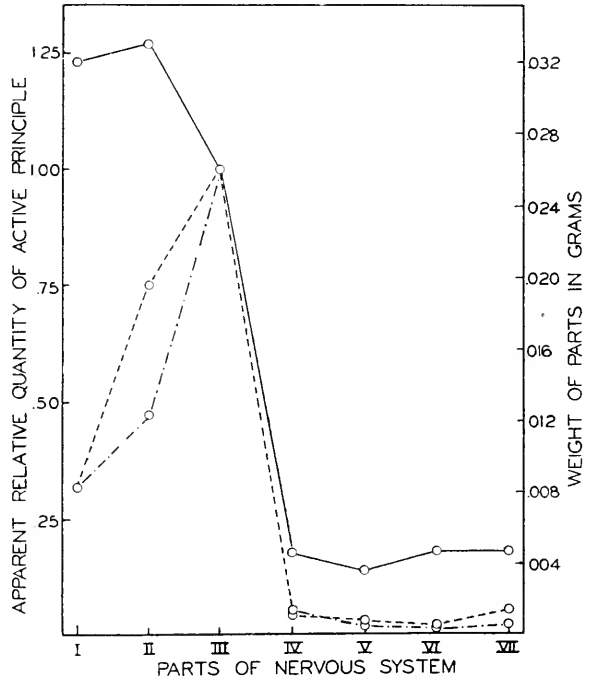

FIG. 3. Plotted together for comparison are the apparent relative quantity of white pigment concentrating principle (dashed line), of black pigment dispersing principle (dot-dash line), and weights of each of the seven assayed parts of the *Limulus* nervous system.

TABLE V

Part	Apparent Rel. Quant. Black Disp. Principle	Apparent Rel. Quant. White Conc. Principle	Weight of Part (gms.)	Rel. Conc. Black Disp. Principle	Rel. Conc. White Conc. Principle	Average
I	0.32	0.32	0.032	10.0	10.0	10.0
II	0.47	0.75	0.033	14.3	22.8	18.6
III	1.00	1.00	0.026	38.5	38.5	38.5
IV	0.053	0.043	0.0045	11.8	9.6	10.7
V	0.045	0.034	0.0035	12.9	9.7	11.3
VI	0.016	0.022	0.0047	3.4	4.7	4.1
VII	0.024	0.054	0.0047	5.1	11.5	8.3

Effects of the Chromatophorotropic Principle of Limulus Nervous System upon Certain Other Chromatophore Types

In the light of the work of Snyder-Cooper (1938) in which she found no apparent chromatophorotropic effects of injection of *Limulus* nervous system extracts upon *Palaemonetes* chromatophores, we believed it worthwhile to repeat her experiments. We were also unable to show any response of either the red or the white chromatophores of *Palaemonetes* to these extracts.

Extracts of the nervous system of *Limulus* were tested upon isolated chromatophores of *Cambarus,* according to the technique of Brown and

TABLE VI

	Uca		*Cambarus*		*Palaemonetes*	
	Black	White	Red	White	Red	White
Sinusgland *Cambarus*	—	—	C	D	—	—
Sinusgland *Uca*	D	D	—	—	C	D
Nervous system *Cambarus*	—	—	C	C	—	—
Corpus cardiacum insects	D	C	C	O	—	—
Brain insect	—	—	O	C	—	—
Nervous system *Limulus*	D	C	O	C	O	O

Meglitsch (1940) and it was found that the chromatophorotropic principle of *Limulus* was very effective in concentrating white pigment, but was entirely without effect upon the red. Furthermore, as Fig. 4 indicates, the relative effectiveness of Parts I through VII was approximately the same upon *Cambarus* white chromatophores as upon *Uca* black and white.

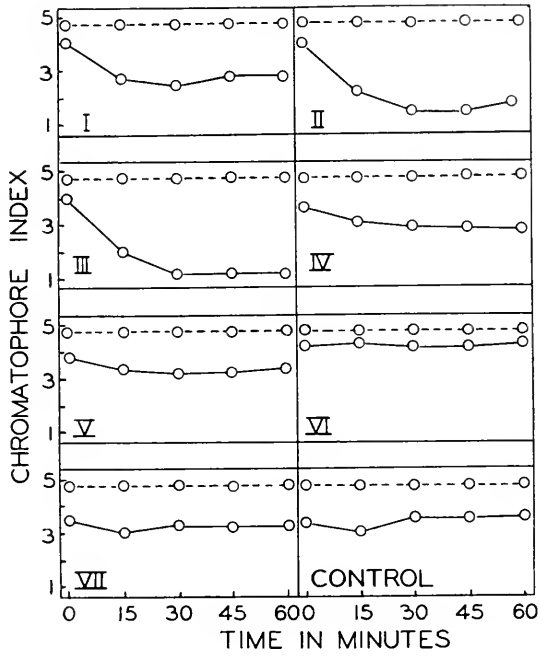

FIG. 4. Relative effects of the parts of *Limulus* nervous system upon crayfish (*Cambarus immunis*) white chromatophores (solid line) and red ones (dashed line). Five upon the ordinate indicates a fully dispersed pigment mass, and one, a fully concentrated pigment mass.

DISCUSSION

Comparison of the Chromatophorotropic Material of Limulus with Chromatophorotropic Materials of Other Arthropods

Table VI has been prepared to show the effects of various arthropod organ extracts upon six types of crustacean chromatophores. This table is admittedly incomplete and although it would be both interesting and instructive to have the gaps filled, it is still possible to draw certain conclusions from it as it is.

In comparing the action of the *Limulus* nerve cord extract with that of the sinus gland extracts of *Uca* and of *Cambarus,* we see that upon *Palaemonetes* red and white chromatophores the *Limulus* extract has no effect, whereas definite and characteristic effects are produced by sinus gland. Upon *Cambarus* red, sinus gland exercises a strong concentrating influence; this is apparently entirely lacking in the *Limulus* extract. Upon *Cambarus* white chromatophores, on the other hand, both the extracts are effective but result in opposite responses of the chromatophore. Similarly, the two extracts have opposite actions upon *Uca* white chromatophores,[3] but upon *Uca* black the activity of the two substances is qualitatively the same.

It seems to us reasonable to assume that this is a similar response of the chromatophore to two chemically different materials, in other words, a non-specific chromatophore reaction. We assume this inasmuch as our experiments suggest that both the white-concentrating action and the black-dispersing action were produced by the same material.

At first it may seem rather extraordinary that *Limulus* should be suspected of having a chromatophorotropic material because of the absence of functional chromatophores in this group of animals, but many other organisms without physiological color change (cockroaches, etc.) possess active corpora cardiaca and sinus glands, and there is abundant evidence accumulated that the chromatophorotropic action of these organs is only one of a number of functions, many of which are far more basic in the life processes of the animals than that of chromatic adaptation. We have utilized the chromatophore response as a test method with a full appreciation of this fact.

There are some who will contend that the materials with which we dealt are nothing more than materials resulting from the mechanical destruction of nerve tissue and possess no normal endocrine function within the organism. This seems highly unlikely considering a number of observations such as the restriction of a specific material to the commissural ganglion of *Crago* and the restriction of a specific action to the corpora cardiaca of insects, and finally, in this research, to a definite demonstration that the material is not uniformly distributed throughout the nervous system, some portions of the nervous system showing roughly ten times the concentration of active principle shown by others. There is some evidence, however, that the material in question in *Limulus* is not produced by a single locus within the nervous system and then

[3] Abramowitz (1937) states that *Uca* eyestalk extract concentrates white pigment in *Uca pugnax*. We have been unable to confirm this observation of Abramowitz and, on the contrary, find that *Uca* eyestalk extract has a definite and striking dispersing action upon white pigment, just as seen in *Palaemonetes* and *Cambarus*.

distributed out from this center by diffusion because it was demonstrated in a brief experiment that the longitudinal commissure connecting the posterior end of the nerve ring with the first ganglion of the longitudinal chain showed significantly lower concentration of active principle (practically no effect) than either the posterior portion of the nerve ring or the ganglion at its opposite end. In the case of diffusion, a smoothly gradual decline in activity would be expected. The increased activity at the posterior tip of the central nervous system also argues against the diffusion of the material out from a single center. Therefore we are inclined to believe that this differential distribution of activity in the nervous system is the result of a differential distribution of cells actively engaged in the production of the substance.

The Probable Number of Hormonal Substances in Limulus Nervous System

If one examines Fig. 3 one is impressed with the parallel nature of quantitative distribution of the black and white pigment-concentrating principles. The differences which occur are not only readily within the experimental error but also are even astonishingly close to one another. On the basis of these data there is no justification for any assumption other than that these two pigments are being affected by one and the same substance. An examination of Fig. 4, showing the effects of the various parts of the nervous system of *Limulus* upon the white chromatophores of *Cambarus*, shows a quantitative gradation of activity of the various parts quite parallel to those shown in Fig. 3 for the *Uca* chromatophores. Again there is apparently no reason for assuming anything other than that the substance active upon *Cambarus* white chromatophores is the same substance responsible for influence on *Uca* chromatophores. We realize the danger of drawing any conclusions upon negative evidence and hence conclude only that there is no suggestion for more than one chromatophorotropic principle in *Limulus* nervous system. However, just as Snyder-Cooper failed to show the presence of any chromatophorotropic principle in *Limulus* using *Palaemonetes* red and white chromatophores, so is it quite possible that utilizing other chromatophores than we have tried will demonstrate the presence of other hormones than we have been able to demonstate.

The action of *Limulus* extract is qualitatively unlike that of extracts of the commissural ganglia and other nervous organs in *Cambarus*, as shown by their effect upon *Cambarus* red chromatophores. The *Cambarus* extracts show an extremely potent activity in concentrating the red pigment while *Limulus* extracts show no effect. On the other hand, the

effects of these two extracts are identical upon *Cambarus* white chromatophores. Two alternative explanations are possible: (1) that the white-concentrating principle from these two sources is similar and that *Cambarus* nervous system possesses in addition a red pigment-concentrating principle, or (2) one could assume that each extract possesses a single principle which is structurally different in the two cases. Again, *Limulus* nerve organ extract differs from extracts of the corpora cardiaca of insects in having a different action upon both red and white chromatophores of *Cambarus*. Finally, in comparing the activity of insect brain and the activity of *Limulus* nervous system, one finds a qualitatively similar action of these two extracts upon both red and white chromatophores of *Cambarus*. Neither possesses an effect on the *Cambarus* red pigment and both exercise a white pigment-concentrating action. Of course it is too soon even to suspect that these latter two substances are identical and further conclusions cannot be drawn until more properties of these two substances have been shown to be identical.

A consideration of these results brings us to a complete realization that a unitary theory of hormonal control of chromatophores in crustaceans—and even more in arthropods in general—is completely untenable. There are undoubtedly several different chromatophorotropic materials found within the various groups, but it is not beyond the realm of possibility that certain threads of similarity or continuity can be woven through various active tissues and their secreted principles in this phylum of animals.

It can be calculated readily that the posterior portion of circumesophageal nerve ring of *Limulus* is still effective when diluted in nearly 5000 times its volume of salt solution. The active secreting cells undoubtedly occupy a very small percentage of the volume of the nervous tissue and consequently, in terms of the ratio of neuro-glandular tissue to volume of extract, the maximal dilution value would be in the hundreds of thousands or even millions.

Summary

1. A principle influencing pigment concentration in *Uca* chromatophores is found in extracts of the central nervous system of *Limulus polyphemus*. This principle is not uniformly distributed through the central nervous system of *Limulus* but is concentrated in the ganglionic masses, with the greatest quantity in the posterior portion of the circumesophageal nerve ring. The lateral portions of the nerve ring show approximately one-half the concentration of the posterior portion and

all the remaining portions of the nervous system show from one-quarter to one-tenth the concentrations of the posterior portion of the nerve ring.

2. The distribution of the principle influencing *Uca* white pigment appears to be identical with that producing dispersion of the *Uca* black pigment and concentration of *Cambarus* white pigment. Hence it is concluded that all three of these effects are brought about by one and the same principle.

3. Certain physiological properties of the chromatophorotropic material from the nervous system of *Limulus* were compared with corresponding properties of certain other invertebrate hormones and it was found that the *Limulus* chromatophorotropic principle is physiologically unlike any other known arthropod hormone substance with the possible exception of insect brain extract.

4. It is calculated that an extract of the posterior portion of the circumesophageal nerve ring is still effective when diluted in nearly 5000 times its volume of salt solution.

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NEUROSECRETION

IV. Localization of Neurosecretory Cells in the Central Nervous System of Limulus

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From an investigation, still under way, of the neuroglandular cells in the central nervous system of the horseshoe crab (*Limulus*), mention of which has been made in a previous review article (Scharrer and Scharrer, 1940), some data concerning the occurrence, localization, and numerical distribution of these cells are reported here. The publication of these data appears to be timely in view of the findings of Brown and Cunningham, reported in the preceding paper of this journal (1941). These authors demonstrate the presence of a chromatophorotropic principle within the nervous centers of *Limulus* and calculate the concentration of this substance in different, separately tested portions of the nervous system. They conclude that the chromatophorotropic principle is probably produced in definite groups of cells within the central nervous system. The agreement between the results of the physiological work of Brown and Cunningham and the morphological findings to be reported here suggests the possibility that in *Limulus* the neurosecretory cells are the source of the chromatophorotropic principle.

Material and Methods

Altogether forty adult male and female specimens of *Limulus polyphemus* were studied. Most of the material was preserved during the summer months of 1937, 1939, and 1940 at the Marine Biological Laboratory, Woods Hole.[1] Additional specimens were obtained alive from the New York Aquarium,[2] and were preserved for histological study at various intervals during the years 1938–1940. Furthermore, a few young specimens of *Limulus polyphemus* and two female *Limulus moluccanus*, one of them from Penang (Malay Peninsula) fixed in the summer

[1] For the use of research facilities during these periods the author is indebted to the Rockefeller Foundation and to the Rockefeller Institute for Medical Research, New York.

[2] I am obliged to Dr. C. W. Coates for his friendly assistance in obtaining this material.

of 1938, the other from the mangrove swamps at Chandipur (Orissa, India),[3] were included in this study.

In order to obtain comparable results, the same histological technique was used in all cases. The central nervous system, which in adult specimens is of considerable size, was carefully dissected out and was fixed in Zenker-formol. It was subsequently embedded in celloidin (nitrocellulose) and horizontal serial sections of 20 μ were stained with Foot's modification of Masson's trichrome method.

The various degrees of neurosecretory activity to be found in different parts of the central nervous system as well as in the different specimens of *Limulus* studied were estimated by counting in every section all cells containing secretory colloid. Thus undoubtedly a certain percentage of the cells was counted more than once because the vacuoles containing colloid are often large enough to appear in more than one section of 20 μ thickness. This is not to be considered an error of consequence, because this method of recording colloid whenever it appears in the sections, even if it belongs to the same cell, takes account of the volume of colloid as a whole rather than of the actual number of cells containing colloid inclusions. For purposes of comparing the secretory activity of different regions of the central nervous system this method appears to be satisfactory.

Observations

The Histological Appearance of the Neuroglandular Cells

It is not intended here to describe the cytological characteristics of cells considered as having a glandular function or to investigate the steps in which the transformation of a nerve cell into a gland cell takes place. In the present study the concern is only with such cells as are believed to represent the fully developed type of neurosecretory cell characteristic of *Limulus*. This cell type is fairly uniform and easily recognizable. The cells contain large masses of a colloidlike substance (Fig. 1) which appears homogeneous in sections treated in the manner described before. The substance stains green with the light green component of the Masson trichrome stain and seems to have physical properties not unlike those of the colloid of the thyroid gland (for corresponding parallels see also Hanström, 1941). This similarity is also suggested by vacuoles in the periphery of the colloid masses in the cells of *Limulus* which remind the observer of similar vacuoles seen in sections of thyroid colloid. Apparently this colloid mass pushes the nucleus and the cytoplasm aside and

[3] The efficient cooperation of Dr. Baini Prashad, Director of the Indian Museum, Calcutta, India, is gratefully acknowledged.

takes up a large space in the cell. These conspicuous colloid-carrying cells were counted.

Cells of this kind have also been found in the ganglia of cockroaches (Scharrer, 1941). However, in the cockroach they represent one of several kinds of neurosecretory cells, whereas in *Limulus* only this one type is encountered. There are in *Limulus* cells the appearance of which suggests that they represent phases preceding the fully developed "mature" neurosecretory cell but their description is not undertaken here, since their relation, if any, to the colloid-containing cells is still undetermined. From a histological point of view they certainly seem insignificant as a possible source of secretion material when compared with the

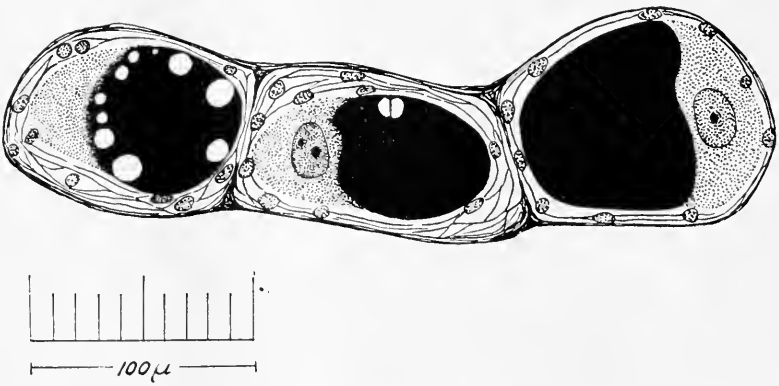

FIG. 1. Three neurosecretory cells, each in its capsule, from the circumesophageal ring of *Limulus polyphemus* with partly vacuolated colloid indicated in solid black. Zenker-formol, nitrocellulose, 20 μ, Masson.

cells containing the large masses of colloid. The contrast between the ordinary nerve cells, including those in which differences in stainability of the cytoplasm etc. suggest that they may be in a state of transformation into glandular elements, and the cells counted here as neurosecretory cells is always so definite that no doubt arises as to which cells should be included in the counts. *Limulus* is particularly favorable in this respect for the kind of investigation carried out here.

It should be repeated that the cells containing a homogeneous mass of colloid with varying numbers of marginal vacuoles are always observed when the histological technique described before is used. The fact that the appearance of the colloid is somewhat different after treatment by different methods is of no concern here where only the amount and distribution of the secretory elements are of interest.

The Localization of the Neuroglandular Cells within the Central Nervous System

The central nervous system of *Limulus* consists of the circumesophageal ring, situated in the cephalothorax, and the abdominal ganglia (Fig. 2). The ring contains the "brain" and a number of thoracic ganglia of the ventral cord, designated in Fig. 2 as Nos. 1–8, beginning with the cheliceral ganglia. There are, in addition, eight pairs of abdominal ganglia, only the first four or five of which are well defined, the remaining pairs being fused together (Patten and Redenbaugh, 1900).

With the exception of the corpora pedunculata which make up about three-fourths of the brain, all parts of the central nervous system of *Limulus* contain neurosecretory elements among the ordinary nerve cells. The distribution of the neurosecretory cells varies, however, with respect to the different ganglia and their total number shows the greatest variations from specimen to specimen.

A most "active" neurosecretory region is the posterior part of the circumesophageal ring, i.e. the area of the thoracic ganglia No. 6 and No. 7. Here the neuroglandular elements are found in clusters which may constitute a considerable proportion of the total mass of cells in the ganglion. These clusters of glandlike cells are arranged symmetrically with respect to the mid-sagittal plane (Fig. 3).

More anteriorly in the ring the neuroglandular cells become less and less frequent. They may even be absent in these portions, particularly in those specimens which, on the whole, show less neurosecretion than others. If present, neuroglandular cells in ganglia No. 1 to No. 5 appear single or in small groups of two or three. Their approximate number and position are the same on both the left and right side of the ring. Of all neurosecretory cells found in the circumesophageal ring only about one-tenth or less lie in the two anterior thirds, the majority being concentrated in the posterior third of the whole ring.

The abdominal ganglia, counted together, contain roughly on the average twice or three times as many neurosecretory elements as the corresponding ring. The numbers of neuroglandular cells counted in the circumesophageal ring and in the abdominal ganglia of four specimens are given here as examples:

Circumesophageal ring	117	206	264	516
Abdominal ganglia	340	442	861	1978

Thus as a rule and within limits, from the degree of neurosecretory activity in a given ring the activity in the abdominal ganglia of the same specimen can be predicted. The individual abdominal ganglia do not

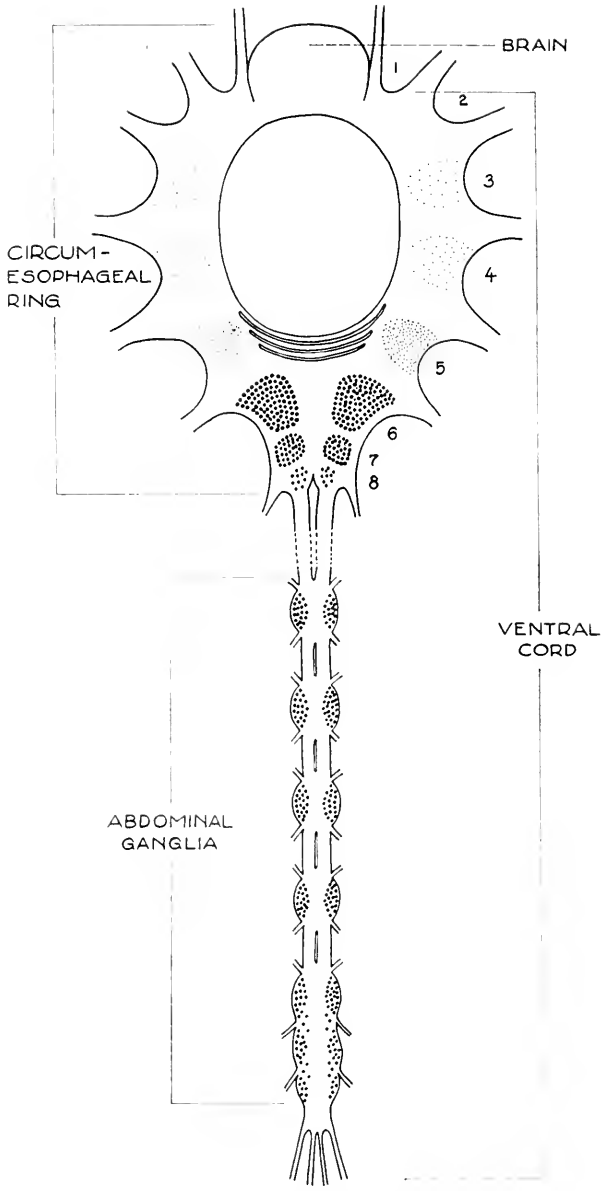

FIG. 2. Diagram of the central nervous system of *Limulus*. Numbers 1–8 are the ganglia of the circumesophageal ring. The areas where neurosecretory cells may be found are dotted. Coarser dots indicate a higher degree of neuroglandular activity than finer dots.

seem to differ significantly from each other in number of neurosecretory cells.

Individual Variations of Neurosecretory Activity

Whereas the central nervous system of a few young specimens of *Limulus polyphemus* (width of carapace from 5 to 8 cm.) does not con-

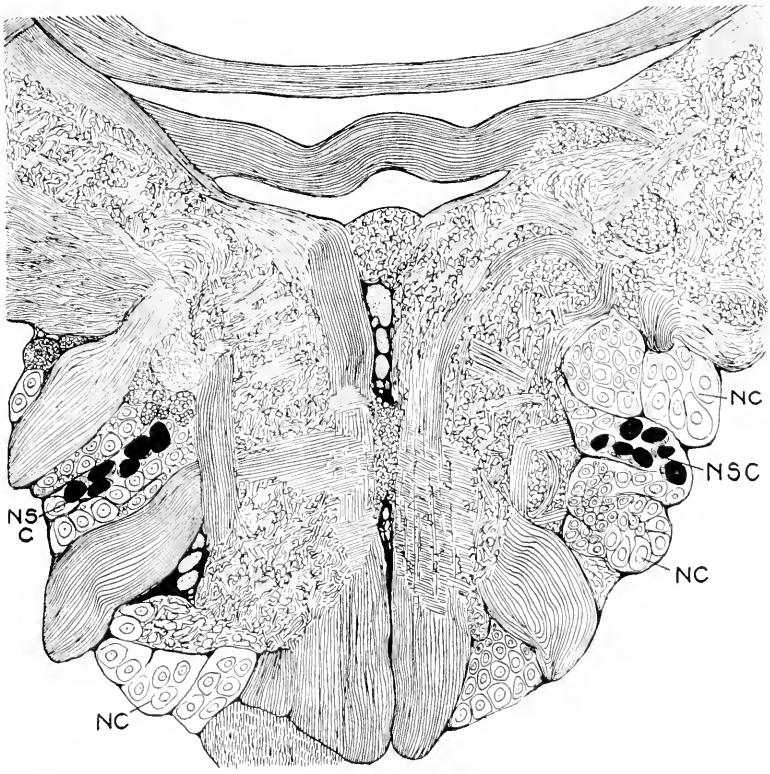

FIG. 3. Horizontal section through posterior part of the circumesophageal ring of *Limulus polyphemus*. *NSC*, neurosecretory cells; *NC*, nerve cells. The colloid of the neurosecretory cells which appears green with the histological technique used is indicated by solid black. Note symmetry of neurosecretory cell groups. Zenker-formol, nitrocellulose, 20 μ, Masson.

tain neuroglandular cells, these cells are present in all adults studied thus far. This concerns animals from different geographical sources as well as from two different species. Furthermore, neurosecretion is found in both male and female *Limulus*.

The degree of neurosecretory activity, however, varies considerably from specimen to specimen. In some, the entire central nervous system is found to contain only one or two neuroglandular cells, in others over

a thousand such cells may be counted by the method described. The highest count thus far made, 2494, was in a large female. Between these extremes are such counts as 457, 648, 1125. These figures are given only to show the wide variation in view of which the errors necessarily involved in the calculation method employed here appear of minor importance.

The question may be asked next whether there exist any relations between the degree of neurosecretory activity and certain known factors. The following observations were made:

(1) A 24-hour cycle of secretion does not seem to exist. The histological appearance of neuroglandular cells is the same in different specimens fixed at various hours of the day.

(2) Neurosecretion in *Limulus* is not restricted to one time of the year, such as, for instance, the breeding season. None of the summer specimens contains more neuroglandular cells than the animals with the highest counts fixed in January, March, or November.

(3) The degree of neurosecretory activity in males and females is not essentially different. On the average, however, smaller numbers of neuroglandular cells are found in males, but this may be explained by the smaller average size of the male central nervous system.

(4) So far the only factor of some importance appears to be the age of the animals, as expressed by the size, i.e. the width of the carapace. As a rule, the larger specimens show a more active state of neurosecretion. The *Limulus* with the highest count of neurosecretory elements (almost 2500) was the largest specimen studied, a female of 32 cm. width which contained many and large eggs. On the other hand, none of five females under 23 cm. width showed more than about five secreting cells. Also among the males the largest specimen examined yielded the highest count but that does not exclude the fact that one or another small male may be encountered in a comparatively active state of neurosecretion. Thus, for instance, in the smallest male among a dozen studied, having a carapace-width of 16.5 cm., the relatively high number of 648 secreting cells was found. For comparison it may be noted that in two other male specimens these counts were made: carapace 19 cm., 5 cells; carapace 20 cm., 205 cells.

Although the existing evidence is not entirely conclusive, the extent to which nerve cells are engaged in secretory activity seems to run grossly parallel with age as expressed in the size of the animal. This fact has to be taken into account in attempts to influence experimentally the ratio of neuroglandular cells. Such experiments as have been carried out up to the present time were unsuccessful. In one extensive series extracts were made from the circumesophageal ring of the central nervous

system of *Limulus* by grinding it with sand and sea water. The extracts from several, for instance five, different specimens of varying sizes and different sex, were pooled and injected into the body cavity of male and female specimens over varying periods of time. Approximate estimates were made of the total number of rings injected into each experimental animal. Thus, for instance, each of several animals got the equivalent of 17 rings over a period of one month. The counts made in such animals kept well within the limits of the normal variation:

♂	21 cm.	1073	♀	23 cm.	1
	21 "	617		23.5 "	22
	22 "	446		28 "	492

From the number of experiments done by the method described it can be safely concluded that sea water extracts from the neurosecretory cells of *Limulus* do not influence the number of cells engaged in secretion. It may be added also that no change in the histological appearance of these cells has been observed.

Similarly ineffectual were injections of pilocarpin. Of a 1 per cent solution in two specimens as much as 19 cc. were administered by means of two injections, both given on one day. Smaller amounts were injected in others. Again, the counts and the histological appearance of the cells gave no indication that pilocarpin acts on the neuroglandular cells in the concentrations used here which, in view of their general effect on the animals, may be considered as near the toxic ones.

Discussion

The data reported here on the occurrence and distribution of neurosecretory cells in the central nervous ganglia of *Limulus* correlate well with the findings of Brown and Cunningham (1941). The two authors describe a chromatophorotropic principle in extracts from all parts of the central nervous system of *Limulus polyphemus*. From the effect on crustacean chromatophores they conclude that the concentration of the active material varies with respect to different, separately tested parts of the central nervous system. The present histological study demonstrates the presence of nerve cells offering the picture of gland cells in all portions of the nervous system tested by Brown and Cunningham. The distribution of these elements in the nervous tissue varies in different regions, and this variation corresponds well with the distribution of the chromatophorotropic material found by these two authors. This correlation becomes particularly evident in the different portions of the circumesophageal ring. Its posterior third for which Brown and Cunningham report the greatest concentration of the active principle con-

tains the majority of neurosecretory elements present in the ring. In the lateral sectors where the relative chromatophorotropic activity shows a considerable decrease, many fewer cells are found to contain secretory colloid. The anterior portion of the ring with relatively the lowest action on crustacean chromatophores contains glandular cells only occasionally. The connectives between the circumesophageal ring and the abdominal ganglia did not yield the chromatophorotropic principle; neither colloid nor neuroglandular cells are found in these connectives.

Considering that the estimate of the concentration of the chromatophorotropic principle in the nervous system of *Limulus* must necessarily be approximate, and that the cell counts may mean little in terms of function, the correlation between physiological and anatomical findings demonstrated here seems all that can be expected.

Within its obvious limitations this correlation consequently suggests that the neurosecretory cells in the central nervous system of *Limulus* may be considered as the source of the chromatophorotropic principle. If this proves to be correct the functional significance of neurosecretion assumes a new aspect. Thus far only one function has, with good evidence, been attributed to neuroglandular cells, namely the production of a hormone controlling molting in insects (Wigglesworth, 1940).

Summary

The occurrence, localization, and quantitative distribution of neurosecretory cells in the central nervous system of *Limulus* have been described. These anatomical findings are in good agreement with the physiological data of Brown and Cunningham (1941), who demonstrate the presence and distribution of a chromatophorotropic principle in the nervous system of this animal. Therefore, the neurosecretory cells of *Limulus* may be considered as the source of a substance influencing color change in crustaceans.

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THE ACTION OF ACETYLCHOLINE, ATROPINE AND PHYSOSTIGMINE ON THE INTESTINE OF DAPHNIA MAGNA

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(From Bard College, Columbia University)

Introduction

The effects of acetylcholine when administered to animals under experimental conditions have recently afforded valuable information which suggests the possibility that acetylcholine is acting as a chemical transmitter of nervous impulses from nerve endings to certain organs of the body. In view of the excitatory action of acetylcholine which has been demonstrated on the heart of crustaceans (Welsh, 1939 *a* and *b*) and the influence of this substance on the autotomy of certain members of this group of animals (Welsh and Haskin, 1939), it appears worth while to investigate the action of this drug on the intestine of a cladoceran, a problem heretofore unexplored. Although there is nothing in Cladocera which corresponds morphologically to the autonomic nervous system in vertebrates, the intestine of these animals is subject to accelatory and inhibitory nervous influences. If the intestine of *Daphnia magna*, for example, is touched with a very fine glass needle at the bend of the digestive tube where the intestine enters the stomach, the heart immediately stops beating and the posterior region of the intestine commences to exhibit powerful intestinal contractions. After a certain period, depending on the strength of the mechanical stimulus applied, the heart renews its activity and the intestine reestablishes its normal muscular contractions. If acetylcholine is involved in the transmission of nervous impulses to this organ, it should be possible to obtain some evidence of the action of this substance and other substances with which it has been said to be associated, when they are administered to this animal.

Methods

Daphnia magna young, when in their second instar, were used exclusively for the experimental work. The animals at this stage measure about 1 mm. in length, they are more transparent than the adult individuals and hence the changes produced in the course of the experimentation can be easily observed under the microscope. The mothers from

which the young were obtained for the experiments were reared at 25° C. in bottles containing the standard amount of the culture medium (Banta, 1921). The daily examination of the animals and the other methods employed in rearing the organisms in the laboratory (Obreshkove, 1930) enabled us at all times in the course of the experimental work to secure animals which were of the same age. A careful selection of the animals was necessary, because of the endeavor which was made to measure the period which elapsed between the addition of the particular chemical substances under investigation and the characteristic changes which they produced. The chemical substances employed were acetylcholine chloride, physostigmine (eserine) and atropine. Care was taken to use freshly diluted chemicals. The acetylcholine was adjusted to pH 5.7.

The animals were subjected to experimentation separately. A single individual was transferred to a micro culture slide with polished spherical concavity 18 mm. in diameter and approximately 3 mm. deep. The culture medium surrounding the animal was removed and immediately after this there was added the chemical substance whose action on the animal was to be studied. The amount of solution employed in each depression slide was kept the same and in each case it was just sufficient to cover the animal without permitting it to carry on extensive locomotive movements. This procedure enabled us to make continuous observations on a single individual under the microscope. The animal is seen at all times to ingest solid particles and fluid with which it comes in contact in the depression slide. With each opening of the mouth, a quick and powerful peristaltic wave of the esophagus forces the ingested material into the stomach, a process which can be easily observed under the microscope. Normally about 40 such peristaltic waves occur each minute. It is suggested, therefore, that the drugs employed in this work were administered orally.

Experimental Results

The intestine of untreated animals usually exhibits movements which are more or less rhythmic in nature. There is a gentle surging back and forth of the nutritive material and only when the animal is in the act of evacuating the contents of the intestine is one able to observe peristaltic and antiperistaltic waves in the musculature of the organ itself. At such times the forward peristalsis becomes more noticeable than the reverse peristalsis, the anus opens and the animal excretes only a small portion of the intestinal contents. This act is repeated at irregular intervals which vary from 30 seconds to more than 1 minute in some cases. At no

time, however, is the intestine entirely empty, for in the depression slide the animal is continuously reëngulfing the materials which it has evacuated.

The Action of Acetylcholine

When a *Daphnia magna* young is treated with acetylcholine, a very distinct change occurs in the intestine. The muscular peristaltic and antiperistaltic contractions of the organ become extremely violent and when stronger solutions are employed the entire contents of the intestine are emptied in a little more than a minute. The time which elapses between the application of the drug and the appearance of the first vigorous muscular contraction varies very definitely with the concentration of the drug employed. From an inspection of Table I it is seen that with acetylcholine 1×10^{-2} this occurs on the average in less than 20 seconds and with acetylcholine 1×10^{-3} this period is increased to 27.4 seconds. There is not a gradual development in the establishment of the violent intestinal activity. When the drug becomes effective, it exhibits its effectiveness to the fullest extent with an abrupt initial powerful contractile wave of considerable amplitude. After treatment with acetylcholine 1×10^{-3} and subsequent transference to water, vigorous forward and reverse peristalsis will continue in some cases for as long as 20 or 30 minutes. Acetylcholine 1×10^{-2} with lapse of time produces high intestinal tone and contracture.

When *Daphnia magna* young are treated with acetylcholine 1×10^{-4}, the time which elapses between the addition of the drug and the first appearance of the characteristic effect produced is on the average 10.7 minutes for the group of experiments presented here (Table I, column 3). With further dilution of the drug this period becomes longer. With acetylcholine 1×10^{-7} the time varies from 50 to 137 minutes (Table I, column 4), showing a definite and considerable increase over the time of reaction obtained with the higher concentrations of the drug.

The Action of Atropine

Atropine was found to antagonize the action of acetylcholine. When *Daphnia magna* are treated with acetylcholine until the characteristic powerful action of the intestine is established and then the solution is replaced by atropine, the effects of acetylcholine are quickly abolished. The powerful contractions, which would otherwise persist for many minutes, not only disappear but in many individuals after the atropine has become fully effective there is no longer any evidence of intestinal muscular contractions. Atropine 10^{-2} abolishes the effect of acetylcholine of

the same concentration in less than 20 seconds, but atropine was found to be effective even in dilutions of 1×10^{-9}. The range of effectiveness beyond this concentration of the drug was not investigated. The results obtained with acetylcholine 1×10^{-3} and atropine 1×10^{-5} are shown in Table II. The rapidity with which acetylcholine 1×10^{-3} produced its characteristic action on the intestine is shown here to be no different from that previously recorded in this paper (Table I). Atropine 1×10^{-5}, on the other hand, repeatedly abolished the effects of acetylcholine within 20 to 52 seconds. Table II also shows that following the abolishing of the powerful intestinal contractions by atropine, a stronger solution of

TABLE I

Onset of vigorous intestinal contractions in *Daphnia magna* after treatment with acetylcholine of various concentrations. The time of action is expressed in seconds or minutes and represents the period elapsing from the addition of the drug to the appearance of the characteristic effect.

Acetylcholine 1×10^{-2}	Acetylcholine 1×10^{-3}	Acetylcholine 1×10^{-4}	Acetylcholine 1×10^{-7}
seconds	*seconds*	*minutes*	*minutes*
20	25	10.8	119
20	35	8.2	137
22	40	8.2	123
19	30	14.4	125
22	30	12.3	74
15	20	9.3	113
16	22	11.7	110
17	25	12.2	124
19	20	9.3	50
24	27	10.8	69
Average 19.4	27.4	10.7	104.4

acetylcholine (1×10^{-2}) reëstablished the previous effect of acetylcholine, the average time for this being 21.8 seconds—a reaction time characteristic for this concentration of the drug (compare with Table I).

Acetylcholine and atropine of the dilutions employed in this work produced no lethal effect on the animals. Likewise atropine, when it was repeatedly administered to the same individual after treatments with acetylcholine, had no paralytic effect on the musculature of the intestine. To test this, a single individual was subjected to experimentation in the following way. The animal was treated with acetylcholine 1×10^{-2}. Immediately after the appearance of strong intestinal contractions, the drug was removed and replaced with atropine 1×10^{-5}. After the abolishing of the muscular contractions, the animal was again treated with acetylcholine and then atropine of the same dilutions as pre-

TABLE II

Onset of vigorous intestinal contractions in *Daphnia magna* after treatment with acetylcholine; the time of abolishing the acetylcholine effect by atropine; and the time of reestablishment of strong contractions by acetylcholine following atropine.

Acetylcholine 1×10^{-3}	Atropine 1×10^{-5}	Acetylcholine 1×10^{-2}
Time of action	Time of abolishing acetylcholine effect	Time of action of acetylcholine 10^{-2} after atropine
seconds	*seconds*	*seconds*
29	30	20
26	30	25
25	40	22
26	52	18
34	22	35
38	36	32
22	38	20
38	20	18
24	38	20
32	34	22
28	35	18
34	42	18
34	38	20
43	42	20
22	40	19
Average 30.3	35.8	21.8

TABLE III

The effects of repeated treatment of a single *Daphnia magna* with acetylcholine and atropine at regular intervals of a few seconds.

Acetylcholine 1×10^{-2}	Atropine 1×10^{-5}
Time of action	Time of abolishing acetylcholine effect
seconds	*seconds*
20	90
20	110
18	80
14	80
16	95
13	70
14	110
14	80
28	52
18	85
Average 17.7	85.2

viously employed. This procedure was repeated on the same individual for ten times and the results which were obtained are shown on Table III. It is evident from an inspection of the table that the drugs continued after each application to produce their characteristic effects. Atropine 1×10^{-5} under the conditions employed in this set of experiments required on the average 85.2 seconds to produce its characteristic effect, in comparison with 35.8 seconds (Table II), which was required for the drug of this dilution to block the effect of acetylcholine 1×10^{-3}. This difference in the reactivity is apparently due to the fact that the treatment with atropine in this particular set of experiments was preceded by a stronger solution of acetylcholine (1×10^{-2}) than heretofore employed in studying the antagonistic effect of atropine.

TABLE IV

Onset of vigorous intestinal contractions in *Daphnia magna* after treatment with acetylcholine (1×10^{-7}) following the administration of physostigmine (1×10^{-4} for 2 minutes) and the action of physostigmine 1×10^{-4} when administered alone.

Time of action of acetylcholine 1×10^{-7} after eserinization	Time of action of physostigmine 1×10^{-4}
seconds	*minutes*
45	9.9
47	10.0
52	10.2
58	10.0
41	9.7
55	10.3
70	9.4
37	10.1
42	9.7
30	9.6
51	9.9
41	10.4
97	10.7
52	9.5
34	9.5
Average 50.1	9.9

THE ACTION OF PHYSOSTIGMINE

Physostigmine (eserine) causes in *Daphnia magna* intensification and prolongation of the effects of acetylcholine. Likewise, after eserinization of animals, the acetylcholine becomes effective on the intestine in a shorter period of time. Fifteen animals which were treated with physostigmine 1×10^{-4} for 2 minutes and then with acetylcholine 1×10^{-7} yielded results which are shown in Table IV. The reaction time for eserinized individuals in the production of vigorous muscular contrac-

tions when treated with acetylcholine 1×10^{-7} is shown to be on the average 50.1 seconds as compared with 104.4 minutes when acetylcholine of the same concentration is employed alone (see Table I).

The intestine of *Daphnia magna* responds to a treatment of physostigmine when employed alone in the same way as it does to acetylcholine. When 15 animals were treated with physostigmine 1×10^{-4}, vigorous intestinal contractions appeared in about 10 minutes (Table IV, column 2). This relatively strong concentration of physostigmine was employed because the utilization of this strength revealed certain manifestations in the course of the action of the chemical substance which were not observed when higher dilutions were employed. The animal under the influence of the drug becomes immediately immobile. The wall of the intestine becomes opaque due to an extreme contraction of the muscular fibers and the intestine enters into a state of contracture. After 2 or 3 minutes the organism gradually begins to recover its normal swimming movements and the intestinal wall commences to reëstablish its normal state. In time there appear extremely powerful intestinal contractions. These contractions with lapsed time become more intensified and persist for a considerably longer period than when acetylcholine alone is administered to the animals. This period was often observed to extend over one hour after the drug is replaced by water.

Discussion

The action of acetylcholine, atropine and physostigmine on the intestine of *Daphnia magna* is such that it strongly suggests the possibility that this organ is controlled by cholinergic nerves. Acetylcholine, when applied in the concentrations employed in this work, was shown to intensify the intestinal activity. This action of acetylcholine was shown to be antagonized by atropine and augmented and prolonged by physostigmine. These and other observations recorded in this paper are in accord with the rôle which has been ascribed to these substances in physiological processes where nervous impulses are involved and where acetylcholine is believed to act as a transmitter of nervous impulses.

The sudden appearance of vigorous muscular contractions of the intestine under the influence of acetylcholine have enabled us to obtain certain data pertaining to the time which elapses between the application of the chemical substance and the onset of the specific effect produced. It is of considerable interest and importance to note that whereas acetylcholine in concentrations of 1×10^{-2} and 1×10^{-3} produces vigorous intestinal contractions in less than 30 seconds, with further dilution of the drug this period is considerably prolonged before the accelerating

response of the intestine to acetylcholine is noted. With acetylcholine 1×10^{-4} the period becomes, on the average, 10.7 minutes and with acetylcholine 1×10^{-7} the time which elapsed between the addition of the chemical substance and the appearance of the characteristic response was shown to be on the average 104.4 minutes. Latent periods of such extreme magnitudes are not in accordance with our present knowledge pertaining to the action of chemical substances which are thought to act as chemical transmitters of nervous impulses.

In view of the observation recorded in this paper it may be assumed that the effectiveness of acetylcholine is dependent on the rate of penetration and diffusion of the drug to the site of action, and on the rate of destruction. Acetylcholine 1×10^{-7}, however, when preceded by physostigmine 1×10^{-4} produces vigorous intestinal contractions in *Daphnia magna* in less than one minute. This indicates that acetylcholine of this relatively weak concentration reaches the site of action quickly and that the rate of penetration and the rate of diffusion in this particular instance are not primarily factors. However, it is possible that the rapid destruction of the acetylcholine when unprotected by physostigmine is responsible for the long delays preceding the onset of its characteristic action.

Artemov and Mitropolitanskaja (1938) have demonstrated the presence in whole *Daphnia* of an acetylcholine-like substance. As yet, however, no one has undertaken to demonstrate the presence or absence of choline esterase in this group of animals. The questions of how acetylcholine, if present in *Daphnia magna,* is bound in the tissues and how it is protected must wait further investigations before they are answered.

Summary

1. Acetylcholine produces in *Daphnia magna* vigorous intestinal contractions which persist for some time after they are established.

2. The period which elapses between the addition of the acetylcholine and the onset of the characteristic effect is definitely dependent on the concentration of the drug employed.

3. Atropine blocks the action of acetylcholine.

4. Physostigmine causes intensification and prolongation of the effects of acetylcholine.

5. Acetylcholine, when it is preceded by physostigmine, causes in *Daphnia magna* a considerable reduction in the time which elapses between the administration of the drug and the appearance of the vigorous intestinal contractions.

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VITAL STAINING OF THE CENTRIFUGED ARBACIA PUNCTULATA EGG

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The stratification and parts of the *Arbacia punctulata* egg obtained by centrifugal force are shown in Plate I (from E. B. Harvey, 1936). The size of the parts and degree of stratification varies with the centrifugal force; the greater the force, the larger the red half and the less marked the stratification (E. B. Harvey, 1941). In any experimental work with the halves and quarters, it is of importance to know exactly what materials are present. This is best done by the use of vital dyes which stain the different materials differentially.

Table I contains a list of vital dyes used, arranged alphabetically, and the effect of each dye on the various materials in the egg. In all cases, the egg was viable after staining, since it could be fertilized and at least begin development. Different brands of the same dye have been found in some cases to differ considerably both in staining capacity and in toxicity. In general, the dyes put out by the National Medicinal Products or the National Aniline and Chemical Co. gave the best results, but for Nile blue sulphate and thionin, Grübler's were better.

It was found better to stain the eggs first by allowing them to stand about one-half hour in a dilute solution of the dye in sea water, and then centrifuge them, because the mitochondrial layer disappears in 5–10 minutes after centrifuging and it often takes longer than that for the dye to be taken up by a centrifuged egg. No accurate measure of the amount of dye used was made, but it was soon learned how deeply the sea water should be tinged for the dye to be efficacious but not toxic. Some of the dyes are readily soluble in sea water, others (Bismarck brown, neutral red, Nile blue, safranin O, thionin) must be dissolved in distilled water and a drop of this added to the sea water; some vital dyes (e.g. cresyl violet, Victoria blue) were found not to be sufficiently soluble even in distilled water. No acid dye was found to enter the cell.

The *jelly* forms a layer around the egg which in *Arbacia punctulata* is 20–30 μ thick. It is invisible under the microscope unless outlined by particles of India ink or stained, since it is of the same refractive index as the sea water. When it is present, the eggs are well separated from each other; when the eggs are contiguous, it means that the jelly

has disappeared, and the eggs are then usually not in optimum condition. The jelly is destroyed by X-rays or by a small amount of acid in the sea water (1 drop of N/10 HCl + 50 cc. of sea water). It is sometimes centrifuged off while the eggs are rotating, though it may remain,

TABLE I

Arbacia punctulata. Vital dyes.

Dye	Jelly	Oil	Clear Layer	Mitochondria	Yolk	Pigment	Remarks
Bismarck brown	0	0	Yellow (upper part more intense)	Yellow	Yellow	Brown	Slightly soluble in sea water
Brilliant cresyl blue	0	0	0	0	Blue	Blue	Very innocuous
Chrysoidin	0	0	Light yellow (upper part more intense)	Light yellow	Yellow	Reddish brown	
Gentian violet	0	0	0	Purple	0	0	
Janus dark blue B	Purple	0	0	0	0	0	
Janus green (=diazin green)	Purple	0	0	Blue	0	0	Rather toxic
Methyl green	0	0	0	Purple	0	0	
Methyl violet	0	0	Upper part violet	Purple	Purple (later)	Purple (later)	
Methylene blue	0	0	0	0	Blue	Blue	Very innocuous
Neutral red	0	0	Pinkish yellow (lower part more intense)	Pinkish yellow	Brick red	Blood red, almost black	Slightly soluble in sea water
Nile blue sulphate	0	0	Light blue (upper part more intense)	Light blue	Blue	Bluish brown to blue black	Slightly soluble in sea water
Rhodamine	0	0	Pink (upper part more intense)	Pink	Pink	Deep red	Very innocuous
Safranin O	Yellow (few cases)	0	0	Pink (after 1–2 hours)	0	Blood red	Not soluble in sea water
Thionin	Pinkish (few cases)	0	0	Lavender (few cases)	0	0	Not soluble in sea water
Toluidin blue	Pinkish lavender	0	Pinkish lavender	Lavender	Lavender	Purple to blue black	More intense if stained after cent.

somewhat elongate, on well centrifuged elongate eggs, or even around the two separated half-eggs when close together, or it may remain around one half-egg. It is best to determine its reaction to dyes on uncentrifuged eggs. The jelly stains *purple* with *Janus green* and *Janus dark blue B,* and *pinkish lavender* with *toluidin blue;* in a few cases it stained *yellow* with *safranin O,* and *pinkish* with *thionin.*

The *oil* cap is not stained by any of the vital dyes. A slight tinge of color was observed in some cases, e.g. with Bismarck brown, chrysoidin and Nile blue, but it is probable that the slight color was in the matrix and not in the oil drops themselves.

The *nucleus* is not stained by any of the vital dyes.

It has been stated that the *clear layer* does not stain in the living egg (Lucké, 1925), and this is certainly true of many dyes. There is no doubt, however, that some dyes do stain the clear layer, not very intensely, while the egg is still living, as could be told by its subsequent development after fertilization. A comparison of the stained egg alongside a control egg in fresh sea water showed whether the clear layer was really stained. The clear layer stains *yellow* with *Bismarck brown* and *chrysoidin, blue* with *Nile blue, pink* with *rhodamine, pinkish yellow* with *neutral red* and *pinkish lavender* with *toluidin blue*. With some dyes there is a decided difference in the intensity of the stain in the upper and lower portions of the clear layer, indicating a stratification of materials within the layer. With Bismarck brown and chrysoidin, which in general act similarly, Nile blue and rhodamine, the upper portion of the clear layer stains more intensely. With neutral red, the lower portion stains more intensely. With *methyl violet*, only the upper portion stains (*violet*). This difference in different regions of the clear layer is more marked when the eggs are stained first and then centrifuged. Although the clear layer is optically empty in the living unstained egg, and no granules can be distinguished in the unstained or vitally stained egg, nevertheless in fixed material, this layer is filled with very fine granules, deeply staining (blue) with Heidenhain's iron hematoxylin (E. B. Harvey, 1940).

The best *mitochondrial* stain is *methyl green*, which stains the mitochondria *purple* and stains no other granules, so that the mitochondria appear as a purple band across the egg. *Gentian violet* also stains the mitochondria differentially (*purple*). *Methyl violet* stains the mitochondria *purple*, like methyl green, but it stains other granules as well. It may be that the purple stain of the methyl green is due to a contamination of this dye with methyl violet or crystal violet, but every brand of methyl green tried has given the same result. *Janus green*, which has been advocated especially by Cowdry as a mitochondrial stain, stains the mitochondria *blue*, but all brands have been found rather toxic, some brands more so than others. *Safranin O* was found to stain the mitochondria *pink* after some time, and *thionin* in a few cases stained them *lavender*. Other dyes stained the mitochondria, but also stained, somewhat more intensely, the underlying yolk (see Table I). As mentioned above, the mitochondrial layer disappears 5–10 minutes after

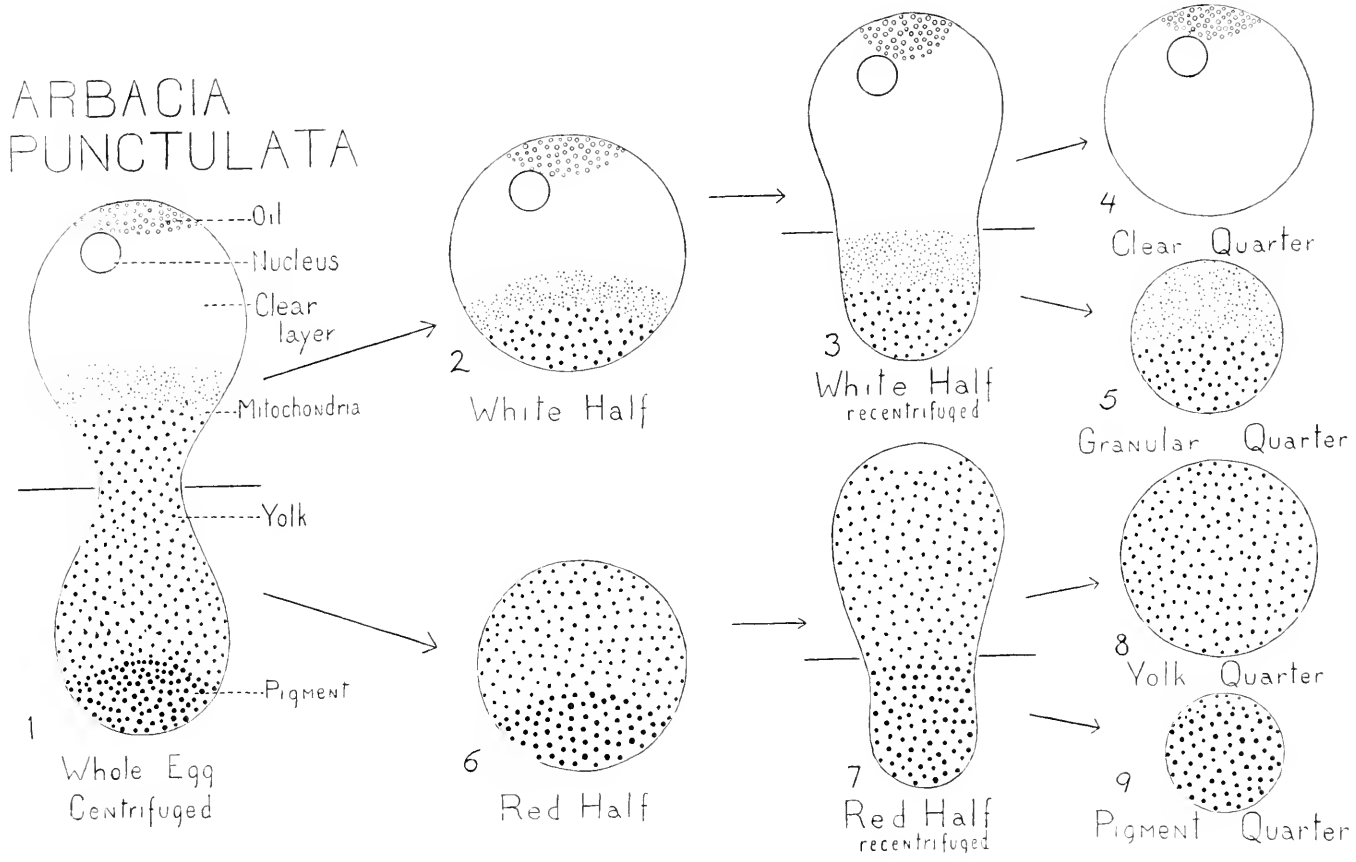

PLATE I

The unfertilized egg of *Arbacia punctulata*, stratified by centrifugal force (about 3 minutes at 10,000 × g.), and the halves and quarters into which it breaks. The drawings are from camera lucida sketches and photographs, made as accurately as possible to scale. Magnified 500 ×.

The clear area in Fig. 7 at the centripetal pole is due to further packing of the granules with longer centrifuging.

removal from the centrifuge, so that observations on it must be made quickly. It is also the last layer to be formed in centrifuging, and in some batches of eggs is not at all sharply defined.

Yolk and *pigment* can be easily distinguished from each other in the unstained egg by the color. They are usually both stained with the same dye, the pigment more intensely and at first more reddish. They are stained *blue* with *brilliant cresyl blue* and *methylene blue,* which in general act alike, and *Nile blue; yellow-brown* with *Bismarck brown* and *chrysoidin; red* with *neutral red* and *rhodamine; lavender-purple* with *toluidin blue.* With *safranin O,* the pigment is stained *blood-red* and the yolk is unstained.

Considerable information as to the chemical structure of the materials in the egg might be obtained from a study of the dye reactions, since the chemical composition of the various dyes is known. For the chemistry and preparation of the dyes and other data, the reader is referred to Rowe's Color Index (1924), Schultz' Farbstofftabellen (1934) and Conn's Biological Stains (1940). For the rate of penetration of the dyes (into gelatin), see Möllendorff's excellent article in *Abderhalden Handbuch der biologischen Arbeitsmethoden,* Abt. V, Teil 2, Heft 2 (1921).

Summary

A table is given of the action of various vital dyes on the different materials in the centrifuged egg of *Arbacia punctulata.* The *jelly* surrounding the egg is stained with Janus green, Janus dark blue B, (purple) and toluidin blue (pinkish lavender). The *clear layer* is slightly stained with Bismarck brown and chrysoidin (yellow), Nile blue (blue), toluidin blue (pinkish lavender), rhodamine (pink), and neutral red (pinkish yellow). The *mitochondrial* layer is differentially stained with methyl green and gentian violet (purple) and Janus green (blue). *Yolk* and *pigment* are stained with brilliant cresyl blue, methylene blue and Nile blue (blue), toluidin blue (purple), rhodamine and neutral red (red), Bismarck brown and chrysoidin (yellow-brown). With safranin, the *pigment* is stained blood red, the yolk is unstained.

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ALLOMETRY IN NORMAL AND REGENERATING ANTENNAL SEGMENTS IN DAPHNIA

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The growth of a part in relation to that of the whole body in many animals follows the law of allometry and may be expressed by the equation

$$y = bx^{\alpha}$$

where y is the size of the part; x, the size of the whole; and b and α are constants (Huxley, 1932 and Huxley and Teissier, 1936). Regeneration has been "regarded as an acceleration of normal growth processes" (Przibram, 1919 and 1926). Regenerative growth might therefore also be expected to follow the law of allometry. Using the data of Zeleny (1908) on the gulf-weed crab, *Portunus sayi*, Huxley (1931) demonstrated that the above equation holds for the size of the chela in relation to the body as a whole and also for the amount of regeneration of the chela during any one instar in relation to the size of the animal during that instar. More recently others, especially Paulian (1938), have shown that in many arthropods the amount of regeneration of a part during any one instar in relation to the size of the body follows the law of allometry. Paulian has also pointed out that normal and regenerating antennae in *Gammarus pulex* and in *Carausius morosus* increase exponentially with time. Inasmuch as the relative growth equation is derivable on the assumption that the parts increase exponentially with time (Huxley, 1932 and Lumer, 1937), we may conclude that regeneration of the antennae in *Gammarus pulex* and in *Carausius morosus* also follows the law of allometry.

In the above cases we are dealing with regeneration that tends to be complete. Do these relations hold in a form where regeneration is not complete? In seeking a solution to the problem *Daphnia magna* is well suited as an experimental animal. After amputation of an antenna regeneration is limited to the restoration of the most proximal segment injured and the formation of setae. The amount of regeneration varies with the level of injury within the segment (Anderson, 1935).

The present study is a determination of the relations of the growth of normal and regenerating antennal segments to that of the animal as a whole in *Daphnia magna*.

Experimental Procedure

Females from a single clone of *Daphnia magna* Straus were used. The culture medium was pond water rich in organic matter. Individuals were isolated within six hours after their release from the mothers and placed in watch glasses with a few drops of culture medium. Just enough of a saturated solution of chloretone was added to make the animals immobile. Each animal was placed on its left side with the left antenna stretched out in front of the body. Those animals that were used to study the growth of normal segments were then drawn with the aid of camera lucida. After the drawings were made the animals were placed in individual vials containing about sixty cubic centimeters of fresh culture medium. In the case of those animals that

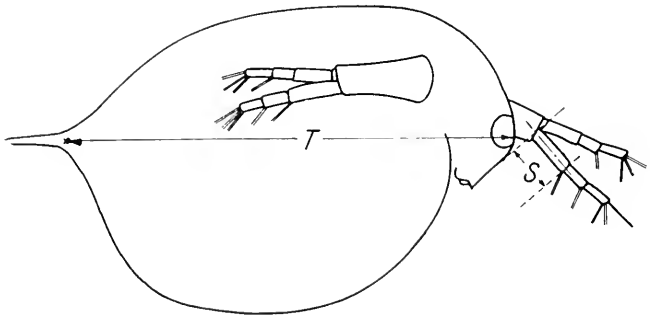

Fig. 1. Diagram showing the method of making measurements. *T*—total length, longest dimension of the body exclusive of the spine. *S*—segment length taken on the central axis of the segment.

were used to study regeneration, the first segment of the ventral ramus of the left antenna was severed by applying pressure with a needle which had been ground to a chisel edge. The level of amputation was varied in each instance. After the operation these animals were also placed in individual vials containing fresh culture medium. Several hours later the operated animals were again placed in watch glasses and immobilized with chloretone. They were then drawn in the same manner as were the unoperated animals. Care was taken to denote exactly the extent of the brown area just proximal to the level of amputation, for this is injured tissue that is cast off at the next molt (Anderson, 1935). After being drawn, they were replaced in their respective vials. From this point on both normal and operated animals were treated alike. Each animal was drawn during each successive instar up to and including the tenth. Inasmuch as the animals change in size only at ecdysis and im-

mediately thereafter (Agar, 1930), drawings were made at any time during the instar. Every time after an animal was drawn it was placed in fresh culture medium. The experiment was run at room temperature (18°–26° C.).

Measurements of the total length of the animals and the length of the antennal segments were made from the drawings. The total length of the animal was taken as the distance from the base of the spine to the most anterior point on the head. The length of the antennal segment was taken on the central axis of the segment. These measurements are illustrated in Fig. 1, and conform to those made by Anderson (1932, 1935) in other studies on *Daphnia magna*.

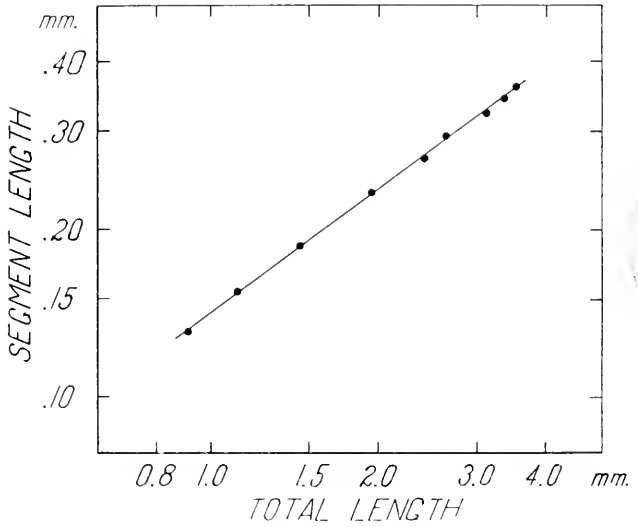

FIG. 2. Double logarithmic plot of the relations between the lengths of the normal segment and the total lengths during each of the first nine instars.

RELATIVE GROWTH OF THE NORMAL ANTENNAL SEGMENT

The relation of the logarithms of the mean lengths of the first segment of the ventral ramus of the left antenna to the logarithms of the mean total lengths of eighteen animals for the first nine instars is shown in Fig. 2. The points fall approximately in a straight line. We may therefore conclude that the law of allometry holds and the relation between the length of the segment and the total length may be expressed by the equation

$$y = bx^{a} \qquad (1)$$

where y is the length of the segment and x is the total length of the animal. The values of the constants b and α are 0.145 and 0.74, respectively. These were determined by the method of least squares. The value of α being less than unity indicates that the antennal segment grows at a lower rate than the body as represented by the total length. The antenna as a whole also grows more slowly than does the body as

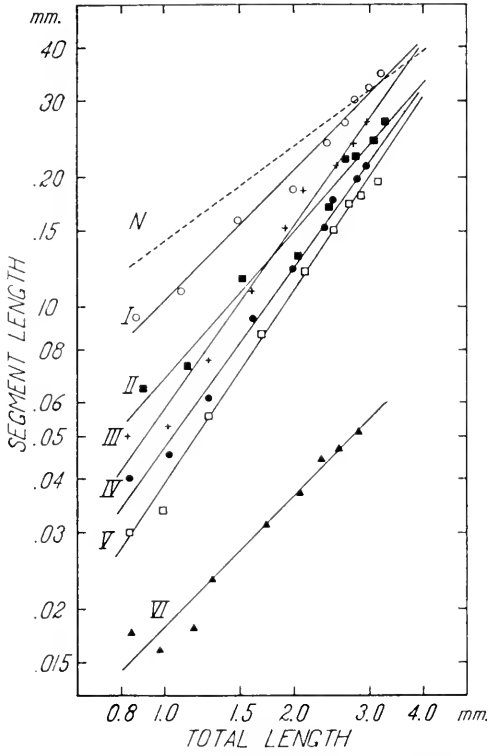

FIG. 3. Double logarithmic plots of the relations between the lengths of regenerating segments and the total lengths during each of the first nine instars for animals with antennae amputated at different levels. The level of injury is designated by the Roman numerals whose values are given in Table I. The dash line designated by N is that for the normal segments shown in Fig. 2.

is evidenced by the fact that the young at birth have antennae whose length is greater in proportion to the body than do the adults. The antennal segments, however, maintain the same proportions to each other throughout life.

Huxley (1931) has shown that the limbs of sheep grow less rapidly than the body after birth. This is also true for the macaques (Lumer

and Schultz, 1941) and probably for all mammals where the young at birth are able to run along with their mothers and perhaps for many other animals that depend on their means of locomotion for protection and/or food-getting.

Regeneration

The nature of regenerated antennae has been adequately described by others and need not be repeated here. Regeneration is limited to the restoration of the most proximal segment injured and the formation of new setae (see Anderson, 1935). The amount of regeneration is quantitatively related to the level of injury within the segment.

The operated animals were divided into six classes on the basis of the length of the intact portion of the segment during the latter part of the instar of amputation. The relations of the logarithms of the mean

Table I

The values of b and α for the relations of the length of the regenerating first segment of the ventral ramus of the left antenna to the total length of the animal.

Class	Level of Injury *	Number of Cases	b	α
I	0.076–0.110	8	0.103	1.01
II	0.060–0.070	7	0.068	1.14
III	0.046–0.053	7	0.058	1.42
IV	0.040–0.042	8	0.047	1.40
V	0.027–0.030	9	0.039	1.49
VI	0.010–0.023	7	0.018	1.02

* Length of the intact portion of the segment in millimeters during the instar of amputation. The values of b and α were determined by the method of least squares.

lengths of the amputated segments to the logarithms of the mean total length of the animals for the instar of amputation and the next eight instars for each class are shown in Fig. 3. While the points for any one class do not fall along a straight line as closely as do those for the relations in unoperated animals (Fig. 2), they do approximate a straight line. The law of allometry may be considered applicable and the relations can be expressed by the equation (1). The values of the constants are given in Table I.

The value of the constant α in the equation (1) is the ratio of the percentage increase of y to the percentage increase in x. Since x always represents the total length of the animals, both normal and operated, in the relations described above, the values of α are directly comparable. Examination of Fig. 3 and Table I shows that the value of α increases

as the level of injury reaches a lower point in the segment until a certain level is reached after which the value of α decreases. This relation is brought out graphically in Fig. 4. Another point worthy of note is that in Fig. 3 the curves with one exception tend to converge at a point where the total length would be about five millimeters, the maximum size that the animals reach. From this it is apparent that as long as the level of injury is above the critical level, the growth rate of the regenerating antennal segments is such that they approach the length of the normal segment simultaneously as full growth of the animals is attained.

Somewhat analogous results have been found by others. Zeleny (1905, 1909) found that the rate of regeneration of an organ in many

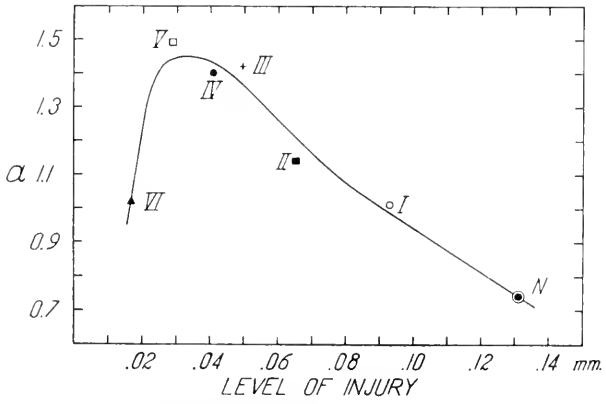

FIG. 4. The relation between the value of α in the equation

$$y = bx^{\alpha}$$

and the level of injury. The symbols correspond to those used in Fig. 3.

animals increases with the degree of injury up to an optimum, after which the rate decreases. Zeleny's work is not directly comparable inasmuch as he was concerned with the rate of regeneration of an organ when that one only was removed in comparison with the rate when several others were removed in addition. The results of Paulian (1938) are more directly comparable. He amputated the antennae of *Gammarus pulex* and *Carausius morosus,* and inspection of his figures (Figs. 14 and 15, pages 320 and 322) indicates that the rate of growth of the antennae increased as the level of amputation approached the proximal end. Whether or not a critical level might be reached beyond which the rate decreases, was not determined in his experiments. Further, the amputated antennae reach the size of the normal at different times, the time taken varies directly with the amount removed.

The Significance of the Constant b

The question of the biological significance of the constants b and α in the law of allometry have been subject to considerable discussion. Huxley (1932) and Needham (1934) have stated that the constant b is of little biological importance. The value of the constant b is that of y when $x = 1$. As a consequence, its value changes with the unit of measure employed while the actual relations of y and x remain the same. Again the unit chosen is usually such that b is an extrapolated value of y. Because of these arbitrary factors the significance of b has remained elusive. Recently Lumer, Anderson, and Hersh (1941) have pointed out that if b is to have biological significance, the unit of measure chosen should be one given by the organism. They suggest that the most satisfactory unit would be the size of a standard part at the beginning of a developmental period, but where this cannot readily be ascertained an approach to it could be made by taking the smallest value of the standard part given by the data as unity. In this way b would be an actual value of y. This is in line with the proposal of Huxley and Teissier (1936) that b should be called the "initial-growth index," for indeed that is what it becomes as far as the data are concerned when the above suggestions are followed. The constant α has presented no such difficulties. Since it is the ratio of the percentage growth rates of the parts y and x, it is constant regardless of the unit of measure used. α has therefore been considered of relatively greater importance than b.

Lumer, Anderson, and Hersh (1941) have shown how the constant b may be made a more tangible entity in that it can be given in terms of the organism. As such it has significance. The question still remains as to the degree of its importance. If the value of b, i.e., the initial ratio of y to x, could be altered experimentally, and if as a consequence the value of α would change, we could conclude that the value of b determines the value of α; b would then have a greater biological importance than heretofore supposed.

This is precisely what we have done in the experiments described in this paper. We have amputated the antenna and so reduced the length of the segment. The ratio of the intact portion of the segment during the instar of amputation to the total length of the animal is given by b, since the total length during that instar is approximately one millimeter and the millimeter is the unit of measure. Following amputation, the growth rate of the antennal segment is changed so that new values of α result. Further, as b decreases α increases until b reaches a particular value, after which α also decreases as is shown in Table I and Fig. 4. The constant b, in the sense in which we have employed it, serves as a measure of the conditions at the beginning of the developmental period.

and as these conditions differ, so also do the consequent rates of development as represented by the constant α.

Summary

The law of allometry

$$y = bx^{\alpha}$$

was found to be applicable to both normal and regenerating antennal segments in *Daphnia magna*.

The growth rate of the regenerating segments increases as the level of injury approaches the proximal end of the segment until a critical point is reached, after which the rate decreases. As long as the level of injury is distal to the critical level, the growth rate is such that the regenerating segments tend to approach the length of the normal segment simultaneously as full growth of the animals is attained.

The significance of the constant b in the law of allometry is discussed.

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EXPERIMENTAL CYTOLOGICAL EVIDENCE FOR AN OUTWARD SECRETION OF WATER BY THE NEPHRIC TUBULE OF THE CRAYFISH [1]

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Introduction

When a crayfish is in freshwater, its normal habitat, it does not drink, but water diffuses into its body through the gills (Maluf, 1937, 1940). An internal aqueous and saline steady state is maintained, in spite of a constant inward diffusion of water, because of the unvarying capacity of its kidneys to manufacture urine that is markedly hypotonic to the blood (Schlieper and Herrmann, 1930; Herrmann, 1931).

The concentration of chloride in the luminal fluid of the coelomosac and labyrinth of the nephron is equal to that in the blood, but that in the tubular fluid is markedly lower than that in the blood (Peters, 1935). The hypotonicity of the urine must therefore be due either to an active resorption of salts by the tubule or to an outward secretion of a hypotonic liquid by the tubule.

The tubule of a 35-gram animal is approximately 3 cm. long and about 2 mm. in greatest breadth.[2] The ventral, i.e., proximal, half of the tubule consists of flat cells without apical secretory globules. The dorsal, i.e. distal, coil is composed of relatively large columnar cells with a distinct mitochondrium and, generally, with large clear apical vacuoles which bulge into the lumen of the tubule (Maluf, 1939).

Because the nephron of the crayfish does not possess a tenuous syncytium, such as the glomerular capsule of the vertebrate nephron, filtration seems unlikely as a major process of urine-formation. Accordingly, this is an attempt to find whether the apical vacuoles of the distal coil of the tubule represent an outward secretion of water.

The experimental attack is partly based on the observation of Herrmann (1931) that, as the salinity of the external medium is raised, the rate of urinary flow falls and the osmotic pressure of the urine simul-

[1] This work was performed when the author was Johnston Research Scholar in the Department of Zoölogy, The Johns Hopkins University. To Professor S. O. Mast much obligation is due for numerous kindnesses.

[2] In the 1939 paper this was misprinted as "2 cm."

taneously increases. As shown by constancy in weight, the total quantity of water in the crayfish is the same in freshwater as in salinities up to 272 mM. NaCl per liter, which is initially hypertonic to the blood. This indicates that the decrease in the rate of urinary flow, with rising external salinity, is not due to a decrease in haemocoelic pressure which, assuming that filtration does occur, might cause a decrease in the rate of filtration. There is, furthermore, no apparent basis for the supposition that the haemocoelic pressure undergoes a localized fall in the vicinity of the kidneys as the salinity of the external medium is raised.

From the above it might be expected that, when the crayfish is in a medium in which inward diffusion of water can be only very small and in which the rate of urinary flow is accordingly depressed, the apical vacuoles of the nephric tubule will tend to disappear.

Methods

The test animals were immersed in 210 mM. NaCl per liter of freshwater, a solution in which they can remain vigorous indefinitely. Although this concentration is somewhat hypertonic to the blood at the outset (see Lienemann, 1938, for the normal osmotic pressure of the blood of *Cambarus clarkii*), some water, probably only a negligible quantity, diffuses inwardly because, as Herrmann (1931) showed, the osmotic pressure of the blood eventually exceeds that of the external medium. Parenthetically, the invariable hypertonicity of the blood, as compared with the external medium, is probably mainly because the urine is always hypotonic to the blood regardless of the osmotic pressure of the external medium (Herrmann). Integumental uptake of salt from the exterior is a relatively minor factor, as can be readily calculated (data of Lienemann, 1938, and Maluf, 1940).

At the end of one to several days the animals were sacrificed and their kidneys removed with minimum handling and fixed in unneutralized formol-sublimate for several hours, washed in running tap-water overnight, dehydrated with dioxane (50 per cent, 75 per cent, and two changes of 100 per cent), imbedded in paraffin with a melting point of about 49° C., sectioned 8 μ thick, and stained with eosin and methylene blue-borax.

Results

A seven-day stay of three animals in 210 mM. NaCl per liter abolished almost all the apical vacuoles from the cells of the distal coil of the nephric tubule (Fig. 1) whereas the majority of the corresponding cells of the three controls, which had been in freshwater, possessed large

apical vacuoles which bulged into the lumen of the tubule (Fig. 2). One of the test animals exhibited an exceptional number of vacuoles for an animal in 210 mM. NaCl but the vacuoles were small and scanty as compared with those of the controls. The photographs were taken from areas at random. The data were analyzed objectively as follows: In this experiment two to three slides were prepared containing serial sections of the pair of kidneys from each individual (16 slides in all); the labels were covered so as to remove every vestige of external identification; the slides were shuffled. The examination of each slide never exceeded one or two minutes and was made under low power (100 ×). In 15 slides out of 16, the identification of the series to which the preparation belonged (freshwater or 210 mM. NaCl) was correct. Measurements did not show a correlation between the height of the cells and the existence of apical vacuoles.

The experiment was repeated with six larger animals and a duration of three days. Here, too, the difference between the three test animals in 210 mM. NaCl per liter (Fig. 3) and the controls (Fig. 4) was pronounced. The objective analysis, identical with that above described, showed a correct identification of 20 slides out of 22. Here, too, extensive measurements indicated no correlation between the height of the cells and the presence of vacuoles. The three-day experiment was repeated with confirmatory results: the two test animals showing practically no apical vacuoles whereas the two controls displayed apical vacuoles in the majority of cells of the distal half of the tubule.

Even a 24-hour stay in 210 mM. NaCl produced a practically complete abolition of the apical vacuoles (Fig. 5) although the interior of the cells was considerably vacuolated. Nearly all of the corresponding cells of the controls in freshwater exhibited large clear apical vacuoles (Fig. 6). Figures 5 and 6 are at a lower magnification than the other photographs and thus exhibit a larger field. The objective analysis showed a correct identification of 8 slides out of 8. Subjection to 210 mM. NaCl for less than 24 hours was not attempted.

After vacuole-formation has presumably been practically abolished by an 168-hour stay in 210 mM. NaCl, the vacuoles reappear upon returning the crayfish to freshwater. In this experiment there were two tests and two controls.

The fact that a large fraction of the cells of the distal half of the tubule of the controls invariably exhibited large apical vacuoles in itself shows that the almost complete absence of such vacuoles in slightly hypertonic NaCl is not an artefact of histological technique. The distal half of the tubule of a live animal was dissected out of the kidney in

crayfish-saline.[3] Fragments teased out of this part of the tubule and suspended in a hanging drop of crayfish-saline on a coverslip, gave an ample picture of the vacuoles.

Historical Statement and Discussion

The nephric tubule of the decapod kidney was first discovered by Neuwyler (1841), who did not understand the function of the "green glands." Only within the present decade have we come to realize the importance of the crustacean kidney in the aqueous and ionic regulation of the bodily fluids. Grobben (1881) was the first to observe that the nephric tubules of freshwater Crustacea and Annelida are markedly longer than those of corresponding marine forms and that length of tubule is not correlated with bodily size. He did not theorize as to the significance of these facts but remarked that, "It therefore appears that the length of the urinary canal goes parallel with life in freshwater." Richard (1891) came to an identical conclusion with regard to copepod Crustacea. Rogenhofer (1905, 1909) confirmed Grobben and found that differences in the nephric dimensions of marine and freshwater Crustacea are not due to differences in cellular size. Rogenhofer failed to alter the length of the tubule of the freshwater isopod, *Asellus aquaticus*, in one generation by gradually bringing the isopod to a salinity of 2 per cent in one year. Della Valle (1893) believed that the differences

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PLATE I[4]

Explanation of Figures

Fig. 1. Epithelium of a portion of the distal half of the nephric tubule of a crayfish which had been in 210 mM. NaCl for seven days. *ha*, haemocoele and blood-vessels; *LU*, lumen of tubule. Triple Mallory's; daylight bulb; Zeiss lens. Animal about 10 grams.

Fig. 2. Control to Fig. 1; animal in freshwater. *va*, large apical vacuoles. Animal about 10 grams.

Fig. 3. Epithelium of a portion of the distal half of the nephric tubule of a crayfish which had been in 210 mM. NaCl for three days. Methylene blue-eosin; red filter; Zeiss lens. Animal about 30 grams.

Fig. 4. Control to Fig. 2; animal in freshwater. Animal about 30 grams.

Fig. 5. Epithelium of a portion of the distal half of the nephric tubule of a crayfish which had been in 210 mM. NaCl for 24 hours. Methylene blue-eosin; red filter; Zeiss lens. Animal about 13 grams.

Fig. 6. Control to Fig. 5; animal in freshwater. Animal about 13 grams.

[3] The saline was based on the most acceptable data on the concentration of inorganic electrolytes in the blood of the crayfish (see Maluf, 1940, for references) and was as follows (g./1.): NaCl, 7.81; $CaCl_2$, 1.31; $MgCl_2$, 0.82; KCl, 0.70; buffered at pH 7.5 with 0.5 cc. M/5 Na_2HPO_4/NaH_2PO_4. A $\triangle$ of about 0.66° C. is assumed (see Lienemann, 1938, and Schlatter, 1941).

[4] The writer is much indebted to Dr. Charles E. Brambel, The Johns Hopkins University, for kind personal instruction in photomicrography.

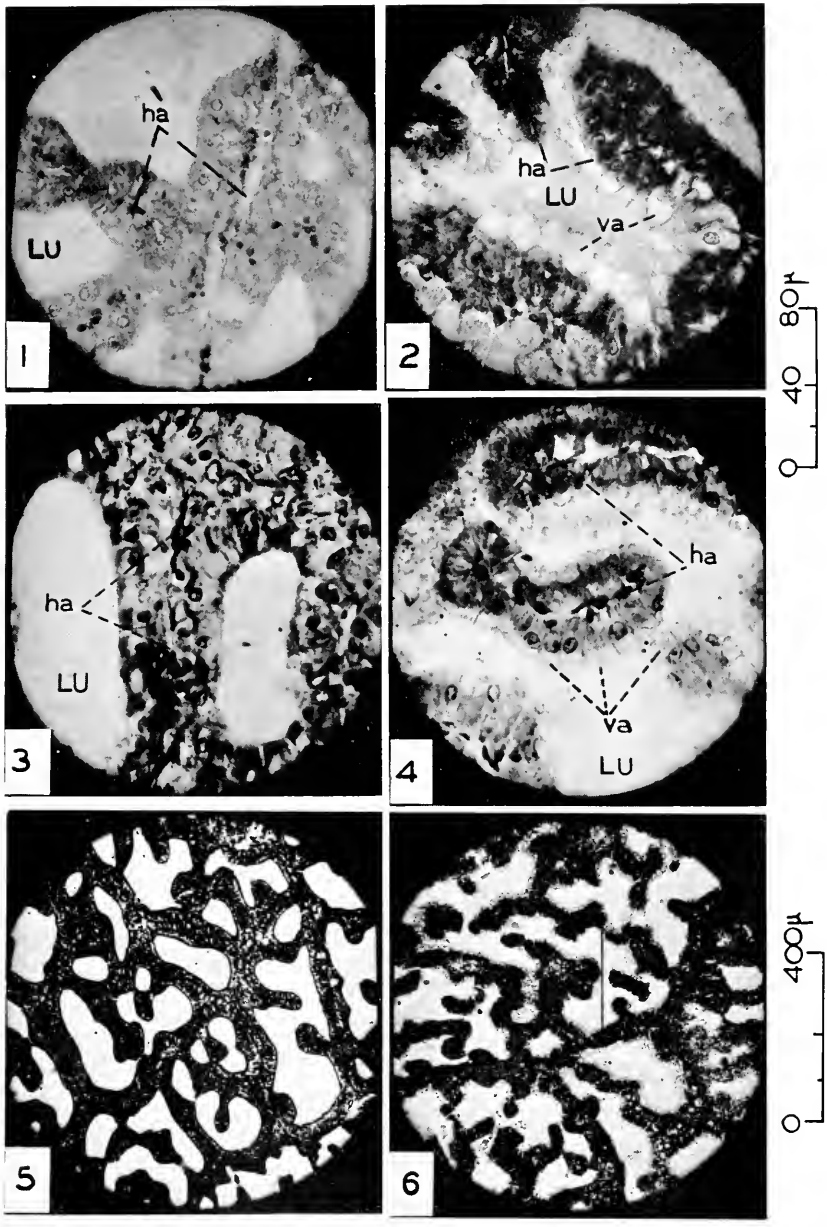

PLATE I[4]

in tubular length are of phylogenetical origin rather than a direct environmental effect. Marchal (1892) observed that the nephrons of the lobster and other marine decapods have no tubule. He suggested that the external medium,—freshwater and sea-water,—may be a determining factor but remarked that the estuarine crab, *Telphusa,* has no nephric tubule even though it frequents freshwater.

In 1930, Schlieper and Herrmann found that the urine of the crayfish is markedly hypotonic to the blood and that the urine of the shore-crab, *Carcinus maenas,* and of the estuarine crab, *Telphusa fluviatilis*—neither of which possess nephric tubules—is isotonic with the blood. They suggested that the nephric tubule is responsible for the hypotonic urine of the crayfish and that it acts by resorbing salts from a filtrate formed at the coelomosac. Herrmann (1931) and Peters (1935), in Schlieper's laboratory, demonstrated that the tubule is of paramount importance in osmoregulation. Peters suggested that the apical vacuoles of the distal coil may indicate a resorption of salts from lumen to blood. Peters' theory presupposes that a filtrate is formed somewhere in the nephron proximal to the tubule. Peters made the important discovery that only in the tubule is the concentration of chloride of the presumptive urine lower than that of the blood. His results do not show, however, in which part of the tubule this is true.

The facts in this paper suggest that the vacuoles represent an outward secretion of water in compensation for that which diffuses inwardly. Physiological data indicate that the crayfish nephron is paramountly if not entirely a secretory organ (Maluf, 1941). The hypotonic urine of this animal may thus be the result of an outward secretion of a liquid markedly hypotonic to the blood and the rate of water-secretion by the tubule may be determined by a hormone.

Summary

The majority of the cells of the distal half of the nephric tubule of the crayfish exhibit large, clear apical vacuoles at their luminal borders when the animal is in freshwater, its normal medium.

If the crayfish remains in a saline medium which is initially slightly hypertonic to the blood, for twenty-four hours or more, these vacuoles completely disappear. The condition is reversible upon return of the animal to freshwater. (The crayfish can maintain its vigor indefinitely in 210 mM. Nacl per liter, which is initially slightly hypertonic to the blood.)

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MICTURITION IN THE CRAYFISH AND FURTHER OBSERVATIONS ON THE ANATOMY OF THE NEPHRON OF THIS ANIMAL

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Preliminary to studies on renal function in the crayfish (Maluf, 1940, 1941*b*), it is necessary to know how urine is retained in the bladders and how discharged. Nothing has been indicated, until the present, as to how urine is retained. There is, furthermore, no adequate study of the anatomical features surrounding the urinary outlet of decapod Crustacea. As a result of this deficit, investigators of renal function in the crayfish have punctured the membranous operculum at the nephropore prior to collecting urine by suction (Marchal, 1892; Boivin, 1929; Herrmann, 1931; Scholles, 1933; Lienemann, 1938). It is not clear why the opercula were destroyed. From Marchal's diagrams it appears that removal of the opercula would tear the ureters and lead into the haemocoele and that, consequently, the urine would be contaminated with blood. Marchal and Boivin, however, stated that the liquid they collected was limpid, clear, and almost colorless and practically uncontaminated with blood. The chemical analyses of Herrmann, Scholles, and Lienemann show that the concentration of inorganic electrolytes in the liquid collected from the excretory orifices was markedly lower than in the blood. The fact that the distal portion of the bladder contacts the base of the excretory eminences at most of its circumference (Fig. 2, *B*) apparently explains how the urine collected by the afore-mentioned investigators did not contain an appreciable quantity of blood. The urine aspirated by Picken (1936), by piercing the operculum with a fine hypodermic needle, was doubtless, at times at least, notably contaminated with blood as shown by the strongly positive xanthoproteic reaction and by the large discrepancies, in this respect, with regard to the urine from both kidneys. Thus, in one instance, the urine from the right kidney gave a negative xanthoproteic test while that from the left gave a strong reaction. The writer found that urine collected from *Cambarus clarkii* by suction from intact nephropores invariably gave a weak xanthoproteic but a negative biuret reaction.

The review of Burian and Muth (1924) may leave one with the impression that the communication between the coelomosac and labyrinth "is closed by a sphincter muscle, and any passage of fluid from the labyrinth into the coelomic sac appears to be prevented by a valve-like arrangement of cells" (Picken, 1936). Examination of the literature left the writer dubious about the existence of a sphincter between the coelomosac and labyrinth. The present paper shows that, at least in *Cambarus clarkii,* there is no sphincter or valve between coelomosac and labyrinth or between nephric tubule and bladder.

Weismann (1874), Grobben (1881), Schlieper (1935), and Peters (1935) believed that a blood-ultrafiltrate is formed in the coelomosac. The writer has made a detailed histological examination of this part of the nephron to find out whether the histological facts support the hypothesis of filtration.

The results in this paper refer to *Cambarus clarkii,* the swamp crayfish.

Micturition

The Retention of Urine

Because the bladders are normally distended with urine and because urine only occasionally leaves the nephropores of undisturbed unheated animals seen under a microscope, urine must be adequately retained in the bladders. The volume of retained urine was sometimes as much as 4 per cent of the fresh weight of the animal.

On the ventral surface of the basal segment of each second antenna is the whitish excretory eminence (Fig. 5, *e*) in the central depression of which is a convex, finely corrugated, flexible, thin membrane, *o,* known as the operculum. Because the operculum does not cover anything external, the name is inaccurate. The convexity of the operculum is maintained by blood-pressure, as the opercula invariably collapse after thoroughly bleeding the animal. In contrast to the rest of the excretory eminence, the operculum is very sensitive to contact as shown by the resulting generalised motor response. The operculum is invaginated at its anterior border, thus forming a narrow crescentic slit (Figs. 2, 3, 5, and 6, *a*) which is the actual excretory orifice, or nephropore. The invagination proceeds at a sharp angle posteriorly, forming the short flat ureter (Figs. 2 and 3, *ur*).

The rounded flexible contour of the operculum is inessential. An animal with both opercula damaged by puncture was under observation for about a month, at the end of which time its opercula were still collapsed. The ureters, however, were not damaged, as shown by subsequent dissection.

The ureter (Figs. 2 and 3, *ur*) is short, dorso-ventrally flattened, and parallel to the operculum. Fine spindle-shaped fibers (Figs. 2 and 3, *f'*) containing elongate nuclei (13 μ long in crayfish-saline) extend from the dorsal wall of the ureter to the basal margins of the excretory eminence. With care, the whole dorsal wall of the ureter, including the fibers, may be dissected and mounted.

The fibers are unstriated (observed at 970 $\times$ while in fresh saline or after being fixed in formalin and stained with haematoxylin or Wright's) and are apparently identical with those which stretch between the distal extremity of the bladder and the integument (Fig. 2, *f, f''*). These ureteral fibers apparently act as a sphincter and their discovery answers the question as to how urine is retained in the bladders. Because a gentle outflow of urine has been seen in deviscerated inverted animals, the bladder must be elastic and the ureteral sphincter evidently normally retains urine in the bladder by tonic contraction.

Similar fibers occur, circularly arranged in considerable numbers, on the haemocoelic surface of the most proximal portion of the bladder, to a very much slighter degree on the main body of the bladder, and also on the main stem of the renal artery. Spindle-shaped unstriated fibers have been observed on the bladder of the American lobster by Waite (1899).

PLATE I

FIG. 1. Dorsal aspect of the opening into the left second antenna and surrounding exoskeleton, showing the distal portion of the bladder wedged between the proximal antennal muscles. *am*, articular membrane between antenna and cephalothorax; *B*, distal portion of bladder; *bas*, basipodite; *c*, lateral wall of the cephalothorax; *comp*, compressor muscles of the antenna; *cox*, coxopodite; dep_{1-4}, depressor branches of the antenna; *lev*, levator muscle of the antenna; *prom*, promotor muscle of the antenna; *rem*, remotor muscle of the antenna; *s*, sternum.

FIG. 2. Sagittal section through the distal portion of the bladder, ureter, and nephropore. *B*, distal portion of the bladder; *c*, connective tissue; *f, f', f''*, unstriated fibers; *f'*, ureteral sphincter; *n*, nephropore; *o*, operculum; *s*, coxopodite; *ur*, ureter.

FIG. 3. Dorsal aspect of the ureter and the depression of the coxopodite which corresponds to the eminence of the ventral aspect. *a*, nephropore, shown in broken lines because it is ventral to the ureter; *f'*, ureteral syncytium; *ur*, ureter.

FIG. 4. Dorsal aspect of the brain. *a*, nerve-stems passing into the lumen of the second antenna; c_1, c_2, individual nerve-fibers issuing from the roots of the former; *lc*, longitudinal connectives; *m*, median nerve; *oc*, oculomotor nerve; *op*, optic nerve; *P*, protocerebrum; *T*, tritocerebrum; *te*, "tegumentary" nerves. The root of the nerve to the first antenna issues from the ventral surface of the brain and is thus not shown here.

FIG. 5. Ventral aspect of the region surrounding the right nephropore. *a*, crescentic nephropore; *e*, excretory eminence of the basal segment of the second antenna; *o*, operculum; *s*, coxopodite; *u*, droplet of urine.

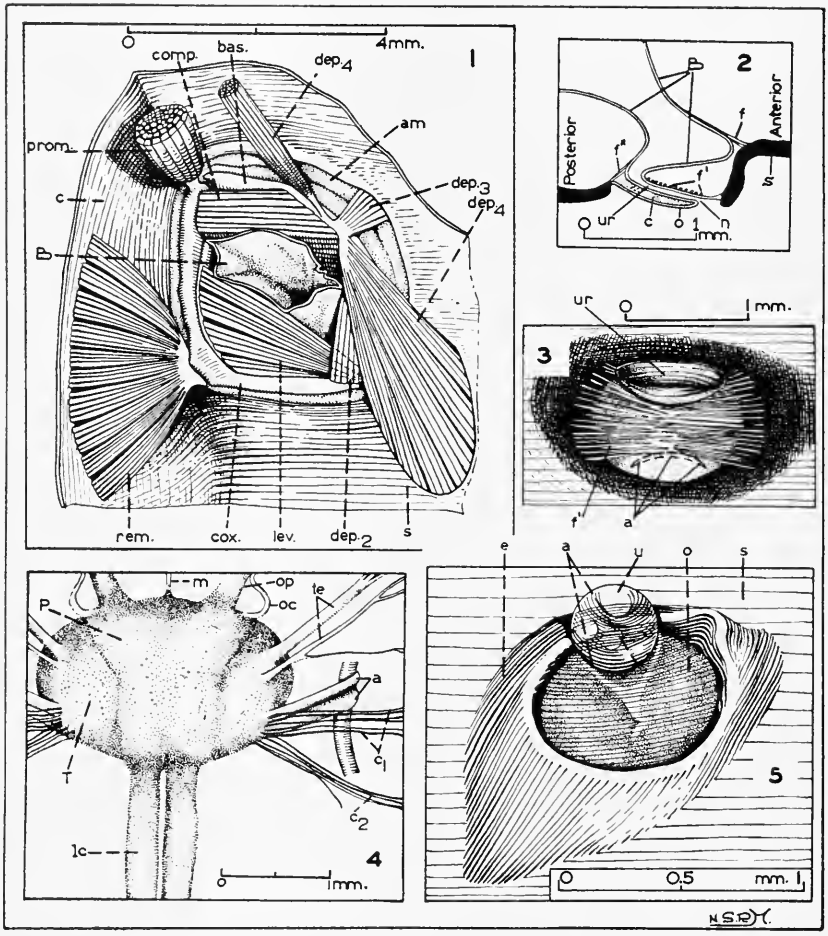

PLATE I

(All figures refer to *Cambarus clarkii;* the animals of Figs. 1, 4, and 5 measured about 7.5 cm. from rostrum to end of telson.)

In spite of numerous careful dissections, the writer has not been able to find any fibers inserting on the operculum. This conforms with Marchal's (1892) observations on the crab, *Maia.* Schmidt (1915), who gave a comprehensive and well-illustrated account of the somatic musculature of the European crayfish, did not mention any special muscles of micturition. The region between the operculum (Fig. 2, *o*) and the ureter, *ur,* is occupied by connective tissue and does not contain spindle-fibers.

At the posterior margin of the excretory eminence the ureter bends sharply anteriorly, enlarges in girth, and continues as the bladder (Fig. 2, *B*). Upon emerging from the excretory eminence (Fig. 2), the bladder passes through a mass of antennal muscles (Fig. 1).

The Expulsion of Urine

The animal was drained of moisture, water was sucked from the branchial chambers, and the anterior border of the chambers plugged with absorbent cotton-wool to prevent remaining water from flowing over the opercula. The opercula were observed under magnifications of 22.5 and 112.5 ×.

The outflow of urine in air occurs anteriad, i.e. in the plane of the ureter. At times the urine issues from the orifice for a short distance and is then sucked back. Slight pressure on the operculum with a blunt instrument frequently induces urinary outflow. A pipette of suitable size and carrying suction (about 10 mm. Hg) may produce urination even for some time after the use of suction. The urine issues as a series of generally spherical droplets. The suction does not injure the operculum. The latter does not undergo movement except for a scarcely perceptible motion only as the urinary droplet attains maximal size. This is doubtless a passive effect. Marchal (1892) stated that, in the crab *Maia,* movements of the opercula accompany the discharge of urine; it is probable that in *Maia,* too, the motion is passive. Marchal stated that muscles do not insert on the operculum of *Maia.* The writer confirms this for the crayfish.

Not infrequently, while the animal was held dorsum down and both nephropores were apparent, fine jets of urine abruptly spurted from both orifices sometimes to a distance of a foot or more. Every jet consisted of droplets in quick succession. On one occasion the occurrence was especially striking in that a series of jets to at least a foot followed one another rapidly. Although the spurts from both nephropores generally were not entirely simultaneous, the writer cannot recollect any instance in which urine spurted from one nephropore and not from the other

within a brief interval of time. Such powerful and sudden jets cannot be accounted for by the very sparsely distributed unstriated fibers of the bladder. Other decapods act similarly. In a single instance the estuarine crab, *Callinectes hastatus,* immediately on being grasped spurted urine to a distance of about 9 cm. from both nephropores simultaneously. Marchal (1892) noted a distance of 2 cm. from a shrimp and Herrick (1909) "an inch or more" from the American lobster on being held. Herrick ascribed the phenomenon to contractility of the bladder but evidently made no observations to support this supposition.

Whether the sparsely scattered vesicular fibers contribute to the discharge of urine is still unknown. The bladder was subjected to electrical induction shocks of high and low frequency, led through fine Ag–AgCl electrodes, both while distended with urine *in situ* and when isolated and under slight stretch in the longitudinal or in the transverse direction between two points in crayfish-saline. Contraction was never observed even under a magnification of 22.5 ×. The induction shocks were capable of causing cardiac tetanus, contraction of the dorso-anterior and -posterior dilators of the crop-gizzard, of the dorso-posterior longitudinal muscles of the crop-gizzard, and of the intact and isolated intestine, and abduction and adduction of the claw of the cheliped. Electrical stimulation of the bladder frequently produced strong generalised somatic muscular contraction; abrupt flexion of the abdomen and contraction of the homolateral remotor of the second antenna (Fig. 1, *rem.*) were among the main effects. Because the latter muscle is contiguous with the latero-ventral surface of the bladder, its contraction generally falsely suggested contraction of the bladder. Marchal (1892) briefly stated that he was unable to elicit contraction of the bladder of *Maia* by electrical stimulation.

Doubtless the major factor in the expulsion of urine is pressure exerted on the bladder by the blood and crop-gizzard. The nephropores, of animals drained from moisture, were often observed to remain dry for forty minutes or more. The injection of 1 to 1.5 cc. of saline into the haemocoele, between the chelipeds, i.e., in the vicinity of the bladders and crop-gizzard, invariably resulted in an immediate outflow of urine from both nephropores simultaneously. Merely puncturing the integument did not produce effects. If urination was occurring slowly, the injection of 1 to 1.5 cc. of saline resulted in a marked increase in the rate of outflow. It is conceivable that in some instances both bladders may be entirely collapsed; urination then would not be expected even upon the injection of any amount of liquid. Bilateral compression of the integument lateral to the bladders often produced an outflow of urine or an increase in the rate of flow. The large crop-gizzard is partly

wedged between the upper surfaces of the bladders. As direct mechanical pressure on the bladders results in their collapse and in the expulsion of urine, the movements of the crop-gizzard must be a factor in urination.

Innervation

Probably because the kidney and bladder are organs of the second antennal somite, all nerve-fibers to the bladder issue from the tritocerebral lobe of the brain (Fig. 4, *T*). The anterior component of the tegumentary nerves, *tc,* sends a branch to the integument beneath the labyrinth; the posterior component sends branches to some of the proximal muscles of the second antenna. About nine fibers issue in the anterior cluster, c_1, which arises from the base of the root of the main antennal nerve-trunks, *a*. Fibers from c_1 innervate the anterior and posterior surface of the bladder. The cluster, c_2, which consists of about five fibers, innervates the posterior surface of the bladder and some of the proximal muscles of the second antenna. Judging from the course of c_2, the sensitive operculum is probably furnished with afferent fibers from c_2 rather than from c_1. The nerve-fibers to the bladder are probably mainly afferent.

Repeated observation could not duplicate, in *Cambarus,* Keim's affirmation (1915; and quoted by Stoll, 1925) that in the European crayfish there extends a nerve-fiber ("nervus glandulae viridis"), bilaterally, from the suboesophageal ganglion to the labyrinth. Keim considered Marchal's description of a renal innervation from the second antennal nerve-bundles as incorrect. Marchal, however, stated that he "could not find a nerve which passed directly to the antennal gland," i.e. without first going to the bladder. Neuwyler (1841) disagreed with the labyrinthic auditory hypothesis of his eminent predecessors, as regards the function of the "green glands," because he could never find a nerve-supply to the glands.[1] Wassiliew (1878), in one of the first papers on the histology of the decapod kidney, stated that no nerves could be seen to enter the kidney. The present writer's observations are in accord with Wassiliew in this respect. The absence of a nerve-supply at the kidney proper indicates that secretion by this organ is not influenced by the

[1] To Ernst Haeckel (1857) credit is due for first suggesting that the green glands are renal organs. Haeckel demonstrated the communication of the bladders with the exterior and with the glands by introducing metallic mercury into the bladders. He pointed out that the existence of an external orifice indicates that the liquid in the bladder is a secretion which is eliminated. This observation, together with Neuwyler's discovery of the tubule and Gorup-Besanez and Will's remark that guanine occurs in the green glands, led Haeckel to term these glands urinary organs. Gorup-Besanez and Will, however, did not state the concentrations of guanine in urine and blood.

nervous system. This is supported by Maluf, Clarke, and Thompson (1939), who were the first to show that, per unit volume of glomerular filtrate, the rate of secretion of various substances is identical in the denervated and normal mammalian kidney.

The Absence of a Valve between the Nephric Tubule and the Bladder

The epithelial cells of the bladder, except those of the most proximal portion, are never columnar. They may be highly vacuolated (Fig. 9, *A* and *B*) or plain (Fig. 9, *C*) in the same bladder. The physiological evidence indicates that the epithelium of the main body of the bladder is non-secretory (Maluf, 1941*b*).

Even though the columnar secretory epithelium of the distal half of the nephric tubule (Fig. 7, *dt* and Maluf, 1939) merges imperceptibly into the epithelium of the main body of the bladder (Maluf, 1939), the tubule as an organ ends abruptly (Fig. 7), since the epithelium of the bladder is not anastomosed and acutely involuted as is that of the tubule (Fig. 8). The distal orifice of the tubule can be readily observed *in situ* (Fig. 8) through the dorsal surface of the translucent distended bladder. There is no valve between the tubule and bladder (Fig. 8) and no evident constriction of the proximal end of the bladder. Sections show no valve or sphincter at the distal orifice of the tubule.

The bladder of a 14-gram animal normally can distend to a diameter of at least 8 mm. The hydrostatic pressure within the bladder must then be somewhat greater than that of a column of water 8 mm. high because the bladder is elastic (see above). This pressure is doubtless too low to interfere with the outward secretion of water for which evidence is presented in an accompanying paper (1941*a*).

The Absence of a Valve and Sphincter between the Coelomosac and Labyrinth

The entire series of sagittal and horizontal serial sections of two kidneys, fixed in formol-sublimate and stained with haematoxylin and methylene blue, was studied. No valve or fibers could be found. A sagittal section at the communication of the lumina of labyrinth and coelomosac is shown in Fig. 10.

The Epithelium of the Coelomosac

The epithelium of the coelomosac, like the rest of the nephron, is single-layered. The appearance of more than one layer throughout a

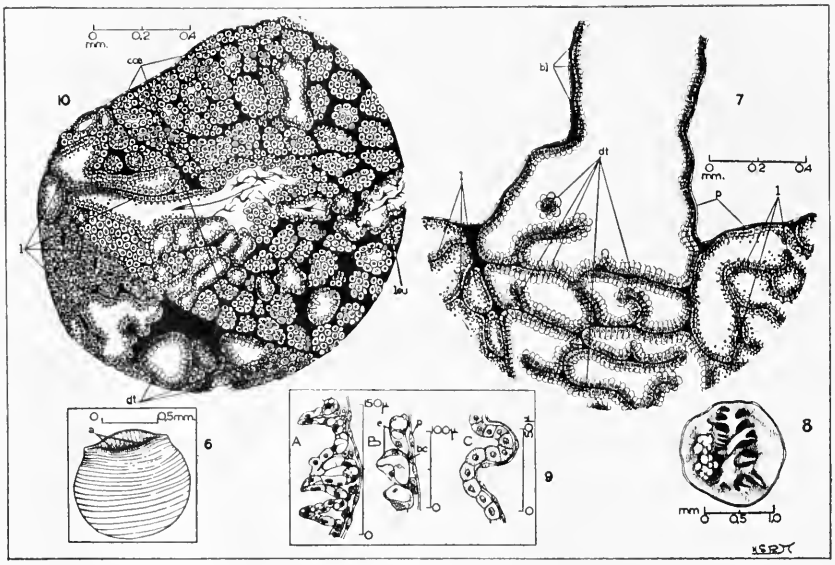

PLATE II

Fig. 6. Dorsal aspect of left nephropore, *a*, and membranous operculum. Note diagonal position of the nephropore. Animal, 22 grams.

Fig. 7. Sagittal section through the kidney showing communication of the distal extremity of the tubule, *dt*, with the bladder, *bl*. *l*, labyrinth; *p*, peritoneum. Solid black areas indicate blood-sinuses and blood-vessels. Animal, 35 grams.

Fig. 8. Dorsal aspect of the distal orifice of the tubule at its communication with the bladder.

Fig. 9. Sections of the main body of a single bladder. *bc*, blood-cells; *e*, epithelium of bladder; *p*, peritoneum. Animal, 35 grams.

Fig. 10. Sagittal section through the kidney showing communication of the coelomosac, *coc*, with the labyrinth, *l*. *dt*, distal portion of the tubule; *leu*, leucocyte. Solid black areas indicate blood-sinuses and blood-vessels. Animal, 35 grams.

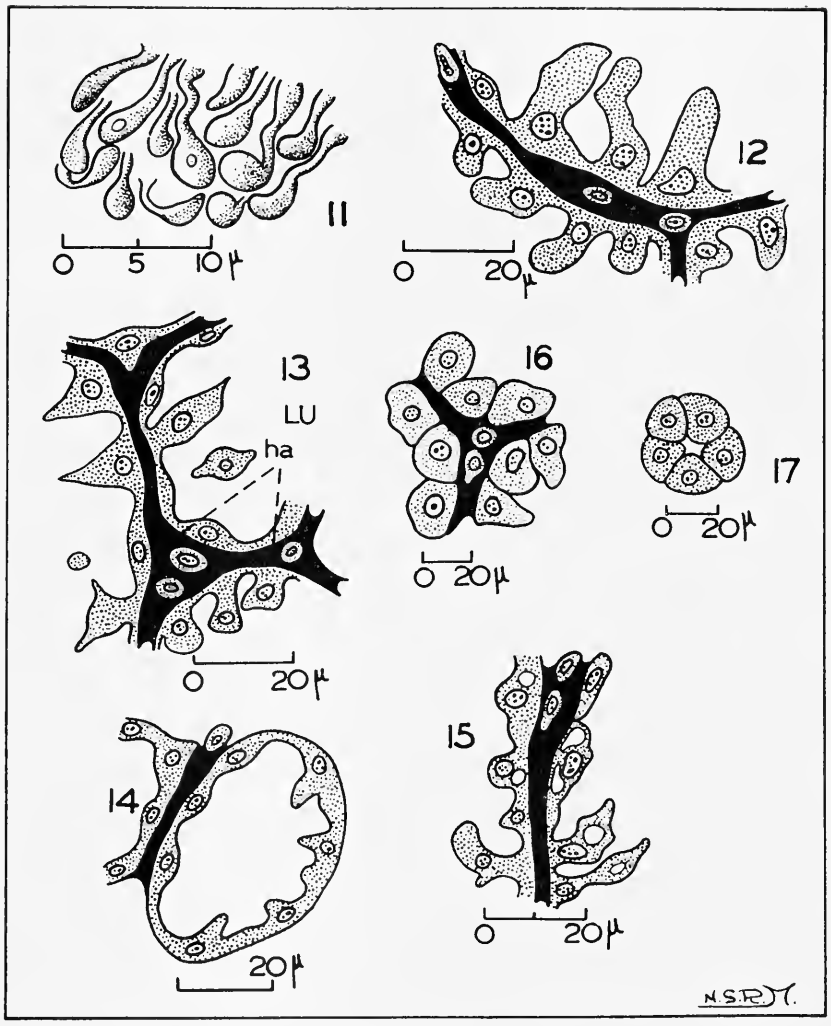

PLATE III

FIG. 11. "Living" portion of the coelomosac teased from the kidney in crayfish-saline and suspended in a hanging drop of crayfish-saline. The apical bulbous protuberances of the cells are shown in relief.

FIGS. 12 to 17. Epithelium of the coelomosac from medium-sized individuals. *ha*, haemocoele; *LU*, lumen of coelomosac. See text.

large part of the coelomosac, labyrinth, and tubule (Fig. 10) is doubtless due to tangential sectioning.

The nephron of the crayfish has no tenuous syncytium, such as the glomerular capsule of the vertebrate nephron. The epithelium of the coelomosac, the most promixal organelle of the nephron, approaches nearer to being a narrow syncytium than any other part of the nephron (Figs. 12–15). In several of the individuals examined, however, the cells were large and rounded throughout the coelomosac (Figs. 16 and 17; and Maluf, 1939).

The epithelium of the coelomosac varies considerably not only from one individual to another but often, too, in a single animal (Figs. 11–17). The cells may be either compact, rounded, and sometimes vacuolated, as in Figs. 16 and 17, or more or less squamous with large protuberances directed into the lumen (Figs. 12–15). The cells at the periphery may be rounded while those toward the center are protuberant; the reverse has never been found. Frequently, either the protuberant or the rounded cells occur exclusively. Both coelomosacs of an individual are always identical.

The histological methods have been described in the previous paper (Maluf, 1939). The protuberant type of cell is evidently not an artefact because it has been observed in teased-out "living" fragments in a hanging drop of crayfish-saline (Fig. 11). The composition of the saline is stated elsewhere (1941*a*).

Grabowska (1930) claimed that the secretion of the coelomosac consists of a discharge of cells in their entirety, i.e. "holocrine" secretion. If the cells are discharged as a whole, one would expect them to be substituted by mitosis. In not one instance, out of numerous coelomosacs examined, has the writer been able to find a mitotic figure. The evidence for a discharge of globules from the apical region of these cells is dubious because where rounded bodies have been seen "free," in the lumen of the coelomosac in sectioned preparations, these may have been merely sections of the bulbous tipped protuberances. In contrast to the rest of the nephron, the main lumen of the coelomosac generally contains numerous leucocytes (Fig. 10, *leu*).

Upon teasing the kidneys of a crayfish on one occasion, the coelomosacs were found packed with hard yellowish-brown irregular concretions the size of which showed that they could not have been intracellular. The largest stone was about 0.2 mm. in length. The material was insoluble in cold and hot water and in absolute ethyl alcohol. The alcohol decomposed the surrounding yellowish-brown organic material and the white stones readily fell apart, upon contact, into minute needle-like crystals which did not dissolve. The stones readily dissolved in

dilute HCl with energetic release of a colorless gas and were slowly soluble in 10 per cent NH_4Cl. There is very little doubt, therefore, that these concretions were $CaCO_3$. The individual had a highly melanized abdominal venter and, hence, must have possessed well-developed calcareous gastroliths. Twenty-one crayfish with gastroliths were examined and only one exhibited a similar condition. This was a single fairly large concretion in the coelomosac of only one kidney; other parts of the kidney did not contain any stones. About thirty animals with a light abdominal venter and without gastroliths were examined and in no instance was any concretion found in the kidneys.

The concentration of calcium in the blood of the crayfish and crabs remains fairly constant even immediately after molting (Paul and Sharpe, 1916; Damboviceanu, 1930), i.e. even when there is occurring, by way of the blood, a rapid transfer of calcium from the hepatopancreas and/or gut to the integument. Oesterlen (1840) has suggested that the formation of gastroliths may be a way of preventing a rise in the concentration of calcium in the blood. The above instances of renal calculi may be exceptions that prove the possible rule that one of the functions of the coelomosac is the secretion of calcium from the blood.

Weismann (1874) suggested that a blood-ultrafiltrate is formed through the coelomosac of the crustacean nephron just as Ludwig (1844) had presumed to occur through the glomerular capsule of the vertebrate nephron. Grobben pointed out that the relatively simple coelomosac of various amphipod crustaceans is attached to the integument by strands; this fact supports Weismann's belief inasmuch as effective resistance to blood-pressure would thereby be offered by the coelomosac, which would otherwise float in the haemocoele. Grobben also suggested that the location of the coelomosac between the antennal muscles in phyllopod Crustacea favors filtration. He nevertheless pointed out that, in copepod Crustacea, the coelomosac lies freely at the entrance to the homolateral second antenna and that these animals have no heart; he also drew attention to the fact that the phyllopod Crustacea have no heart and that it is therefore questionable whether, in such instances, filtration can occur and he ascribed the formation of urine in copepods and early-instar phyllopods to secretion by the tubule—a conception which had just begun to gain favor due to Heidenhain's (1874) experiments with the mammalian kidney.

Certain teleological evidence contra-indicates filtration through the coelomosac. Marshall and Smith (1930) and Marshall (1934) pointed out that when fishes migrated from freshwater, where they evidently arose, into the sea they had to conserve water. Some succeeded in losing their glomeruli while others are still doing so. The crayfish, how-

ever, has probably descended from a marine ancestor and is capable of compensating for water which diffuses inwardly through the gills (Maluf, 1937) by manufacturing a hypotonic urine through the agency of its nephric tubule. The crayfish nephron has a coelomosac but so does that of the lobster—a strictly marine relative. Because the osmotic pressure of the blood of the lobster is slightly hypertonic to that of the surrounding sea water (Cole, 1940), the lobster, unlike the crayfish, does not absorb water by diffusion from the exterior and hence does not have to maintain a steady state by an outward secretion of water. The lobster has either lost its nephric tubule or has never owned one. If the coelomosac were a filtration-organelle one would expect it to show some signs of regression in the lobster; but the coelomosac of this crustacean exhibits no evidence of being on the decline. Physiological evidence (Maluf, 1941*b*) indicates that the nephron of the crayfish is paramountly if not entirely a secretory organ.

Summary

1. The internal anatomical features surrounding the urinary outlet of the crayfish are described in detail for the first time.

2. Urine is retained in the bladders evidently by the ureteral syncytium, which is here described for the first time. There is no other way, conceivable to the writer, by which urine can be retained. Fibers do not insert on the operculum of the nephropore.

3. Urine is discharged by a localized rise in the haemocoelic pressure and can be expelled by direct action of the crop-gizzard on the bladders. Adequate electrical stimulation cannot cause contraction of the bladder but often evokes generalized motor activity.

4. Occasional abrupt spurts of urine, which were almost simultaneous from both nephropores, extended to the distance of a foot or more.

5. Destruction of the opercula before urinary collection has no rationale.

6. The bladder is innervated by fibers from the tritocerebral lobe of the brain. These fibers are doubtless mainly if not entirely afferent. The kidney is not innervated.

7. There is no valve between the nephric tubule and the bladder.

8. There is no valve or sphincter between the coelomosac and the labyrinth.

9. The epithelium of the coelomosac, the most proximal portion of the nephron, has been studied in detail. "Holocrine" secretion evidently does not occur because no mitotic figures could be found. The histological, chemical, and phylogenetical data contra-indicate filtration through the coelomosac.

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CHROMATIN BRIDGES AND IRREGULARITY OF MITOTIC COÖRDINATION IN THE PENTATOMID PEROMATUS NOTATUS AM. AND SERV.

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Introduction

A specimen of the pentatomid species *Peromatus notatus* obtained in 1937 presents such significant modifications of the orthodox course of meiosis, that a description and consideration of the most striking features seem warranted. As will be seen, the individual in question clearly is an exceptional case, but its departure from the normal is based on fundamental changes that have altered its mitotic mechanism in a very definite way. Apparently it is chiefly the relative timing of the various mitotic processes that has been affected, and the chromosomes and the spindle apparatus are, so to speak, out of step with each other. Their behavior under these conditions is of some interest in the analysis of mitosis in general.

Material

The specimen was caught on Barro Colorado Island in the Panama Canal Zone, in March, 1937. Its testes were fixed in B 15 within two hours of capture. It was close to the maximum size recorded for the species, in good condition, and very active.

Peromatus notatus, like the other six species of the genus, is strictly neotropical in its distribution. Examination of the sixteen specimens in the collections of the U. S. National Museum and the American Museum of Natural History shows that the species is subject to considerable variation in form and color. Variability in form is, however, more or less superficial and chiefly due to differences in the size and shape of the pronotal spines. The usual chestnut-brown color is replaced by green in some individuals from Panama (identified and labeled in the American Museum collection by H. G. Barber). It is a specimen of the latter type caught on Barro Colorado Island in 1941 that has served for comparison in the present study. It offers a typically pentatomid spermatogenesis which is almost indistinguishable from that of a specimen of *Peromatus truncatus* obtained in the same locality.

Spermatogonia

The spermatogonial divisions of the exceptional individual show no unusual features. The spindles conform to the common type, the chromosomes divide normally, and successive spermatogonial cell generations show no variation in chromosome number. The latter comprises the usual set of 14 chromosomes, in which one pair is a little larger and one pair somewhat smaller than the rest. The X is intermediate in size, whereas the Y is about as large as a member of the smallest pair (Fig. 1).

Meiotic Prophases to Diakinesis

Up to late diakinesis, the meiotic prophase stages conform to the usual pentatomid behavior. The sex chromosomes are heteropycnotic and frequently, though not always, appear joined from leptotene to diakinesis. There is a plasmosome which dwindles rapidly after the pachytene stage.

It is not until toward the end of diakinesis that the first unusual feature is encountered. Just as in other pentatomids, the two centers at this time move toward opposite sides of the nucleus. Both are in contact with the nuclear membrane and when they have reached their final position, the membrane underneath them is pulled or bulged outward.

This and the oval form of the nucleus, assumed in the direction of the centriolar axis, have frequently been noted (as early as 1891 by Henking). The point to be noted in this instance, however, is that the centers in the majority of cases are not on truly opposite points of the nucleus but are closer to each other on one side than on the other (Fig. 2). It is, of course, true that in other pentatomids also the position of the centers is not always geometrically exact, but the position here clearly is not accidental. This is borne out by the metaphase conditions that immediately follow the breakdown of the nuclear membrane.

The First Metaphase

The equator of the first spindle is in almost all cases displaced to one side, so that a line through the two centers does not represent the symmetrical axis of the mitotic figure as in other cases. In many cells all the chromosomes form a plate that lies to one side of the centriolar axis and hence the half spindle components are similarly displaced (Fig. 4). A few continuous fibres can sometimes be seen to stretch between the centers without such displacement, indicating their relative independence of the chromosomes. The latter rarely form a circle or round

plate, but constitute a semicircle with the two sex chromosomes usually but not always lying on the concave side (Fig. 3).

Despite this distortion of the mitotic apparatus, the tetrads divide in orderly fashion (Fig. 6) and the sex chromosomes undergo an equation division, just as in the normal *Peromatus* and other pentatomids. The peculiar configuration of the chromosome plate, however, is mirrored in the two daughter groups and may persist until middle anaphase (Fig. 5).

The initial movement of the dyads seems to occur without reference to the center and hence shows no effect of their askew position (Fig. 4). This is, of course, what might be expected since in nearly all cases known these first division stages of the chromosome appear to be autonomous.

The configuration of this first spermatocyte spindle challenges several interpretations concerning the mitotic mechanism. If the poles of the spindle are established by a mutual repulsion of two centrioles, it is very difficult to conceive of anything but a symmetrical spindle structure resulting therefrom. If the chromosomes assume their metaphase position because they react to forces from the poles, it is again not easy to understand why they should take such an "off center" position as they do. The conclusion is unavoidable that the mitotic conditions are affected by factors which normally are not present at this time.

First Anaphase to Second Anaphase

In the normal *Peromatus* as well as in most other pentatomids so far investigated, each of the centers carries two centrioles already at diakinesis. These two centrioles usually remain closely associated until telophase, though occasionally they have separated by some 15° before the end of anaphase (see, for instance, Paulmier's Fig. 29, 1899). The movement is quickened at telophase and before the second division is begun, the two centrioles are separated by 180°. There appears to be no exception to the rule that in Heteroptera the polar axis of this second division is at right angles to that of the first. This relation is especially striking in those cases where the interzonal connections of the first division continue to stain intensely, as in *Pachylis* (Fig. 8, and also those of other Heteroptera by Henking, 1891; Montgomery, 1898; and Paulmier, 1899).

The course followed in the present case is characterized by either one of two departures from the normal procedure just described. In about 75 per cent of the cells there is a marked precocity in the movements of the centrioles. Starting with little more separation than in normal cases, they diverge quickly after the early anaphase and in most cases have separated by 40°–45° before the anaphase movement of the chro-

mosomes has been completed (Fig. 7). Among the remaining cells about half show no such precocious separation of the centrioles, but the center as a whole may shift as much as 90° from the axial position of the first division (Fig. 9). In short, in such cases both centrioles assume the position of one of the poles of the second division, though the chromosomes are still in late anaphase of the first.

It was a matter of some surprise to find that in every such instance both centers moved to the same side of the anaphase cell. But this may simply be the consequence of the asymmetry of the first spindle which puts both centers closer to one side than the other to begin with. The two extremes of centriolar behavior are bridged by intermediate conditions which are not always easy to interpret. Thus the centrioles may succeed in separating after the center as a whole has begun to shift, or else one of the centrioles is for some reason held at the first pole and only the other moves toward its position for the second division (Fig. 7).

Whatever the type of variation may be, one point is held in common by all these cells. The processes that establish the achromatic figure of the second division are decidedly in advance of the corresponding steps in normal cells.

The precocity of the centers has marked effects on the behavior of the chromosomes. This is, perhaps, no more than might be expected, since they are still in the anaphase of the first division when the centers are already in process of establishing the mechanism for the second. The chromosomes show a definite response to the two poles which is manifested most strikingly in a tendency to divide again at this early

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PLATE I

Drawings made with Zeiss, 90 × objective and 20 × ocular. They were reduced ⅙ in reproduction.

FIG. 1. Three plates showing the 14 spermatogonial chromosomes.

FIG. 2. Diakinesis. The two centers are closer to each other on one side than the other.

FIG. 3. First metaphase. Autosomal tetrads arranged in semicircle, with *X* and *Y* on the inside.

FIG. 4. Side view of early anaphase, showing asymmetrical spindle. The autonomy of the initial separation of chromosomes is attested by lack of orientation toward the centers.

FIG. 5. Polar view of two sister groups in first anaphase, still showing typical arrangement.

FIG. 6. Middle anaphase of first division. The centrioles at each pole are separated less than usual.

FIG. 7. Upper pole of a late anaphase of first division. The two centrioles already have separated by about 45°, and there is no collocation of the chromosomes.

FIG. 8. Interphase in the coreid *Pachylis*, to show the characteristic relation of the second to the first spindle in the Heteroptera.

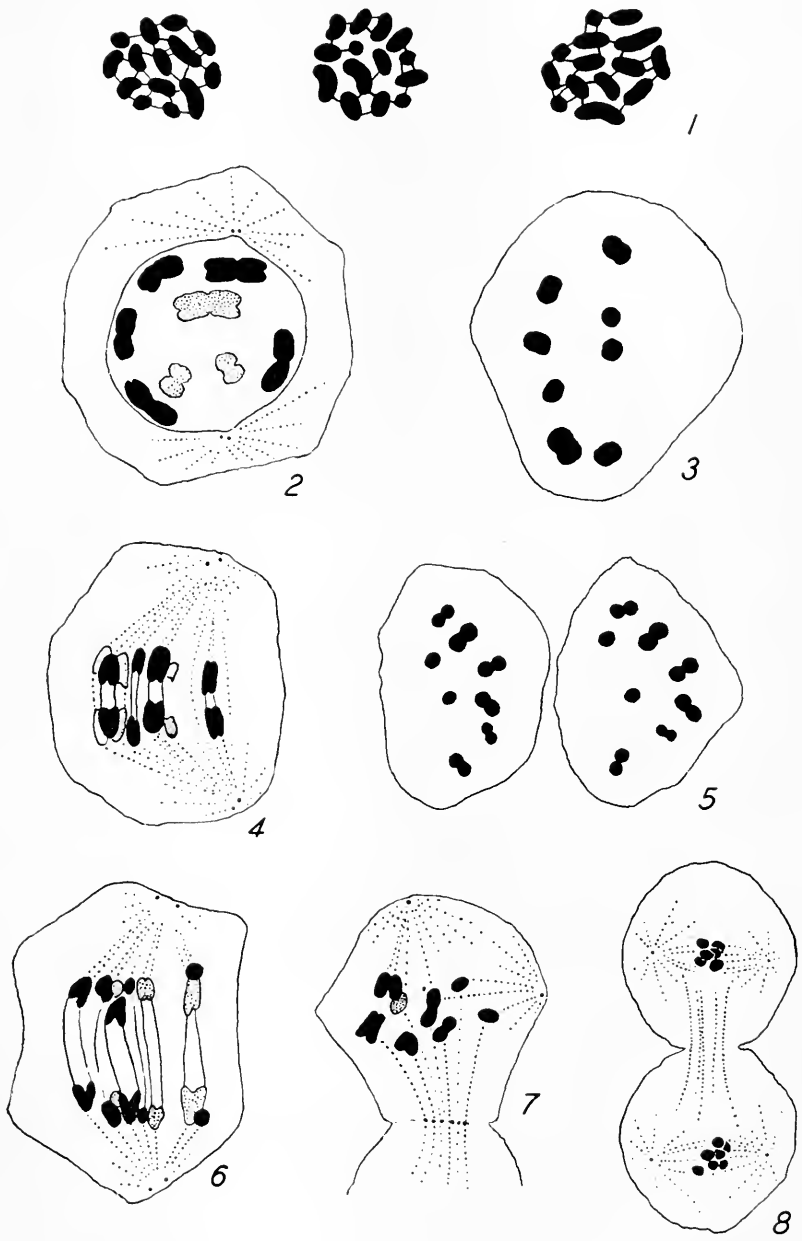

PLATE I

stage (Fig. 10). If this occurs before they have become dissociated from the interzonal connectives, such peculiar configurations as shown in Fig. 12 may result. In these as well as in less extreme cases the significant feature lies in the marked elongation of the chromosomes.[1]

This occurs despite the fact that the two chromatids of each dyad move in opposite directions toward the centrioles which are establishing a new axis. In other words, though the demarcation between the two chromatids is clearly indicated—as indeed it already is in diakinesis—and though the attenuation of the chromatids evidently betokens forces that tend to move them apart, they do not succeed in separating from each other (Figs. 10–13). The attenuating process continues until the chromosome body is torn into two pieces. The break apparently occurs at random and usually not in the natural line of separation between the chromatids (Figs. 13 and 14). Hence the amount of chromosome material distributed to each pole is variable and certainly not normal.

During this time the centriolar movement is completed. As a result the flexion that characterizes the spindles during the early part of this division disappears and the spindles of the late second anaphase are perfectly straight (Fig. 13).

PLATE II

FIG. 9. Late anaphase of first division. Each of the centers (both show two centrioles) has moved through 90° toward one pole of the second division.

FIG. 10. Centrioles of second division acting on chromosomes which are still in the condition of the first anaphase. (In Figs. 10, 11, and 12 only one of two sister cells is shown.) The demarcation between the chromatids is evident in several dyads.

FIG. 11. Second division showing attenuation, with chromatid demarcation showing in several dyads.

FIG. 12. Second division. The centrioles have separated relatively little, and the whole figure is strongly flexed as a result. Trace of interzonal connections of first division still showing at lower left.

FIG. 13. Late anaphase of second division. The spindle has straightened out. Chromatid demarcation still present in two of the dyads.

FIG. 14. Telophase of second division. There is no trace of collocation. The abnormality of the chromosome division is evident.

FIG. 15. Late telophase. Chromosomes still scattered and already becoming diffuse.

FIG. 16. Spermatid with four micronuclei, one Nebenkern, and one tail filament.

[1] It will be seen that the side of the chromosome presented toward the pole in the first division does not correspond to that of the second. This puzzling feature is, however, encountered in all Heteroptera and does not constitute a peculiarity of the present case. The explanation may lie in the fact that in the Hemiptera we are dealing with a "diffuse" instead of a localized kinetochore, as Hughes-Schrader and Ris (in press) have recently established.

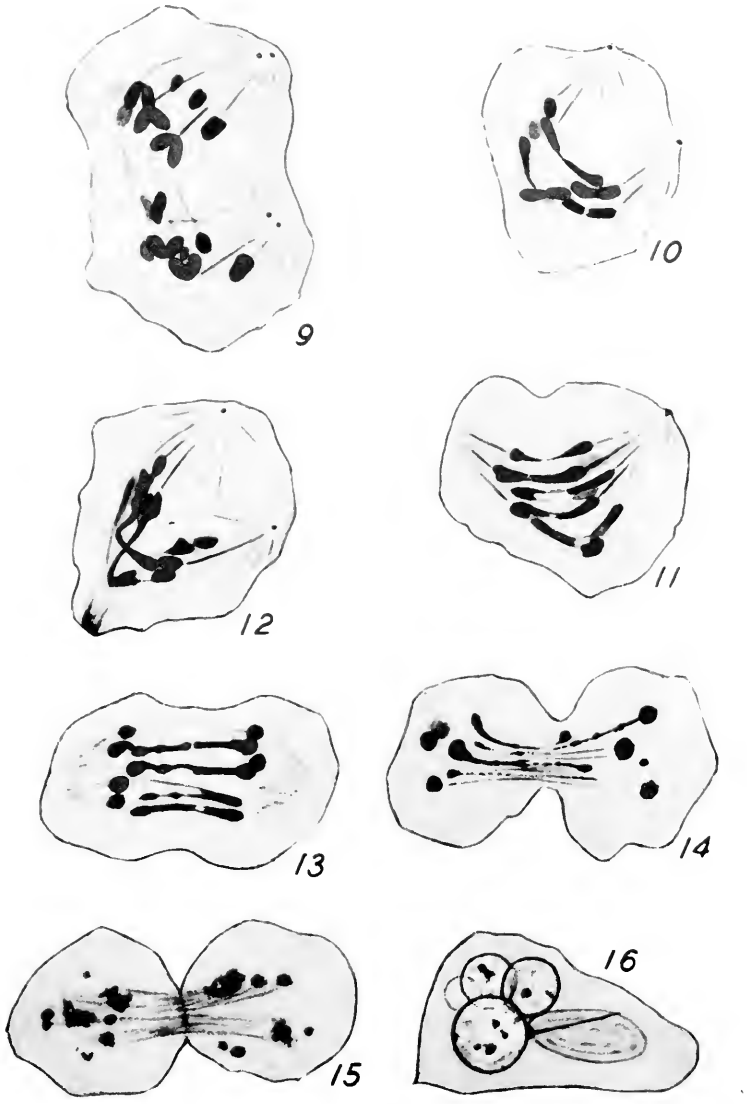

PLATE II

Second Telophase to Spermatozoa

Since the chromosomes of the first division are subject to the forces of the second division while they are still in anaphase, nothing can be said of their behavior under telophase conditions. Since there is only one centriole at each pole of the second division, no comparable centriolar disturbance takes place there and the chromosomes reach the telophase in every case.

Instead of the collocation of chromosomes that is typical of normal telophases, the chromosomes here actually tend to move further apart or to repel each other (Fig. 14). This tendency is not overcome even by the time that the chromatin becomes diffuse and as a result the products of the division lie more or less scattered in the cell (Fig. 15). Separate, small nuclei are formed from such masses of chromatin, and the spermatid is always a multinucleate cell (Fig. 16). In most cases only one Nebenkern is formed though in some instances two have been encountered. In no cell, however, does one find more than one axial filament and middle piece. These are associated with one of the nuclei which is not necessarily the largest one.

Apparently even the smallest of the nuclei takes steps toward the elongation that characterizes the formation of the sperm head. Later, however, there is much degeneration, though some of the sperms appear more or less normal.

The relative independence of mitotic phenomena in the cytoplasm and in the nucleus is attested by the fact that all the manoeuvers of the centers and the chromosomes do not hinder the division of the cytoplasm. Separate and complete cells, more or less equal in size, are found both after the first as well as the second division.

Discussion

The relationship of the significant features of this case is not always entirely clear, though it seems safe to assume that they are interconnected. They may be listed as follows: 1. The asymmetry of the first division figure. 2. The precocity in the behavior of the centers. 3. The attenuation and irregular division of the chromosomes in the second division. 4. The formation of multinuclear spermatids.

(1). The asymmetry of the first division figure is difficult to explain. If bipolarity is brought about merely by a mutual repulsion of two centers, the latter should be separated by 180° on the diakinetic nucleus and in the first metaphase. Again, the location of the chromosome plate, if it rests on a system of repulsive or attractive forces correlated with

those of the centers, should be on the axis formed by the latter. To explain the askew position of the chromosomes, it might be suggested that a primary spindle, comprised of fibres extending from pole to pole, arises before the chromosomes have formed a metaphase plate. This spindle then constitutes a core into which the chromosomes do not penetrate and hence they are disposed in the form of a semicircle around it. But such a hypothesis does not touch the root of the matter, which lies in the asymmetrical position of the centers themselves. And for this nothing more can be said than that a factor or force, probably extraneous to centers and chromosomes, is responsible.

(2). The extreme degree of separation of sister centrioles during the first division is clearly an indication of precocity in their cycle. Not so pertinent to this conception are those instances where the entire center, including both centrioles, moves to one of the poles of the second division (Fig. 9).

This might be attributed to the elongation of the spindle which pushes both centers around the periphery to one side. Precocity would there be expressed only in the development of astral rays and half-spindle fibres which actually appear to be growing at a time when in normal cases they are waning.

If, however, the movement of the undivided center is not thus accidental, its shift to the axis of the second division must mean that this pole is predetermined. This would imply that the centers are only secondarily concerned. The evidence hardly permits of extensive hypothetical considerations, but the early establishment of such a pole might involve forces that also are responsible for the asymmetry of the first division.

(3). But whether or not the centers are the primary agents in the determination of polarity, their direct influence on the chromosomes is not to be denied. This is strikingly shown in the premature second division, where it appears that the precocity of the centriolar processes is correlated with an exertion of forces that are normally not in evidence until a later stage. Their influence is indicated by the fact that the mitotic movement of chromosomes is toward the two centers from the very start. The autonomous separation of chromatids which takes place without reference to centers and which always comprises the first step under normal conditions, does not take place at all.

The attenuation of the chromosomes suggests that they are subjected to tensile forces. The failure of the chromatids to dissociate from each other under such conditions must then indicate that they are not yet completely ready when the centriolar forces are exerted thus precociously.

The lag does not lie in the chromosome proper, for in this as well as in normal cases all the chromatids are sharply demarcated from each other already in the preceding diakinesis (Fig. 2). That this demarcation persists into the second division is clearly shown in Figs. 10, 11 and 13, and the conclusion hence is unavoidable that a separation is prevented by other factors. The latter can be sought only in either the sheath or the matrix of the chromosomes, and it is therefore this constituent which is not yet ready for the division and holds the chromatids together.

It may be pointed out that the attenuation of chromosomes during division is not at all rare and that its cause is by no means always the same. It has been reported in cells that were subjected to X-ray or radium treatment. It is then usually correlated with a tendency of chromosomes to clump, and secondarily to translocations and inversions. Such cases are difficult to analyze since so many of the mitotic processes seem to be affected.

It has been described in tapetum cells (Steil, 1935) which show signs of degeneration. The attendant irregularities may well arise from an upset in the timing of the various mitotic processes as in the present case, but the necessary details of behavior that would justify such a conclusion are not available.

Bauer (1931) has reported it in *Tipula* and ascribes it to the presence of supernumeraries. The disturbance is there correlated with an adhesion of the chromosomes to each other.

It results from changes in the physical condition of the chromosomes, which in at least one case arise from the mutation of a single gene (Beadle, 1932). The "stickiness" which there characterizes the chromosomes seems to be caused chiefly by changes in the matrix and it is not impossible that the frequent attenuation during division is closely akin to that observed in *Peromatus*.

Lastly, it is a well-recognized characteristic of chromosome inversions which have resulted in dikinetic or dicentric chromosomes. Such "chromosome bridges" have played so striking a rôle in recent cytogenetic investigations that there has been a tendency to forget that not all chromatin bridges need be of the same nature. Thus Gentscheff and Gustafsson (1940) in their excellent study of meiosis in *Hieracium* utilize Beadle's conception that fragmentation of his maize chromosomes results from changes in the matrix, but quite ignore his explanation that his chromosome bridges were due to stickiness and increase in viscosity. Instead, they ascribe the very similar bridges in *Hieracium* to inversions and thus seem to agree with Darlington (1937, p. 320), who does not accept Beadle's convincing interpretation and states that "at anaphase

several bridges are found, showing that the changes include inversions."

It need hardly be pointed out that in the present case of *Peromatus notatus,* an explanation that rests on inversions is not tenable at all. This is already strongly indicated by the fact that the first division shows no bridges whatever, whereas they characterize all second divisions. To explain this on the basis of inversions would necessitate that two crossovers of a very specific type take place, and that these occur in the meiotic prophase of all cells. This would, moreover, result in a chromosome fragment which most assuredly is not present. Further, such a hypothesis would assume an orthodox, localized kinetochore, whereas here we are dealing with one of the diffuse type (Hughes-Schrader and Ris, in press). Finally, it must be remembered that an inversion bridge arises because bipolar tension is exerted upon a portion of a chromosome which does not include the natural line of demarcation between chromatids and which therefore can be divided only by tearing. In contrast, the bridges in *Peromatus* include the region where two chromatids, sharply demarcated from each other, are placed end to end. Dissociation therefore should and would follow quite normally without attenuation, if it were not hindered by the matrix or the sheath.

(4). Multinucleate spermatids arise because of the upset in the timing of mitotic processes. The chromosomes of the second division arrive at telophase when, in a sense, they are still in the anaphase condition. The mutual repulsion that characterizes them at the normal metaphase and anaphase, is therefore still encountered here when they have arrived at the poles. Hence there is the reverse of the usual collocation, the chromosome bodies are scattered singly or in small groups through the cell, and several micronuclei are found. The case for an irregularity in the timing of the centers is further supported by the fact that the actual division of the centrioles, albeit their movements are precocious, is in itself quite normal and only one middle piece and one axial filament are encountered in every multinucleate spermatid.

Conclusion

The nature of the case makes it rather futile to speculate on the origin of the meiotic abnormalities just described. Practically nothing is known about the ecology of the genus, and the possibility of interracial and interspecific crosses is purely hypothetical.

Clearly, however, the case is an exceptional one for the species. The conditions basically affect the production of normal sperms and can have no survival value. Indeed, the rather orthodox course of spermatogenesis in other specimens of *Peromatus* renders this certain.

But so far as this individual is concerned, the abnormality is a deep-seated one since the absence of normal spermatids indicates that it has persisted for some time. The conditions strongly suggest that at least one of the mitotic processes has fallen out of step and that coördination with the other processes becomes progressively more difficult in the successive cell generations, from spermatogonia to spermatids. The disturbance has no visible effect on the spermatogonia; has a well-defined influence on the spindle mechanism of the first division without, however, upsetting the essential aspects of orderly chromosome division; renders impossible a normal distribution of chromosomes in the second division; and culminates in spermatids that are definitely abnormal.

Summary

1. The abnormal course of meiosis in a specimen of *Peromatus notatus* is characterized by a series of well-defined irregularities.

2. The spindle of the first division shows both centers to one side of the geometrical axis and the metaphase plate displaced to the opposite side.

3. Before the chromosomes of the first division have reached the poles, they are subjected to the forces involved in the second division.

4. The effect on the chromosomes is to attenuate them without bringing about a normal division. The resulting configurations simulate inversion bridges, though that is quite clearly not their nature.

5. The spermatids receive varying amounts of chromosome material and are multinucleate.

6. It is suggested that this abnormal meiosis is due to an irregularity in the timing of one of the mitotic processes. The indications are that this process involves the movement of the centers.

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THE RESPONSES OF CATFISH MELANOPHORES TO ERGOTAMINE

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Some eight years ago Bacq (1933), on the basis of experimental evidence, reached the conclusion that ergotamine contracts denervated catfish melanophores and expands normally innervated ones. It is now known that the so-called contraction of catfish melanophores is the result of a neurohumor from the concentrating nerve-fibers, very likely adrenaline, and that their expansion is due to two agents, intermedine from the pituitary gland and a neurohumor, probably acetylcholine, from the dispersing nerve-fibers (Chang, Hsieh, and Lu, 1939; Parker, 1940). In consequence of these new discoveries it seemed desirable to repeat Bacq's experiments with the view of bringing his rather remarkable results into relation with this newly acquired information.

The ergotamine tartrate used by Bacq is fortunately still to be had in the American market. It is dispensed in 1 cc. glass ampules under the name of gynergen (Sandoz Chemical Works) and in this form it is extremely convenient for experiments on fishes. Three sets of catfishes (*Ameiurus nebulosus*) were prepared for these tests,—pale, intermediate, and dark. The pale fishes, three in number in the initial set, were kept in white-walled, illuminated vessels. Two caudal bands were cut in each fish. By the end of six days these fishes were very pale and their caudal bands were almost fully blanched. The axis and tip of each band, however, were noticeably dark as observed and figured by Bacq (1933). The three fishes of intermediate tint were kept in a gray, illuminated vessel. Their caudal bands after six days were very slightly darker than the rest of their darkish tails. The three fishes of the dark set were rendered very dark, coal-black, by complete blinding. It is well known that catfishes assume this intense shade on double enucleation. Notwithstanding the great depth of tint thus produced, the caudal bands in these fishes were a shade darker than the rest of their very dark tails.

Six days after the cutting of the caudal bands in the pale fishes these bands were recut a little distal to the original incision. Since the part of the band distal to the new cut did not change in tint as a result of the recutting, it was concluded that so far as color changes were concerned the nerves of such bands had degenerated. As the three fishes in any given set, pale, intermediate, or dark, were very similar in color, one in each set was reserved as a control and the other two were subjected to tests. Two injections each of 0.25 cc. of gynergen separated by an interval of about a quarter of an hour yielded the best results. These injections were at times supplemented by a third. Two injections of 0.5 cc. of gynergen with an interval of fifteen minutes between them gave more vigorous responses than the weaker injections, but they were usually followed some hours later by the death of the fish. I was unable to obtain unquestionable responses with only a single injection of 0.25 cc. of gynergen as reported by Bacq. The catfishes used by me weighed each about 50 grams. Bacq makes no statement as to the weight of his specimens. Possibly he had smaller individuals than I had and therefore obtained satisfactory responses with less ergotamine. In my procedure any given catfish must have received into its body from the two injections ordinarily given a total amount of 0.25 mg. of ergotamine tartrate judged from the formula published by the Sandoz Chemical Works for their preparation of gynergen.

Bacq's tests, which were carried out only on pale catfishes, consisted in injecting into such a fish with a blanched caudal band 0.25 cc. of gynergen whereupon the fish as a whole became dark, but the band remained pale or even took on a somewhat lighter tint. My repetition of such a test gave almost identical results. When a pale fish with two blanched caudal bands was injected with the usual two doses of gynergen, 0.25 cc. each, with an intervening quarter of an hour, the fish began to darken noticeably in about half an hour after the first injection and in an hour to an hour and a half it had reached a full intermediate tint, its maximum color change under the circumstances. As the tail darkened the bands appeared to become paler as noted by Bacq, but whether this was an actual blanching or a contrast phenomenon could not be settled except by close scrutiny. When the bands on the tail of an injected fish were closely compared with those on the uninjected pale control, the two sets of bands were found to be in very close agreement. This was particularly well seen under a low power of the microscope. In both sets of bands the pigment masses in the macromelanophores were rounded bodies with short, blunt protuberances on their sides marking the roots of the pigmented processes of the dispersed stage. The pigment masses in the injected fishes appeared to be in no sense less dispersed than those

in the control fish, and yet when the bands were inspected by the unaided eye those in the dark fishes appeared to be paler than those in the pale control. In my opinion this apparent difference is purely an illusion due to contrast. The dark surroundings of the pale bands in the injected fishes made these bands appear paler than the pale bands in the pale control. I therefore conclude that, contrary to Bacq's view, ergotamine has no effect on denervated melanophores with concentrated pigment. This agent, however, does induce pigment dispersion in innervated color cells, as stated by Bacq.

In one other respect my observations do not agree with those of Bacq. In the majority of caudal bands that have been blanched in pale fishes for some six days the axes and tips of these bands, as already stated, are slightly dark. This feature was described and figured by Bacq, who noted further that when catfishes showing these peculiarities were injected with ergotamine the pale bands not only became paler but their dark axes and tips also blanched. In my experience such was not the case. After full doses of ergotamine had been allowed to act on the two pale catfishes tested by me, the dark axes and tips in their caudal bands were as visible after the injection as they had been before it or as they were in the control.

In making these several comparisons the individual catfishes in the course of inspection were necessarily much handled. As is well known, this treatment induces such fishes to darken temporarily and it might be supposed that this darkening could in some way have influenced the results just described. But both the control fish and the two injected individuals were handled to about the same degree and therefore should have shown the same amount of change as a result of this treatment. Moreover, it has been demonstrated in a recent paper (Parker, 1940) that the darkening already alluded to is a response mediated by the dispersing nerves. Consequently it ought to play no part in the activities of a denervated area such as a caudal band. There is therefore no reason to suppose that the ordinary darkening of catfishes from handling could have had any influence on the results herein recorded.

The tests on the three pale catfishes just described were repeated on two other sets of pale individuals, one of two fishes and the other of three. In both these sets the pale bands of the injected fishes showed no more change in tint than did those of the first set and their bodies in general darkened to intermediate. This agreement in three sets of results justifies the conclusion that, as Bacq maintained, ergotamine excites innervated melanophores in catfishes to disperse their pigment to a point where the fish attains an intermediate tint. It shows further that, contrary to Bacq's opinion, this agent does not induce a concentration of

pigment in denervated melanophores whereby caudal bands in pale fishes would become still paler. Ergotamine apparently has no influence whatever on denervated melanophores with concentrated pigment. It does induce pigment dispersion in innervated melanophores.

When catfishes with caudal bands cut in their tails are kept for some six days in a gray, illuminated vessel they take on, as already stated, an intermediate tint in which the bands are as a rule slightly darker than the rest of the fish. If these fishes now receive the usual injections of ergotamine, two doses of gynergen, 0.25 cc. each, separated by a quarter of an hour, they will either show no noticeable change in tint at all or darken very slightly. This rule held for all three sets of catfishes tested, including a total of nine individuals. In no instance was there any evidence of the blanching of the denervated bands, but, contrary to what might have been expected from Bacq's statements, these bands remained usually a little darker than the rest of the tail. Bacq apparently never tested fishes of intermediate tint with ergotamine. Had he done so, he surely would have observed that when the ergotamine excited any change at all it was a very mild darkening in the region of the innervated color cells and not only no blanching but no change of color whatever in that of the denervated cells.

What has been stated for catfishes of an intermediate tint may be affirmed in general for those that are fully dark. As noted previously, the caudal bands in such fishes are as a rule very slightly darker than the rest of the tails in these individuals. After the usual injections of ergotamine these color conditions either showed no change or the fish as a whole darkened very slightly. In two instances this general darkening was sufficient to make the tail slightly darker than the bands. As comparisons with the control fish showed, this was a real darkening of the tail and not a slight blanching of the band. Hence we are justified in concluding, as in the instance of the intermediate fishes, that ergotamine induces pigment dispersion in innervated melanophores but has no effect on denervated ones.

From these several tests on pale, intermediate, and dark catfishes it seems fair to conclude that ergotamine acts only on innervated melanophores by inducing them to disperse their pigment and has no effect whatever on denervated melanophores. The assumed blanching of these color cells by this agent, as described by Bacq, is purely illusory. So far as the end result is concerned, ergotamine is like intermedine or acetylcholine in that it causes catfishes to darken. But it does not act in the same way as these two neurohumors do. They act directly on the melanophores (Parker, 1941), for they will induce denervated caudal bands to darken. Ergotamine acts on melanophores indirectly, that is through

nerves, in that it excites at some central nervous station the dispersing nervous elements which in turn excite the appropriate nervous terminals to discharge acetylcholine. This neurohumor causes the melanophores to disperse their pigment whereby the fish darkens. Ergotamine is a good example of an indirect melanophoric agent as contrast with direct ones such as intermedine, acetylcholine, and adrenaline. These will activate denervated melanophores in caudal band. Ergotamine is incapable of this activity.

Summary

1. Ergotamine acts on only innervated melanophores by inducing them to disperse their pigment. It is without effect on denervated melanophores either with dispersed or with concentrated pigment.

2. It acts on innervated melanophores only indirectly, that is, through their nerves. These are excited by ergotamine centrally to produce at their melanophore terminals acetylcholine which causes the color cells to disperse their pigment.

3. Ergotamine is a good example of an *indirect* excitant of melanophores as contrasted with *direct* excitants such as intermedine, acetylcholine, and adrenaline, all of which act directly on these color cells.

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SEXUAL PHASES IN WOOD-BORING MOLLUSKS

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It has been demonstrated previously that the well-known shipworm *Teredo navalis* is typically protandric, nearly all individuals functioning as males when young and later changing to the female phase (Coe, 1936; Grave and Smith, 1936). Under favorable conditions the female phase may be followed by an additional sequence of male and female phases.

In no other species of the wood-boring mollusks (Teredinidae) is the sexual sequence fully known, although Yonge (1926) recognized protandry in *Teredo norvegica* and Siegerfoos (1908) concluded that it was present in "*Xylotrya gouldi*" = *Bankia fimbriata* (Jeffries).

During the past few years the writer has had the opportunity of investigating the biology of three species, *Teredo navalis, T. diegensis* and *Bankia setacea,* with particular reference to the development of the gonads and the sequence of the sexual phases. The results of this study may be briefly summarized and compared with previous reports on the sexuality of the shipworms.

Sexual Phases of Teredo navalis

This widely distributed species occurs on both the Atlantic and Pacific coasts of the United States and has been particularly destructive in past years in San Francisco Bay. Individuals in the female phase are larviparous, the fertilized eggs developing through about half the larval period in the maternal gill chambers. A free-swimming period follows the discharge of the larvae into the water. After settling upon a piece of wood, transformation to the adult condition takes place and boring into the wood begins (Kofoid and Miller, 1927; Grave, 1928).

The primary male phase becomes functional within four to six weeks after the completion of the free-swimming larval condition in the warmer season of the year or in warm localities, but may be delayed for six months or more under colder conditions. The body is then only 20 to 30 mm. in length and about 2 mm. in diameter. The female phase may begin at the age of eight to ten weeks.

[1] Contributions from the Scripps Institution of Oceanography, No. 145.

Growth is rapid under favorable conditions. At the end of one year the body may have attained a length of 10 to 40 cm. and a diameter of 4 to 9 mm. In the meantime the individual has normally transformed to the female phase and has produced perhaps 1,000,000 young. A second sequence of male and female phases may have occurred.

Because of the long breeding season and the sequence of sexual phases, the proportion of the two sexes in each piece of wood will obviously depend upon the ages represented in the colony. In a recently attacked timber nearly every individual will be in the male phase. A few weeks later, after the sexual transformation has occurred, 60 to 90 per cent of the original colony will be functioning as females.

But in the meantime there may have been daily additions of recently arrived young from other pieces of wood, resulting in a continuing supply of male-phase individuals. These, together with the few so-called true males, and a small proportion of second male-phase individuals, are then available for the fertilization of the eggs produced by such individuals as are at that time functioning as females. Because of this overlapping of sexual phases all the older colonies are at all times represented by both sexual phases in varying proportions. Most of the smaller and consequently younger individuals will be functioning as males, while most of the larger, older individuals are in the female phase.

A rhythmical sequence of four sexual phases may be considered to represent the normal life cycle but this is seldom realized because of an earlier death due to parasites or to the exhaustion of the wood supply or to other unfavorable environmental conditions. Most individuals die after only two of these phases have been completed and many others survive only the primary male phase.

There are some variations in this sequence, however, because a second female phase may sometimes follow the first without an intervening male phase. Other individuals, known as true males, retain the male phase long after their contemporaries have changed to the female condition, and this may sometimes mean throughout their entire lifetimes.

Sexual Phases in Teredo diegensis

This species, like *T. navalis,* is protandric and larviparous, but the two species differ considerably both in the conditions of sexuality and in larval development.

T. diegensis Bartsch occurs abundantly and causes considerable damage along the coast of Southern California, and has been reported as far north as San Francisco (Kofoid and Miller, 1927). It is also found at the Hawaiian Islands. On the coast of Southern California this spe-

cies breeds through all except the two or three cooler months of the year and through the entire year when the winter is warmer than usual. At the lower temperatures the larvae may remain within the maternal gill chambers for several months before they are discharged.

Ovulation occurs at intervals of a few weeks, the later broods of larvae often becoming established in the gills before an earlier brood has left. This condition occurs throughout the year. With the exception of young individuals and a few "true males" a sexually mature individual without a brood of larvae is seldom found.

Large individuals, 120 mm. or more in length, may have more than 1000 larvae in the gill chambers, while dwarfs may have less than 100. The bivalve larvae reach a shell length of 0.35 to 0.38 mm. before leaving the gills. In this species, as in *T. pedicellata* (Roch, 1940), the larvae remain within the gill chambers until nearly ready for metamorphosis. The free-swimming stage consequently lasts but a few hours if wood is available for attachment. The total period of larval development is about four weeks and is therefore of about the same length as in *T. navalis* (Coe, 1933*a*). In this latter species, however, only about two weeks of this time are spent in the maternal gill chambers, followed by a free-swimming period of about the same duration.

Shortly after being set free in the water the larvae of *T. diegensis* attach themselves to any available piece of wood but do not immediately penetrate the surface. Some of them may remain two weeks or more on the surface of the wood before beginning to bore. Their stomach contents show that minute particles of organic food materials are ingested in the meantime. Because there is no necessity for feeding during the brief free-swimming stage, this species may be reared from generation to generation in the aquarium. By supplying a fresh piece of wood occasionally the stock may be continued for at least several years. The aquarium water evidently contains sufficient materials to supplement the wood as sources of nourishment. After penetrating the wood the young teredos grow rapidly and reach the primary male phase within four to five weeks. The body is then about 8 to 12 mm. in length.

The primary gonad is composed of branching follicles filled with large, vacuolated cells and having a few proliferating germinal cells scattered along the walls of the follicles as shown for *Bankia setacea* (Fig. 1, *A*). This condition is closely similar to that described by Coe and Turner (1938) for the developing gonads of *Mya*. As the germinal cells increase in number they become differentiated into the two sexual types of gonia and then further differentiated into ovocytes and spermatocytes.

By their rapid proliferation and differentiation the spermatogenic cells encroach upon the spaces occupied by the vacuolated follicle cells and eventually fill the entire lumen of the follicle (Fig. 1, *B*). In some individuals the follicle cells contain numerous fragmenting and degenerating nuclei, representing a kind of atypical spermatogenesis, as described by Coe and Turner (1938) for *Mya*.

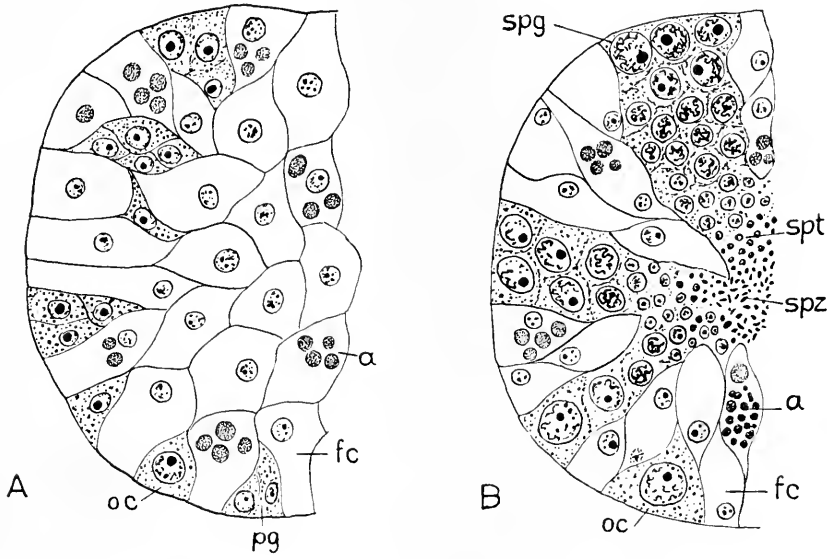

FIG. 1. *Bankia setacea*. Development of the primary ambisexual gonad. *A*, portion of section of young follicle, showing large vacuolated follicle cells (*fc*), with a few primary gonia (*pg*) and a single young ovocyte (*oc*) peripherally; several follicle cells contain atypical, degenerate nuclei (*a*), derived from original primary gonia. *B*, portion of follicle in early male phase, with a few remaining vacuolated follicle cells (*fc*) and various stages of spermatogenesis; *a*, atypical degenerate nuclei; *oc*, ovocyte; *spg*, spermatogonia; *spt*, spermatids; *spz*, spermatozoa.

There is considerable variation in the number and size of the ovocytes which are always present on the walls of the follicles during spermatogenesis. Some individuals, corresponding with the so-called true males of *T. navalis*, have only a few small ovocytes in each of the follicles (Fig. 3, *A*), while others show a preponderance of ovocytes in some or all of the follicles before the spermatozoa are fully ripe (Fig. 2).

Occasionally all the spermatozoa are discharged before ovulation occurs, resulting in a distinctly female phase (Fig. 3, *B*), but more frequently a functionally hermaphroditic condition is found. Both spermatozoa and ova may ripen at the same time and evidently both

may be discharged simultaneously. Under experimental conditions self-fertilization occurs readily; this is followed by the formation of the polar bodies and cleavage, but only as far as the blastula and gastrula stages. For the normal processes of larval development the environmental conditions peculiar to the maternal gill chambers appear to be necessary.

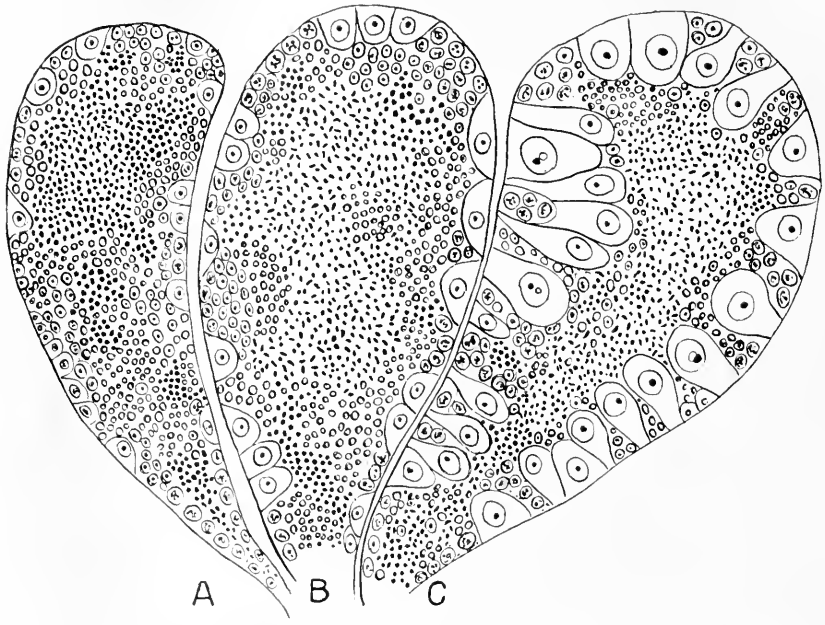

FIG. 2. *Teredo diegensis*. Sections of three follicles from gonad of second male-phase individual which had branchial brood pouches distended with larvae. *A*, immature follicle, principally in male phase, with relatively few ripe spermatozoa and with only small ovocytes in basal layer of germinal cells. *B*, more nearly mature follicle in male phase, distended with spermatogenic cells and many ripe spermatozoa; numerous half-grown ovocytes in basal layer of germinal cells. *C*, ripe follicle in hermaphroditic male phase; lumen filled with ripe spermatozoa and with nearly mature ovocytes densely crowded on periphery.

During some seasons this species becomes particularly injurious by boring in mooring ropes. A similar habit has been reported for *T. navalis* (Coe, 1933). Under such conditions only dwarf individuals are produced but many of these are nevertheless capable of forming a small number of ripe gametes. A single change of sexual phase, from male to female, may occur, although many individuals are killed by the disintegration of the rope before even the primary male phase is completed.

Sexual Phases of Bankia setacea

This species differs from *Teredo diegensis*, with which it is often associated in the cooler waters on the coast of southern California, in being oviparous rather than larviparous. On reaching the female phase vast numbers of minute ova are produced and these are discharged directly into the water. Fertilization of these ova by sperm of other individuals takes place in the water. Then follows a free-swimming larval period of perhaps four weeks before the larva is ready to settle on a piece of wood and transform to the adult condition.

It is evident that all individuals pass through a functional male phase a few weeks after entering the wood. The two types of males are more

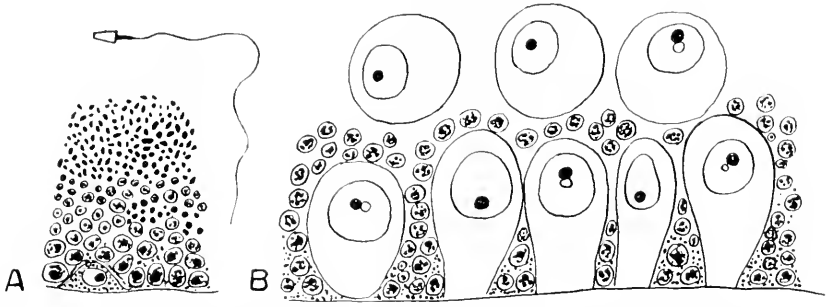

Fig. 3. *Teredo diegensis*. *A*, portion of gonad of young individual in "true male" phase, showing spermatogenesis and a single, more highly enlarged spermatozoön; a single ovocyte is shown at the base of the spermatogenic cells. *B*, portion of gonad in female phase, with ripe ova; those ova still attached to wall of follicle are surrounded by follicle cells with undifferentiated gonia basally.

easily distinguished than in either *Teredo navalis* or *T. diegensis*. About half of the young, male-phase individuals are apparently hermaphroditic; these complete spermatogenesis early and then change to the female phase. These are evidently genetically protandric females, while an approximately equal number retain the primary male phase much longer. The latter presumably represent the "true males" of other species (Coe, 1933*a*, 1936). Fully adult individuals, measuring 20 to 50 cm. in length, usually have the appearance of being either males or females, with little indication of ambisexuality. Only occasionally do their gonads reveal distinctly their essentially hermaphroditic nature. Careful examination of the gonad in full spermatogenesis, however, usually reveals a few ovocytes on the walls of the follicles and these may be considered as an indication that a change of sexuality may later occur. In the female phase likewise indifferent gonia show the potentiality of a sex change.

The sexual conditions in this species are therefore similar to those of the oyster *Ostrea virginica* (Coe, 1938), which is seasonally of separate sexes but in which the sexual phase of any season cannot be predicted from the sexual condition of the preceding season. In *Bankia*, however, many of the females experience but a single change of sex, from male to female, while the true males may retain the male phase throughout life.

On the coast of southern California some individuals of *B. setacea* are found in spawning condition throughout the year. The majority of individuals, however, spawn only during the autumn and spring months, with resting periods in the winter and summer. Consequently wooden blocks and timbers become much more quickly and more heavily infested in the spring and autumn than at other times of the year.

Johnson and Miller (1935) found that settlement of this species in Puget Sound occurred principally from October to December and less abundantly from March to September. Kofoid and Miller (1927) also observed that in San Francisco Bay settlement of this species was confined to the cooler months of the year.

When removed from the body the eggs of *Bankia* develop rapidly to the free-swimming larval condition after artificial fertilization by sperm from another individual. The eggs of juvenile protandric females are sometimes capable of self-fertilization.

Conclusions

It is evident that the sexuality of these three species of pelecypods represents a graded series of ambisexual or hermaphroditic conditions intermediate between such dioecious forms as *Mya* or *Mytilus*, which are almost strictly of separate sexes, and those that are uniformly monoecious, as the larviparous oysters, *Ostrea edulis* or *O. lurida*. In all the dioecious pelecypods of which the sexuality has been extensively investigated hermaphroditism is found occasionally and this may include a large or only a small portion of the gonad.

Even the monoecious species usually have some dioecious tendencies, with some individuals ("true males") showing a preponderance of masculine characteristics, while others are more nearly feminine. A sequence of functional male and female phases is of common occurrence and in the case of long-lived species this may constitute an alternating rhythm. The wood-boring mollusks are of this type.

Protandry is characteristic of many species. This represents a juvenile type of sexuality and often occurs when the individual is very young and when the body has reached but a small fraction of its definitive size.

As mentioned above for *Bankia* and as Loosanoff (1937) found in *Venus,* the genetic females pass through a juvenile male phase before adult sexuality is realized. This has been reported for other species. In the oviparous oyster *Ostrea virginica* the proportion of the genetic females which pass through a functional male phase during their first breeding season depends both upon the particular local race concerned and upon the environmental conditions. This juvenile male phase is more frequently aborted or omitted under conditions favorable to rapid growth, thereby increasing the proportion of juvenile females. This species also shows a rhythmical tendency toward seasonal change of sex in later life (Coe, 1938).

In all the examples mentioned and in many others belonging to the various classes of mollusks, the sex-differentiating mechanism is so delicately balanced between the two sexual tendencies that relatively slight differences in environmental conditions may be potent in determining which of the two contrasted aspects of sexuality shall be realized. In some the entire population functions as male when young and as female when fully adult. An intervening functionally hermaphroditic phase may occur.

Summary

1. The three species of wood-boring mollusks *Teredo navalis, T. diegensis* and *Bankia setacea,* are all protandric, with a strong tendency toward rhythmical changes of functional male and female phases.

2. Each species differs as to the degree of ambisexuality characteristic of the primary male phase and of the subsequent sexual phases.

3. The primary gonad in all three species develops from branching follicles filled with large vacuolated follicle cells and having the primary gonia scattered along walls of the follicles.

4. In each of these species the gonads of young animals indicate that there are two types of primary male-phase individuals: (1) ambisexual males or protandric females, characterized by many ovocytes on the walls of the spermatic follicles, and (2) true males with few ovocytes. In those of the former type the male phase is of short duration, while true males retain the male phase longer or in some cases indefinitely.

5. In *T. navalis* the first female phase does not usually become functional until nearly all the sperm of the primary male phase have been discharged. Functional hermaphroditism is not usual, although the gonad is histologically ambisexual during the change of sexual phase in both directions.

6. In *T. diegensis,* on the other hand, functional hermaphroditism is of usual occurrence and the sexual phases are not sharply demarcated.

7. In *B. setacea* functional hermaphroditism occurs only occasionally in the primary male phase; the subsequent sexual phases are clearly differentiated, often with a resting stage intervening between two sexual phases. The sexual phases are of the alternative type in that any sexual phase, after the first, may be followed by either a male or female phase if the length of life suffices. The relatively short life of many individuals, however, allows but a single change of sex, from male to female, in the genetic females, and none at all in true males.

8. In all of the three species the eggs begin development after artificial fertilization. In *Bankia* the larvae may be reared to the free-swimming veliger stage, but in the other two species the larval stages require the peculiar environmental conditions of the maternal gill chambers. Under experimental conditions self-fertilization and apparently normal cleavage occurs readily in the two species of *Teredo* and occasionally in *Bankia*.

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REGENERATION OF COENOSARC FRAGMENTS REMOVED FROM THE STEM OF TUBULARIA CROCEA

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The rôle of environmental factors in the regeneration of hydroids has been studied extensively, the evidence all pointing to the extreme lability of hydroid systems. The literature on this subject has recently been reviewed by Barth (1940*b*). Morgan (1903) found that, when the cut end of a *Tubularia* stem was placed in sand, regeneration was inhibited at that end. Regeneration is inhibited also when the sectioned ends of a *Tubularia* stem are ligated. The reason for this inhibition was made clear by experiments in which *Tubularia* stems were exposed to a differential of oxygen in the sea water. Barth (1938*a*) was able to reverse the normal polarity by placing the distal end of the stem in a glass capillary, and he attributed this reversal to a lack of oxygen at the covered end. Miller (1937) reversed the polarity of *Tubularia* stems by exposure of the proximal end to a higher oxygen tension. Further, the rate of regeneration was shown to be dependent upon the oxygen tension (Barth, 1938*a*). All of these experiments have been interpreted as meaning that regeneration in *Tubularia* is dependent upon the availability of oxygen (Barth, 1940*b*).

Experiments designed to determine the origin of polarity in regenerating *Tubularia* stems are complicated by the presence in these stems of an already existing polarity. This polarity is evidenced by a gradient in the rate of regeneration and a gradient of oxygen consumption in the stems (Barth 1938*b*, 1940*a*), and by the dominance of distal over proximal levels (Barth, 1938*b*). Direct exposure of the coenosarc to sea water provides a sufficient stimulus for regeneration (Zwilling, 1939), and since the process of regeneration involves reorganization of cells, it was indicated that exposure of the entire coenosarc surface to sea water might result in sufficient reorganization to obliterate the existing gradients in the stem. The coenosarc fragments could then be subjected to carefully controlled environmental differentials in an attempt to determine the rôle of the environment in regeneration.

The experiments were therefore designed to ascertain: (1) the nature of the structural changes which occur during the development of coeno-

sarc fragments; (2) whether there is a gradient of oxygen consumption in coenosarc taken from different levels of the stem; (3) the polarity exhibited by coenosarc fragments during regeneration.

Methods

During June and July the experiments were performed on *Tubularia crocea* collected from the wharf piles at the Marine Biological Laboratory. From September through December colonies were collected from floats in the Far Rockaway channel in New York City. Uniform, clean stems 5 to 8 cm. in length were chosen for the experiments. Segments 10 mm. long were used, the hydranth plus the first 5 mm. being discarded. The cuts were made with iridectomy scissors. Holding the perisarc at one end of the stem segment with a jeweler's forceps, a needle was passed gently over the perisarc, and the coenosarc expressed at the opposite end. During the summer most satisfactory survival and regeneration were obtained when the coenosarc fragments were kept in running sea water which had been filtered through absorbent cotton. The coenosarc fragments were placed on agar (2 per cent agar in sea water) in Syracuse dishes, and the latter transferred to a large glass aquarium through which the filtered sea water constantly flowed. The fragments were kept one-half inch from the surface of the water by elevating the Syracuse dishes in the aquarium. This was done to insure the availability of oxygen. The coenosarc fragments were moved around in the dish every few hours to insure uniform healing. Twenty-four hours after removal, they were transferred to Syracuse dishes which contained no agar. The coenosarc fragments removed from stems collected at Far Rockaway during the fall and winter were very hardy, satisfactory viability and regeneration being obtained using filtered sea water. Agar and continuous circulation of the sea water were not necessary. The operations and observations

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Explanation of Plate I [1]

Figs. 1–3, and 8–11 (× 15); Figs. 4 and 6 (× 160); Figs. 5 and 7 (× 950).

1. Coenosarc fragment (above) and empty perisarc (below) immediately after expression of the coenosarc.

2 and 3. Coenosarc fragment two hours (Fig. 2) and twenty-four hours (Fig. 3) after removal from the perisarc.

4 and 5. Section of coenosarc fragment immediately after removal.

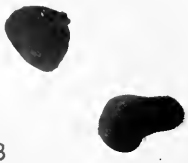
6 and 7. Section of coenosarc fragment two hours after removal.

8–11. The types of regenerants obtained from expressed coenosarc fragments. Unipolar (Fig. 8); bipolar (Fig. 9); bipolar-unipolar (Fig. 10); multipolar (Fig. 11).

[1] The authors wish to thank Mr. Jack Godrich for his assistance in the preparation of the photomicrographs.

1

2



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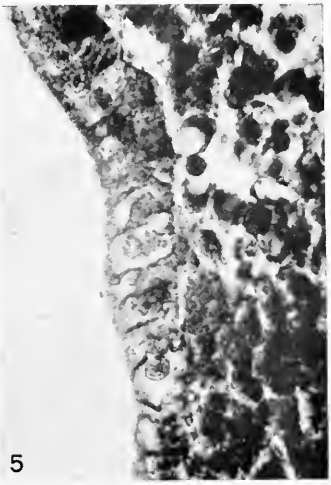

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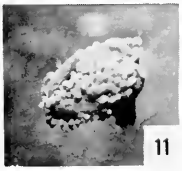

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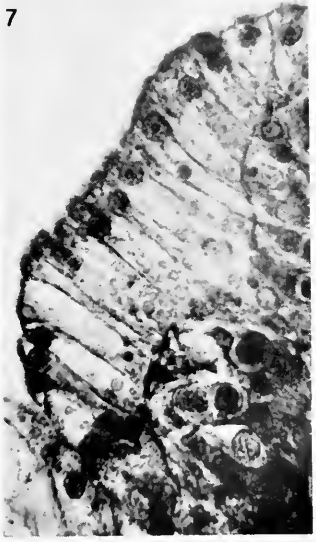

7

PLATE I

were made with the aid of a binocular microscope. The coenosarc fragments were fixed in Bouin's picro-formol-acetic fixative. They were sectioned at five microns, and stained with Delafield's haematoxylin.

The Development of Coenosarc Fragments

When coenosarc is removed from the perisarc, the tissues undergo a series of structural changes resulting, finally, in regeneration of hydranths. For convenience of description, the process may be divided into six stages based on characteristic morphological relationships.

Stage 1. The coenosarc has just been removed from the perisarc. There has been some morphological disturbance due to the mechanics of the operation. Plate I, Fig. 1, shows the condition of the coenosarc, above, and the empty perisarc, below. When examined histologically (Pl. I, 4), it may be noted that the ectodermal and endodermal layers are well defined, although the coelenteron has been obliterated. The nuclei of the ectodermal cells are centrally located and there is no trace of perisarc present (Pl. I, 5).

Stage 2. Two hours after removal the fragment has begun to contract along the original distal-proximal axis (Pl. I, 2), and the interior has become somewhat vacuolated (Pl. I, 6). The ectoderm is still well defined, but the nuclei of the ectodermal cells are located peripherally and the cells are somewhat elongated and swollen (Pl. I, 7). No perisarc is present. The atypical appearance of the ectodermal cells may be an indication of cellular degeneration, which is followed by a sloughing off of the original ectodermal cells. This loss of cells from the coenosarc may be observed, with the aid of a binocular microscope, from the time the perisarc is removed until a new perisarc is formed.

Stage 3. Twenty-four hours after removal, the coenosarc has undergone further contraction and is now somewhat spherical (Pl. I, 3). The outer layer is not well defined, and the inside of the spherical mass consists of numerous, closely packed cells (Pl. II, 12, 13). Traces of new perisarc may be noted around the periphery of the tissue mass (Pl. II, 13).

Stage 4. Thirty-six hours after removal, the center of the tissue

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Explanation of Plate II

Figs. 12, 14, and 16 (× 160); Figs. 13, 15, and 17 (× 950).

12 and 13. Section of coenosarc twenty-four hours after removal from the perisarc.

14 and 15. Section of coenosarc thirty-six hours after expression.

16 and 17. Section of coenosarc sixty hours after expression.

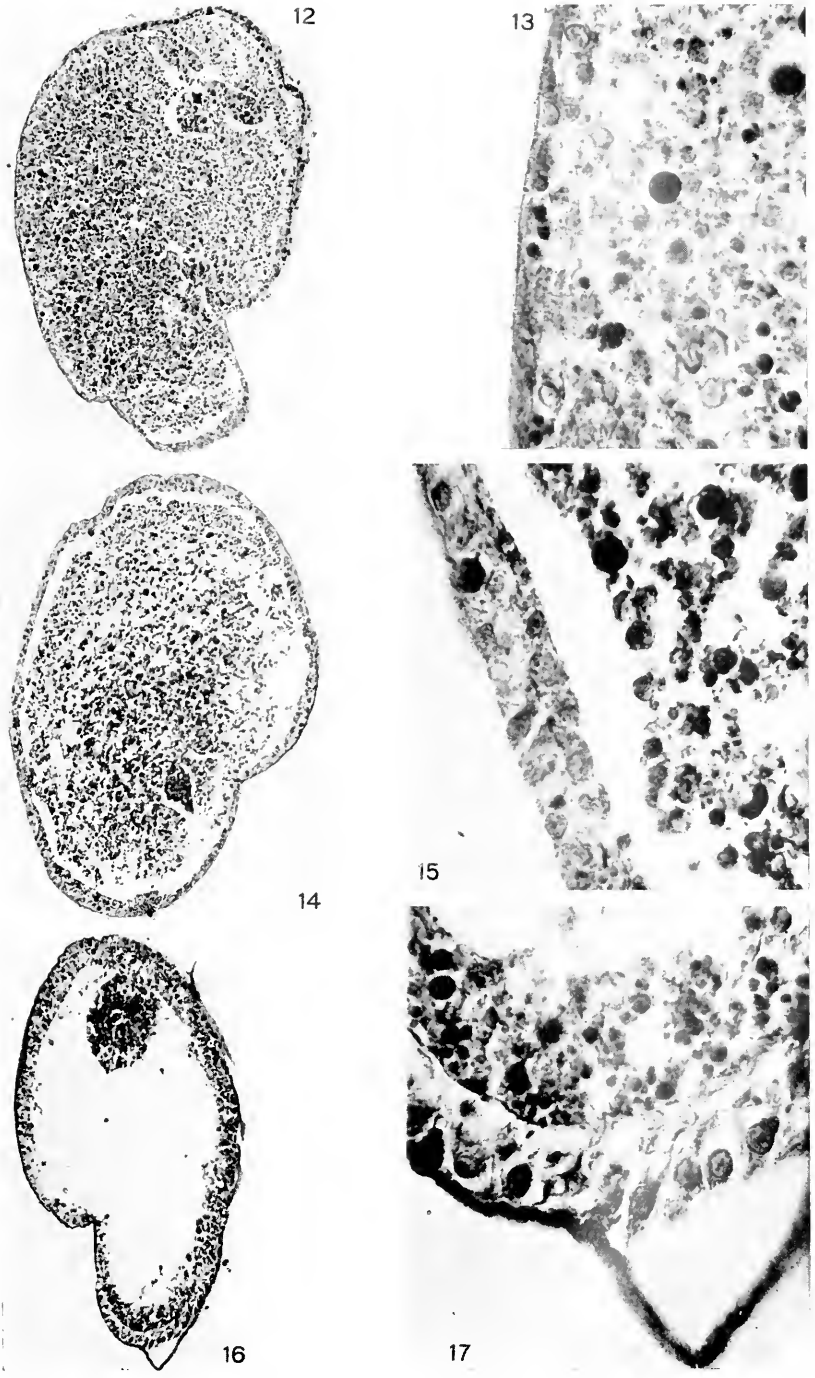

PLATE II

mass is less solidly packed with cells, and ectodermal cells have started to become arranged around the periphery (Pl. II, 14, 15).

Stage 5. Sixty hours after removal, the center of the mass of tissue is hollow. Well-defined ectodermal and endodermal layers have been formed around the periphery (Pl. II, 16, 17). The ectodermal cells are smaller than the ones in Stage 2 (Pl. I, 7).

Stage 6. Seventy-two to ninety-six hours after removal of the coenosarc fragments from the perisarc, regeneration occurs. This stage is characterized by the formation and emergence of new hydranths (Pl. I, 8–11). Hydranth formation is preceded by an aggregation of cells and later of pigment at the point of regeneration. Some of the fragments, although healthy in appearance, do not regenerate. They develop as far as Stage 5 (Pl. II, 16), at which time a heavy perisarc is secreted and development stops.

The changes which occur after removal of the coenosarc from the perisarc result in a general morphological dedifferentiation, namely, the formation of a spherical mass of coenosarc tissue in which the original ectoderm and endoderm are no longer clearly defined. Cellular dedifferentiation was not observed, no evidence being found that the cells return to an embryonic condition. The morphological dedifferentiation is followed by a redifferentiation, involving the reorganization of ectoderm and endoderm, formation of new perisarc, and subsequent regeneration. It is of interest to note that the time required for regeneration of the fragments is longer (72–96 hours) than the time required for intact stem segments (approximately 36 hours) at the same temperature. The additional time required for regeneration of the fragments is understandable when the time required for the initial dedifferentiation and early reorganization is taken into account.

The Rate of Oxygen Consumption of Coenosarc Removed from Different Levels of the Stem

In these experiments young unbranched stems were selected. The segments of stem used were 10 mm. long. The distal segments were taken from the region extending from 5 to 15 mm. proximal to the hydranth, and the proximal segments 20 to 30 mm. proximal to the hydranth. The coenosarc fragments were removed from these stem segments and kept in running filtered sea water until ready to be placed in the Warburg manometers. The fragments were placed in the manometers from 17 to 24 hours after removal from the perisarc. At this time the fragments have reached their greatest morphological dedifferentiation (Stage 3, Pl. II, 12). They have become spherical and are

protected from the mechanical shaking of the Warburg manometers by a thin, newly secreted perisarc. The rate of oxygen consumption was calculated on the basis of $mm.^3O_2$ used per hour per ten mg. of dry weight. The results are summarized in Table I. In most of the ex-

TABLE I

Oxygen consumption of distal and proximal coenosarc fragments removed from the perisarc. Rate = $mm.^3$ O_2/hr./10 mg. dry weight. The temperature of the sea water during the experiments was 18.5±.02°C.

Description of coenosarc fragments			Oxygen consumption			
Exp.	No.	Region	O_2	Time	Dry weight	Rate
			$mm.^3$	hours	mg.	
RE 1	19	distal	48.5	10.75	0.494	91.0
	19	proximal	37.2	10.75	0.391	88.5
RE 2	15	distal	19.8	9	0.46	48.0
	15	proximal	23.4	9	0.69	37.6
RE 3	4	distal	6.6	22	0.224	13.4
	14	proximal	41.2	22	1.294	14.4
RE 4	18	distal	10.5	9	0.720	16.2
	17	proximal	10.8	9	0.735	16.3
RE 5	14	distal	13.1	8	0.378	43.5
	15	proximal	15.2	8	0.482	39.5
RE 6	19	distal	24.3	7	0.729	47.7
	19	proximal	19.6	7	0.694	40.5
RE 7	15	distal	18.2	11.5	0.764	20.7
	15	proximal	17.0	11.5	0.721	20.4

periments the distal rates are approximately the same as the proximal rates. The averages of the distal and proximal rates for the seven experiments are 40.1 and 36.7 respectively. The distal and proximal regions of stem segments with perisarc, however, show a distal-proximal gradient in rate of oxygen consumption (Barth, 1940*a*). This difference in rate is present after the stems are cut, and persists from 24 through 36 hours after cutting. The distal and proximal coenosarc fragments, however, show only slight difference in rate 24 hours after removal from the perisarc. This means that the coenosarc fragments must have lost the differential during the first 24 hours. Thus, the reorganizational changes in the coenosarc fragments involve a dedifferentiation of the physiological gradient present in the intact stem.

Apparently then, exposure of the coenosarc to sea water has an effect on the rate of regeneration. In order to clarify the nature of this effect, the rates of distal coenosarc fragments and distal stem segments may be compared. The average rate of oxygen consumption of distal coenosarc fragments is 40.1 as compared with 21.8 for distal stem segments, the latter average being calculated from data presented by Barth (1940*a*). If the dry weight of the stem perisarc represents even as high as 50 per cent of the dry weight of the stem, the rate of distal coenosarc fragments would still be as high as the distal rate of the stems. Thus, the distal coenosarc fragments consume oxygen at a rate at least as high as the distal stems. Since, in addition, distal and proximal coenosarc fragments respire at about the same rate, the effect of exposure of the coenosarc to sea water and the resultant dedifferentiation is to increase the rate of oxygen consumption of coenosarc from proximal levels of the stem up to the higher rate of the distal levels.

Polarity of the Regenerated Coenosarc Fragments

The polarity relationships exhibited by the regenerated coenosarc fragments are worthy of examination for comparison with stems, where only unipolar and bipolar forms regenerate after cutting. Uniform exposure of the coenosarc to sea water removes the possible complication of an environmental differential created by the presence of the perisarc. The regenerated fragments may be classified using the general terminology employed by Child (1927) for *Corymorpha,* the groupings being based upon the axial pattern developed by the fragments. The regenerated coenosarc fragments may be classified as unipolar, bipolar, bipolar-unipolar, multipolar, and apolar. The unipolar forms have regenerated a single hydranth on the rounded mass of tissue (Pl. I, 8). Bipolar regenerated fragments have formed hydranths at two opposite poles of the coenosarc mass (Pl. I, 9). The bipolar-unipolar forms have regenerated two hydranths from the same region of the coenosarc (Pl. I, 10). These may be two independent hydranths or two partially fused hydranths. The category "multipolar" is used to designate fragments which have regenerated more than two hydranths. This group includes regenerated fragments ranging from tripolar forms, to forms in which the entire surface of the coenosarc has become covered with tentacles (Pl. I, 11). In each of these categories are included forms in which the regenerated hydranths are not complete. Thus, a hydranth may be lacking in the number of oral or basal tentacles or lacking in any of the structures necessary for a complete hydranth. For the purpose of this analysis, however, it was not deemed necessary to subdivide the various

categories with respect to these irregularities. Many of the hydranths which form do not emerge from the new perisarc. These may be readily classified in the above groups, so that no distinction is made between emerged and non-emerged hydranths. The apolar forms are those fragments which fail to regenerate. They develop as far as Stage 5 (Pl. II, 16), form a thick perisarc, and remain in that condition.

The classification of the regenerated coenosarc fragments is arranged in Table II. The unipolar and multipolar forms make up the greatest percentage of the regenerants (78.6 per cent). The bipolar and bipolar-unipolar forms comprise a much smaller percentage of the total (21.4

TABLE II

Classification of the regenerated coenosarc fragments obtained after the removal of the perisarc. The observations were made at 96 hours. Percentage regeneration equals the number of a particular kind of regenerated coenosarc fragment divided by the total number of fragments which formed hydranths. The temperature of the sea water was 19±2°C.

	Description of the regenerated coenosarc fragments				No regeneration	
	Unipolar	Bipolar	Bipolar-unipolar	Multipolar	Apolar	Dead
Number	73	26	9	56	50	166
Percentage Regeneration	44.5	15.9	5.5	34.1		

per cent). Since exposure of the naked coenosarc to sea water is a sufficient stimulus for hydranth formation (Zwilling, 1939), the formation of hydranths should be enhanced when the entire coenosarc is naked, and therefore in more direct contact with sea water and oxygen dissolved in the sea water. That this may be true is demonstrated by the relatively high percentage of multipolar regenerants (34.1 per cent) obtained after removal of perisarc. These multipolar forms are never obtained when the perisarc is left intact. The appearance of a high percentage of unipolars (44.5 per cent) suggests that in these cases the mass of tissue may have been exposed to a uniform gradient of oxygen in the sea water, for many of them became attached to the bottom of the dish at an early stage and the hydranths always formed away from the attached end. This interpretation is supported by Child's experiments with *Corymorpha* (1928) in which hydranths were regenerated at the upper surface of undisturbed cell aggregates. Coenosarc fragments develop in the same way, irrespective of the level of the stem from which they are removed.

Data for the regeneration of coenosarc fragments removed from three different levels of the stem are summarized in Table III. The coenosarc fragments of all three levels of the stem give rise to regenerants having similar types of polarity relationships. In addition, it may be noted that 96 hours after removal of the coenosarc from the perisarc, approximately

Table III

Classification of the regenerated coenosarc fragments removed from different levels of the stem. The observations were made at 96 hours after removal of the fragments from the perisarc.

	Description of the regenerated coenosarc fragments					No regeneration	
	Unipolar	Bipolar	Bipolar-unipolar	Multi-polar	Total	Apolar	Dead
Distal	10	4	2	12	28	14	18
Middle	12	3	1	13	29	19	12
Proximal	15	6	2	12	35	19	6

the same number of regenerants appear at all three levels. Thus, the gradient in the rate of regeneration present in *Tubularia* stems (Barth, 1938*b*, 1940*a*) apparently disappears when the coenosarc fragments are removed from the perisarc.

Discussion

One of the chief difficulties in any attempt to analyze the rôle of the environment on regeneration is the inability to work with homogeneous systems. That hydroids have a gradient of metabolic activity has been well established. This gradient is developed, apparently as the result of an environmental differential, early in the development of the organism. Thus, Child (1925) has shown that a metabolic gradient is probably established as a result of the nature of the orientation of the egg during its growth. Further, once a gradient has been established, it may maintain itself in a uniform environment, and the gradient has therefore become a function of localized differences within the tissues themselves. Barth (1938*b*) demonstrated a gradient in the rate of regeneration along the length of the stem of *Tubularia*, distal segments regenerating at a higher rate than proximal segments. There is likewise a gradient of oxygen consumption of the parts of the stem (Barth, 1940*a*). Barth (1938*b*) also suggested that the dominance of distal over proximal levels of *Tubularia* stems might be interpreted as a compe-

tition for substance "S," the success of which is dependent upon the activity of enzyme "E." Thus, an organism living in a uniform external environment may nevertheless maintain its own heterogeneity once this heterogeneity has become established.

In order to determine more accurately the rôle of environmental factors on the origin of new gradients in regeneration, it is of importance to obliterate first any existing gradients in the animal tissues themselves. Some attempts in this direction have been made. Child (1928) found that cells of *Corymorpha*, when dissociated mechanically, will aggregate and establish new polarity relationships. *Corymorpha* stems, when subjected to toxic agents, may lose their established polarity relationships and form new gradients of regeneration (Child, 1927), the new gradients being produced by a differential exposure to the environment.

The experiments with expressed *Tubularia* coenosarc indicate an obliteration of the original polarity after the coenosarc is removed from the perisarc. This is borne out by the reorganizational changes which the coenosarc undergoes after removal. There is, at first, a morphological dedifferentiation, in which the mass becomes spherical and the ectoderm and endoderm are no longer clearly defined. Cellular dedifferentiation, however, was not observed. The morphological dedifferentiation is followed by a redifferentiation. The ectoderm and endoderm are reorganized and a new perisarc is formed. Subsequently, regeneration occurs. If the polarity of an organism is dependent upon a gradient of activity of some enzyme, as suggested by Barth (1938*b*), then it is quite likely that the activity of this enzyme is radically changed during the process of reorganization. That the initial polarity is lost, is evidenced also by the appearance of regenerated hydranths at the free surface of fragments which have become attached to the bottom of the dish, the regenerated hydranths having no necessary relation to the original polarity. It is further substantiated by the appearance of a high percentage of multipolar forms in which appear numerous and apparently unrelated polarity relationships. The appearance of these multipolar forms must mean that the original polarity is no longer extant; the exposure of the coenosarc to the sea water being sufficient to stimulate regeneration at many points on the uniform mass of tissue.

Correlated with the disappearance of the original polarity is the disappearance of the difference in oxygen consumption of tissues removed from distal and proximal levels of the stem. The sharp gradient of oxygen consumption found along the length of the stem (Barth, 1940*a*) is not exhibited by the excised fragments at the time when morphological dedifferentiation has reached its peak (17–24 hours after removal of the tissue). At this time the rates of oxygen consumption of dedifferenti-

ated coenosarc fragments from both distal and proximal levels of the stem are at least as high as the rate for distal stem segments. The loss of the gradient of oxygen consumption is due, therefore, to a general increase in rate. This increase in rate is probably stimulated by a high availability of oxygen to the coenosarc fragments, made possible by their removal from the perisarc. Thus, the removal of the coenosarc from the perisarc results in a reorganization involving not only morphological dedifferentiation but also a dedifferentiation of the physiological gradient. The end product of this process is a more homogeneous mass of *Tubularia* cells. The localized differences in the ability to regenerate, found in stems covered with perisarc, also disappear as a result of the morphological and physiological dedifferentiation. Coenosarc fragments removed from distal and proximal levels of the stem regenerate at the same rate and develop similar types of polarity relationships.

Expressed coenosarc, therefore, if used at the time when dedifferentiation is greatest (approximately twenty-four hours after removal from the perisarc), offers good biological material for studies of the environmental factors stimulating regeneration and for an investigation of the origin of polarity in regeneration.

Summary

The morphogenesis of coenosarc expressed from the perisarc of *Tubularia* stems is described. A series of structural changes occurs in the coenosarc, there being first a dedifferentiation of histological structure, followed by a redifferentiation culminating in the regeneration of new hydranths.

The gradient of oxygen consumption present in the stem of *Tubularia* disappears when the coenosarc is removed from the perisarc. This physiological dedifferentiation represents an increase as well as an equalization of oxygen consumption by coenosarc fragments from distal and proximal levels of the stem.

Concomitant with the morphological and physiological dedifferentiation, differences in the ability of distal and proximal levels of the stem to regenerate disappear. Distal and proximal coenosarc fragments regenerate at the same rate and develop similar types of polarity relationships.

The different kinds of regenerants obtained are described and classified on the basis of their polarity relationships. Evidences were given that these polarity relationships are new, and have no relation to the original polarity in the intact stem.

The value of using expressed coenosarc to study the effect of the environment on regeneration and on the origin of polarity in regeneration is discussed.

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THE RÔLE OF FERTILIZIN IN THE FERTILIZATION OF EGGS OF THE SEA-URCHIN AND OTHER ANIMALS

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Introduction

The striking phenomenon of the specific agglutination of spermatozoa by a substance obtained from the eggs has been described in a number of species of marine animals (see Lillie, 1919; Just, 1930; Tyler, 1940*a*). Lillie considered this substance, which he called fertilizin, to play a central rôle in the fertilization process, and developed a theory of the mechanism of fertilization based on the ability of fertilizin to combine with the spermatozoön and with some substance within the egg. One of the principal arguments for his views was the evidence that eggs of the sea-urchin which had been deprived of fertilizin lost their capacity for fertilization. In his first experiments (1914) the fertilizin was removed by prolonged washing of the eggs (*Arbacia*), combined in some cases with shaking to remove the jelly layer which he had shown (1913) to be heavily charged with fertilizin. Loeb (1914, 1915) raised the objection that the decrease in fertilizability was due to the aging and death of the eggs during the washing period of one to three days. He showed, on the other hand, that fresh eggs of *Strongylocentrotus purpuratus*, that had been deprived of their jelly layer (which he considered identical with fertilizin) by means of acidified sea water, would still give 100 per cent fertilization. Lillie (1915) repeated the acid sea water experiments with *Arbacia* and found the capacity for fertilization (per cent fertilized) to be much reduced as a result of the treatment. He also noted that some fertilizin could be obtained from the acid-treated eggs although the jelly layer appeared to be completely gone. Later (1921, footnote p. 16), with *Strongylocentrotus*, he found that acid-treated jellyless eggs could still be fertilized although there could not be obtained from these eggs sufficient fertilizin to agglutinate the spermatozoa. He interpreted that result to mean that "an amount of fertilizin insufficient for sperm agglutination is yet adequate for fertilization."

The present experiments resolve these differences as apparently being due to differences in amount of sperm employed for insemination. The

results show that jellyless, fertilizinless eggs can be fertilized but that they must be inseminated with much higher concentrations of sperm than are necessary for untreated eggs. The present, as well as some of the earlier work of the author (1939, 1940), also lends support to Lillie's view that fertilizin is concerned in the fertilization process, and some suggestions are made here as to its rôle. It is further shown that the sperm agglutinating property of fertilizin can be destroyed without altering its ability to combine with the sperm. An interpretation is offered of the temporary nature of the agglutination reaction in the sea-urchin and its more permanent nature in forms like the keyhole limpet. Evidence is also presented that fertilizin is not merely confined to those species of animals whose egg water causes iso-agglutination of sperm, but is more generally distributed and may very likely be universal.

Identity of Fertilizin with the Gelatinous Coat of the Egg

It has been shown (Tyler, 1940*a*) in experiments with the sea-urchin *Strongylocentrotus purpuratus* and the keyhole limpet *Megathura crenulata* that the sperm agglutinin (fertilizin) is located in the jelly layer surrounding the egg. On the rather reasonable assumption that the material of the jelly is a single substance, this means that fertilizin is identical with the jelly. In any event, the evidence showed that fertilizin is a component of the jelly layer and is not secreted by the ripe eggs. Removal of the jelly layer was readily accomplished by means of sea water acidified to between pH 4.5 and 3.5 and also by means of a 1 per cent solution of chymotrypsin in sea water. No fertilizin could be obtained from such jellyless eggs even after prolonged standing. When ripe eggs are allowed to stand in sea water, the jelly slowly goes into solution and the concentration of fertilizin in the solution increases. But this, it was shown, does not increase the total amount of fertilizin that can be obtained from the eggs. In other words, extraction of freshly shed eggs with acid sea water gives just as much fertilizin as that in the acid extract of eggs that had stood for some time in sea water plus that in the supernatant sea water.

Hartmann, Schartau and Wallenfels (1940) support the view that fertilizin is identical with at least a part of the material of the jelly layer. They found in *Arbacia pustulosa* that fertilizin is given off in repeated changes of sea water as long as remains of the jelly layer are present on the eggs. They also removed the jelly layer by means of a sperm extract containing an antifertilizin (see Frank, 1939; Tyler, 1939*a*, 1940*b*; Tyler and O'Melveny, 1941) and obtained no fertilizin from the treated eggs. Further evidence for this view is given by their finding that *Ar-*

bacia sperm agglutinate on the surface of the jelly layer of *Astropecten* eggs but fail to do so if the eggs are first treated with the sperm extract which forms a precipitation membrane on the surface of the jelly.

Additional evidence along this line is contained in some experiments by Evans (1940). He found that Roentgen radiation caused an immediate disappearance of the jelly from around the *Arbacia* egg. Using Janus green as a test for the presence of jelly in egg water, he found that after an irradiation of 59,000 *r* or more it could not be demonstrated in the egg water. He also noted that the agglutinating power of the egg water is greatly reduced after irradiation, and this agrees with Richards and Woodward's (1915) earlier results.

Fertilization after Removal of Fertilizin

The primary question concerning the rôle of fertilizin is whether or not its complete removal in a non-injurious manner renders the eggs non-fertilizable. This question was examined in some experiments with eggs and sperm of the west coast sea-urchin *Strongylocentrotus purpuratus*. Since, as the evidence shows, fertilizin is identical with, or at least a component of the gelatinous coat of the egg, its removal involves the dissolution of this coat. In *Strongylocentrotus,* the jelly is rapidly dissolved by placing the eggs in sea water acidified to between pH 3.5 and 4.5 (Tyler and Fox, 1940). If the eggs are not allowed to remain too long in the acid sea water, there is no visible sign of injury.

Although the jelly is colorless and transparent, its absence is readily noted by the fact that the eggs can then be brought into contact with one another by their surfaces (Tyler, 1940*b*, Fig. 1, *d*). When eggs of *Strongylocentrotus* are deprived of their jelly coat and washed, no detectable (by agglutination of sperm) amount of fertilizin can be obtained either by allowing them to remain for prolonged periods in sea water or by macerating and extracting them with various solvents (Tyler and Fox, 1940).

Upon insemination the jellyless eggs are capable of fertilization to the extent of 100 per cent, as Loeb (1914, 1915) and Lillie (1921) had reported for eggs of *S. purpuratus*. One typical experiment may be cited. Two 20 cc. samples of a 0.1 per cent suspension of fresh *S. purpuratus* eggs in sea water were taken and one of them acidified to pH 4.0. After 5 minutes both dishes of eggs were given a set of four washings with a total of 100 cc. of sea water, allowing the eggs to settle and 1 cc. of suspension to remain in the dishes between washings. The acid-treated eggs were observed to be deprived of their jelly. The addition of 0.05 cc. of a 1 per cent fresh sperm suspension gave 100 per cent

membrane elevation and cleavage in both the acid-treated and control eggs. Similar results were also obtained when the jelly was removed with chymotrypsin.

It may be concluded from this evidence that fertilizin is not essential for fertilization. However, such a conclusion is only valid if the fertilizin has in fact been completely removed from the treated eggs. That this may not be the case is indicated by other evidence and considerations presented below. But, even if it be assumed for the present that fertilizin is not essential for fertilization, the question may still be raised as to whether or not it is an aid to fertilization.

Fertilizin as an Aid to Fertilization

It is well known that the number of spermatozoa required for fertilization is in general much greater than the number of eggs present in the suspension, and as the number of spermatozoa employed for insemination is decreased, the percentage fertilization decreases. The factors responsible for this fact, that many more than one spermatozoön per egg must in general be present in the suspension in order for fertilization to succeed, have been examined by several investigators (Glaser, 1915; Lillie, 1915; Cohn, 1918; Morgan, 1927, p. 27 et seq.), and will not be discussed in any detail here. The present question is whether or not more spermatozoa are required for fertilization when fertilizin is removed from the eggs. This question was investigated with eggs and sperm of *Strongylocentrotus purpuratus* and the results do, in fact, show a decrease in "fertilizability" (increase in amount of sperm required for fertilization) upon removal of the jelly.

Table I lists the results of nine experiments in which the jelly was removed by means of acidified sea water or chymotrypsin. In all cases the eggs were washed after treatment and the pH brought back to that of normal sea water. In the table, cleavage rather than membrane elevation is taken as an index of fertilization inasmuch as the treated eggs often fail to form or to elevate fertilization membranes but may nevertheless cleave (see Tyler and Scheer, 1937). The amounts of sperm added are for convenience all given on the basis of a 0.01 per cent sperm suspension although actually the larger amounts of sperm were taken from more concentrated suspensions. In the different experiments there are, as the table shows, considerable variations in the amount of sperm required to give the same percentage fertilization of the control eggs. This may be due to variations in the condition of the sperm and eggs, in aging of the sperm at various dilutions, in temperature, etc. For the point in question, however, it suffices to compare simply the jellyless with

the control eggs in each horizontal line. The results show that with the larger amounts of sperm the jellyless eggs give practically the same percentage fertilization as the controls. But with smaller amounts there are considerable differences. Thus, with small amounts of sperm that give between 75 and 100 per cent fertilization in the control eggs, only 0 to

TABLE I

Fertilization of jellyless and normal eggs of *S. purpuratus* inseminated with various amounts of sperm. The egg suspensions contain 200 to 400 eggs in 5 cc. of sea water.

Experiment	Treatment for removal of jelly coat	Amount of 0.01 per cent sperm suspension added	Percentage cleavage	
			Jellyless eggs	Control eggs
		cc.		
1	30 min. in pH 4.5 sea water	0.1	5	95
		0.5	35	98
		2.0	99	99
2	30 min. in pH 4.0 sea water	0.05	0	20
		0.5	15	90
		5.0	95	95
3	5 min. in pH 3.5 sea water	0.2	0	25
		1.0	55	95
		5.0	100	100
4	10 min. in pH 3.7 sea water	0.2	10	85
5	10 min. in pH 3.9 sea water	0.1	20	100
		1.0	100	100
6	30 min. in 1 per cent chymotrypsin in pH 8 sea water	0.05	0.2	90
		1.0	45	95
7	30 min. in 1 per cent chymotrypsin in pH 8 sea water	0.1	0.1	75
		2.5	100	100
8	10 min. in 1 per cent chymotrypsin in pH 6 sea water	0.25	15	100
		2.5	100	100
9	10 min. in 1 per cent chymotrypsin in pH 6 sea water	0.5	45	95
		5.0	95	95

20 per cent is obtained in the jellyless samples. To get the same percentage fertilization as in the controls, the amount of sperm required for the treated eggs is roughly five to ten times greater. While the variations in the results do not permit an exact figure to be given for this ratio, it is clear that the differences are all in the same direction in each experiment and are well outside the limits of error. It should

also be noted here that, since sufficient sperm gives as much fertilization in the treated eggs as in the controls, there is no particular injurious action of the treatment involved.

Fertilizin as a Barrier to Fertilization

It appears then that the presence of fertilizin on the eggs is an aid to fertilization in that smaller amounts of sperm are required than in its absence. It might be supposed, then, that restoration of the fertilizin would eliminate the difference and that addition of fertilizin to normal eggs would lower the amount of sperm required for fertilization. Unfortunately, no way is as yet known by which the fertilizin can be restored in its normal state; that is, in the form of a gelatinous coat around the egg. When the jelly is dissolved in acidified sea water it does not go back on to the eggs upon neutralization of the suspension but remains in solution. One might, however, enquire whether or not the presence of fertilizin in solution in the egg suspension increases the fertilizing power of the sperm. This was examined with both jellyless and normal eggs, and it was found that, instead of increasing the fertilizing power of the sperm, the presence of fertilizin in solution had the opposite effect. In one experiment the fertilizin was restored in its original amount (but in solution) and in roughly ten times that amount to suspensions of naked eggs. Various amounts of sperm were used for insemination. The lowest quantity of sperm that gave 100 per cent fertilization in the jellyless controls gave only 15 per cent in the sample with original fertilizin content and 0 per cent in that with the ten-fold concentration. In an experiment with normal eggs the smallest amount of sperm that gave 100 per cent fertilization, gave about 35 per cent when an amount of fertilizin roughly equivalent to the content of the eggs was present in solution and 0 per cent when ten times that amount was present.

The presence of fertilizin in solution evidently acts as a barrier rather than an aid to fertilization. This action, it appears, is due to increase in amount of agglutination of sperm that occurs with increase in amount of fertilizin present in the solution. It is not merely the temporary locking up of the sperm in the agglutinates that causes the decrease in fertilizing power, but, as the next section shows, it involves a permanent effect of the fertilizin on the sperm.

Loss of Fertilizing Power as a Result of Agglutination

F. R. Lillie (1913) showed that the agglutination of sea-urchin sperm by egg water (fertilizin) is temporary. On testing the sperm after reversal of agglutination, he found them to have about half the fertiliz-

ing power (fertilized half the percentage of eggs) of the control sperm suspension. He also noted (1919) that after reversal of agglutination the sperm cannot be re-agglutinated. I have confirmed these findings with *S. purpuratus* and have obtained a much greater reduction in fertilizing power of the sperm.

In twelve experiments that were run, the sperm was agglutinated with sufficiently strong egg water, so that further addition of egg water, after reversal, gave no visible agglutination. The agglutination usually lasted 30 to 40 minutes. Insemination with amounts of sperm that were well above the control minimum for 100 per cent fertilization gave in all tests with the agglutinated and reversed sperm between 0 and 3 per cent fertilization. To obtain the same percentage fertilization with the control as with the treated sperm was found to require between a forty- and a two-hundred-fold reduction in the amount of control sperm used for insemination. The possibility was also examined that the reversed sperm might be more capable of fertilizing jellyless eggs, but the results were negative.

Along with this reduction in fertilizing power of the sperm there is no visible sign of any injurious effect after reversal of agglutination, nor is there any reduction in the activity of the sperm. In fact, the egg water, as is well known, increases the activity of the sperm and as measurements of respiratory rate showed (Tyler, 1939*b*) the increase persists long after the reversal of agglutination. The experiments show, then, that sperm which have been agglutinated are, after spontaneous reversal, incapable of fertilization. The small percentages of fertilization that result when large amounts of treated sperm are used are evidently due to the fact that some spermatozoa in the treated suspensions may escape being agglutinated.

It may be concluded, then, that some change is produced in the spermatozoa, as a result of their reaction with fertilizin, which, although essentially non-injurious, renders them incapable of fertilizing normal eggs. This change might occur during the initial reaction or upon the spontaneous reversal of the agglutination.

The Spontaneous Reversal of Sperm-Agglutination in Sea-Urchins

The temporary nature of the agglutination reaction exhibited by sea-urchin sperm in egg water is an exceptional affair. In the usual serological reactions, the agglutination of various types of cells (blood cells, spermatozoa, bacteria, etc.) by their antisera does not spontaneously reverse, but persists indefinitely. Natural agglutinins, too, such as the

blood group agglutinins in humans, give permanent agglutination which can only be reversed by special treatment. It is of interest, then, not only in connection with fertilization, but in regard to the nature of agglutination reactions in general, to consider the possible causes of the spontaneous reversal.

We shall use as a basis of the present discussion the lattice or framework theory of Heidelberger (1938) and Marrack (1938). This theory postulates that in agglutination as well as precipitation reactions the antigen and antibody are structurally complementary and both are multivalent in regard to their combining groups. Thus one molecule of antigen may combine with more than one molecule of antibody which in turn may combine with more than one molecule of antigen and so build up large aggregates. Where both of the complementary substances are in solution, precipitation results. Where one is present as the surface of the cell, agglutination occurs. The following interpretations may then be suggested for reversal of agglutination in the sea-urchin. (1) The fertilizin molecules plus the combined antifertilizin split off from all of the spermatozoa, leaving neutralized fertilizin in the suspension. (2) They split off at some, rather than all, combining sites in such a way that each (completely neutralized) fertilizin molecule remains attached to not more than one spermatozoön. (3) The fertilizin molecules are split by the action of the sperm leaving univalent fragments combined with the antifertilizin on all the spermatozoa.

All three of these interpretations can account for the failure of reagglutination and the loss of fertilizing capacity on the part of the reversed sperm. Attempts were made to eliminate one or another of these possibilities but the experiments were inconclusive and need not be described here. However, some new findings and further consideration of earlier work lend support to the third interpretation.

It was shown (Tyler and Fox, 1940) that fertilizin of the keyhole limpet is much more resistant than that of the sea-urchin to inactivation by heat and proteolytic enzymes and that this greater stability correlates with the more permanent nature of the agglutination reaction in that form. That the difference is not due to differences in the relative amounts of fertilizin involved is evident by the fact that the reaction is of long duration in the keyhole limpet even when weak fertilizin solutions are employed, whereas it does not in the sea-urchin surpass a maximum of very much shorter duration when the strongest available fertilizin solutions are added. This suggests then that, in the sea-urchin, the combined fertilizin may be broken down fairly rapidly by action of the sperm. It has also been noted that when fertilizin solutions are heated or treated with proteolytic enzymes there is at first a small but definite

increase in activity followed by the gradual inactivation. This suggested the possibility that the fertilizin is first split into smaller but still multivalent molecules. Such behavior is not unique for it has been frequently noted with immune antibodies (see Marrack, 1938; Zinsser, Enders and Fothergill, 1939; Petermann and Pappenheimer, 1941) and the altered agglutinin is termed "agglutinoid." It seemed possible then that, by careful inactivation of fertilizin solutions, univalent fragments might be obtained. The "univalent" fertilizin should be incapable of causing agglutination, but should inhibit subsequent agglutination by untreated fertilizin. It should also be expected to be effective in destroying the fertilizing power of the sperm. As will be shown in the next section, both of these effects have been obtained with heat-treated fertilizin solutions. This, then, lends support to the third interpretation of the spontaneous reversal of agglutination; namely, that the fertilizin molecules are split and the univalent fragments remain attached to the combining groups on the sperm.

"Univalent" Fertilizin

In five experiments concentrated solutions of *S. purpuratus* fertilizin that had been purified by previously described methods (Tyler and Fox, 1940) were heated at 90° to 100° C. just up to the time at which the agglutinating activity had practically disappeared. Sperm was then added to samples (at room temperature) of (*A*) the heated solutions, (*B*) the control solutions, and (*C*) sea water, the relative amounts being such that complete agglutination (no reaction to additional fertilizin after reversal) occurred in the control solution. When unheated fertilizin was added to samples of the sperm in *A*, there was either a very weak reaction or no visible agglutination at all. After reversal of agglutination in *B*, normal eggs were inseminated with various amounts of the sperm suspensions. When amounts of sperm were used that, in the case of the sea water controls, *C*, were near the minimum for 100 per cent fertilization, the *A*-sperm gave 0 to 5 per cent (av. 0.5 per cent) and the *B*-sperm gave 0 to 1 per cent (av. 0.2 per cent) fertilization. A further control was run in those experiments where *A*-sperm showed a weak agglutination reaction upon addition of unheated fertilizin. This was done by diluting the control fertilizin to a concentration giving a similar reaction and adding sperm to the dilute solution at the same time and in the same relative amounts as employed in the other solutions. The fertilizing capacity of the sperm in the diluted fertilizin was found to be only slightly lower than that of the sea water control sperm. An absorption experiment was also performed by the addition of excess

sperm to a sample of the heated fertilizin solution and, after centrifugation, the active agent was found to have disappeared from the supernatant solution.

The results show, then, that the agglutinating property of fertilizin can be destroyed without altering appreciably its capacity to combine with the sperm. The heated fertilizin is usually somewhat weaker than the control in its ability to prevent subsequent agglutination and in its ability to destroy the fertilizing power of the sperm. This most likely means that a small amount of the fertilizin is more completely degraded during the heat treatment. It is clear, however, that by careful heat treatment a modified (non-agglutinating) fertilizin can be produced that differs only slightly, if at all, in its ability to combine with the sperm. Since according to the modern theory a specific agglutinating substance is assumed to be multivalent in respect to its specific combining groups, it is reasonable to consider the non-agglutinating substance in this case univalent.

The formation of univalent fertilizin may be assumed to involve the splitting of the molecule into fragments each of which contains a single combining group or it might involve the splitting off of the combining groups alone. In the latter instance the active agent would be expected to be of small molecular size. Dialysis tests showed, however, that the active agent is incapable of passing through a cellophane membrane. The first assumption appears then to be the more likely one. Other properties of the active agent have not as yet been studied except for a preliminary test of its inactivation by heat. It is inactivated in about one and one-half to three times the time required for destruction of the agglutinating property of the original fertilizin.

Fertilizin in Animals not Exhibiting Iso-Agglutination of Sperm

Lillie (1919) and Just (1930) assumed that eggs of all species of animals possessed fertilizin, although they, themselves, had shown that in many species there is no detectable agglutination of sperm by homologous egg water. They regarded the agglutination reaction simply as an indicator for the presence of fertilizin, but they did not offer any evidence or tests that would demonstrate an analogous substance in the absence of the clumping reaction. The present concept of univalent fertilizin has led to the demonstration of specific sperm-combining substances in species in which the agglutination reaction is lacking. If, in a particular species of animal, the fertilizin obtained in the egg water is univalent, then it should give no agglutination of homologous sperm, but it should destroy their fertilizing capacity.

This point was examined in the starfish *Patiria miniata* and in the gephyrean worm *Urechis caupo*. In the starfish, concentrated egg water causes no agglutination of homologous sperm. In *Urechis* there may occasionally be a weak reaction. Concentrated egg waters were prepared from eggs of these two species by extraction with pH 4 sea water. Sperm was then added to the neturalized homologous and heterologous egg waters as well as to sea water and after a few minutes various amounts were taken for insemination of the homologous eggs. In all cases there was found to be a great reduction in the fertilizing capacity of the sperm treated with homologous egg water, while that treated with heterologous egg water showed approximately the same fertilizing capacity as the sea water controls. A typical experiment may be cited. Concentrated *Patiria* and *Urechis* egg waters were prepared from 10 per cent egg suspensions. One part of a 1 per cent *Patiria* sperm suspension was added to nine parts of (*A*) *Patiria* egg water, (*B*) *Urechis* egg water and (*C*) sea water. The same was done with a one per cent suspension of *Urechis* sperm. Insemination of homologous eggs (approximately 200 eggs in 5 cc. of sea water) with 0.05 cc. of these mixtures gave for *Patiria* no fertilization with *A*, 100 per cent with *B* and 99 per cent with *C*. For *Urechis* the results were 100 per cent with *A* and *C* and 0 per cent with *B*.

These results then lend support to the view of Lillie and Just that fertilizin is of general distribution in animals. When appropriate material is available, the investigations will be extended. For the present it is evident in two species of animals that a specific sperm-combining substance is obtainable from the eggs and, since the substance has no agglutinating action on homologous sperm, it may be termed univalent fertilizin.

Discussion

It has been shown that fertilizin, when present in the form of a gelatinous coat, is an aid to fertilization in the sea-urchin. It would also appear from the experiments that fertilizin is not entirely essential to fertilization. But this assumes that all of the fertilizin is removed upon removal of the jelly. While no detectable fertilizin is obtainable from the jellyless eggs, it is quite conceivable that it may be present in combined form on the surface of the egg. It has been shown (Tyler, 1940*b*) that there is an antifertilizin below the surface of the egg and it would be reasonable to assume that the surface of the egg is composed of a fertilizin-antifertilizin complex. Upon removal of the jelly, this combined fertilizin would remain as a monomolecular layer with free spe-

cific combining groups on its outer surface. In support of this view may be cited the observation of Frank (1939) that jellyless as well as normal sea-urchin eggs can be agglutinated by means of an antifertilizin obtained from the sperm. The possibility may then be admitted that fertilizin is indispensable for fertilization but further evidence along this line would be desirable before any attempt is made to develop a theory of fertilization with it as an essential agent.

In regard to the manner in which fertilizin may act as an aid to fertilization there are several possibilities. In the first place it is clearly not merely the greater volume due to the presence of the jelly that is involved, since the spermatozoön must, in any event, reach the surface of the egg for fertilization to ensue. It is possible that the gradient produced, as the jelly slowly goes into solution, exerts a chemotactic effect on the sperm. There is, however, still no general agreement as to chemotaxis. Hartmann (1940) reports demonstrating such action of fertilizin by means of the Pfeffer capillary method, whereas Cornman (1941) could obtain no positive results with that method.

Another possibility is that the jelly serves as a trap for the sperm. This appears reasonable on the basis of the fact that the spermatozoön reacts with fertilizin in solution. One may suppose that, while most of the fertilizin is in the form of a jelly, some of it is in solution in the interstices; or that even as a gel there are some free combining groups available. The formation of a precipitation membrane on the surface of the jelly by the action of antifertilizin (Tyler, 1940*b*) is more readily explainable on the basis of the latter assumption. Trap action would help to explain how fertilizin (as a jelly) acts as an aid to fertilization, since it would restrict the random movements of the spermatozoa to a small volume and thereby increase the chance of fertilization. However, other and more quantitative experiments are needed before decision can be made as to whether or not it alone can account for greater fertilizability of the normal in comparison with the jellyless eggs.

Another possibility is that some structural property of the jelly causes the sperm to approach so that its long axis is normal to the surface. While observations (*see* Morgan, 1927; Chambers, 1933) indicate that a radial approach is more favorable for fertilization, it has not definitely been shown that oblique approach and contact with the surface results in failure of sperm entry.

The possibility should also be considered that the greater fertilizability of the normal eggs is due to the activating effect of fertilizin on the sperm. But before decision can be made as to the value of this factor, it would be important to know that there is no corresponding decrease in the fertilizable life of the sperm.

In connection with these possibilities, it must be recalled that after the sperm has reacted with fertilizin in solution it is incapable of fertilization and that, probably because of this, the presence of fertilizin in solution in a suspension of eggs acts as a barrier to fertilization. Thus excess sperm is required to take up the fertilizin in solution and leave uncombined sperm available for fertilization. It is evident that in normal fertilization the spermatozoön must reach the surface of the egg before the inhibiting action of the fertilizin surrounding the egg has taken place. If, as suggested above, fertilizin in the form of a jelly has only a few superficial combining groups available, it is quite conceivable that they may serve as the initial trap for the sperm but would not be sufficient to neutralize all of the reacting groups on the sperm before the latter has reached the surface of the egg. The increased activity of the sperm upon reaction with fertilizin would also aid its reaching the surface before the fertilization-inhibiting reaction went to completion. While this seems to be the most likely interpretation, it requires considerably more experimental support. Also, it appears that the information so far available does not warrant a detailed discussion of Lillie's theory of fertilization, nor of the recent views of Hartmann (1940), nor of the development of a new theory of the exact function of fertilizin and other specific substances.

It has been shown in the present work that appropriate treatment of sea-urchin fertilizin converts it into a non-agglutinating agent that is still capable of reacting specifically with the sperm. On the basis of the lattice theory of agglutination reactions this altered fertilizin may, quite legitimately, be designated a univalent substance. It was also shown that the egg waters of certain species of animals that do not contain specific sperm agglutinins nevertheless contain specific sperm-combining substances which may likewise be designated univalent. The absence of the agglutination reaction in many species of animals does not, then, mean the lack of fertilizin, if by that term we mean simply a substance that reacts specifically with the sperm.

This concept may also be extended to problems in general immunology. It is well known that certain animals, such as the rabbit and the horse, readily produce upon immunization, specific agglutinins and precipitins. Others, such as the mouse and the rat, produce little or none but do form protective or neutralizing antibodies. It may be suggested, then, that the antibodies produced in the latter species are principally or entirely of the univalent type. This possibility can be readily tested experimentally;—cells treated with the univalent antibodies should be rendered incapable of being agglutinated by the specific agglutinating antibodies obtained in the former species.

Summary

1. It has been shown in the sea-urchin that the presence of fertilizin, in the form of the jelly coat of the egg, serves as an aid to fertilization. In solution it acts as a barrier to fertilization.

2. Confirmation is presented of Lillie's finding that sea-urchin sperm cannot be re-agglutinated after reversal of an initial agglutination. It is also shown that the reversed sperm are incapable of fertilization.

3. Appropriate heat treatment converts fertilizin into a substance that does not cause sperm agglutination but still combines with the sperm as shown by the inability of the sperm to be subsequently agglutinated by ordinary fertilizin and by loss of fertilizing power. In accordance with the assumption of multivalency in the lattice theory of agglutination, the modified fertilizin is assumed to be univalent. It is found to be non-dialyzable.

4. In the starfish and in *Urechis* the egg water is shown to contain a specific sperm-combining substance (univalent fertilizin) that is incapable of causing iso-agglutination of sperm.

5. Of various interpretations of the spontaneous reversal of agglutination in the sea-urchin, a splitting of the fertilizin into univalent fragments is considered the most likely.

6. Reasons are presented for holding open the possibility that fertilizin plays an indispensible part in fertilization. Various possible explanations as to the manner in which it serves as an aid to fertilization are discussed and that involving trap action is considered the most likely.

7. It is suggested that some species of animals produce upon immunization only, or principally, univalent antibodies and a method of determining this point is offered.

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SPECIFICITY AND HOST-RELATIONS IN THE TREMATODE GENUS ZOÖGONUS [1]

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The genus *Zoögonus* was erected by Looss (1901) to contain *Z. mirus,* (as type), and *Distomum viviparum* Olsson, 1868. *Zoögonus mirus* was described from two specimens found in the intestine of *Labrus merula* at Trieste. The worms measured 1.55 mm. in length and about 0.45 mm. in width. The following year, Odhner (1902) designated *Zoögonus viviparus* (Olsson, 1868) Looss, 1901 as type of a new genus *Zoögonoides.* In this paper he redescribed *Distomum rubellum* Olsson, 1868, and transferred the species to *Zoögonus.* Like Olsson, he found the parasites in *Labrus berggylta* (syn. *L. maculatus*) from the west coast of Sweden, but the examination of twenty fishes at the Zoölogical Station of Kristineberg disclosed only two infections and only a few worms were obtained. The specimens were yellowish in color, 1.1–1.4 mm. in length and 0.45 mm. in greatest width. *Zoögonus rubellus* was distinguished from *Z. mirus* on the size of suckers and length of the miracidia. Although Looss and Odhner were two of the ablest students of the trematodes, their observations on *Zoögonus* were limited to the study of very few specimens.

The specific identity of *Z. mirus,* questioned by competent investigators, still remains an unsolved problem. Goldschmidt (1902, 1905), after comparing specimens collected at Trieste with the descriptions of *Z. mirus* and *Z. rubellus,* stated that there were no morphological differences. The dimensions of suckers and miracidia, characters used by Odhner to separate the species, were found to be variable and hence invalid as specific criteria. Furthermore, Goldschmidt was unwilling to differentiate the species on the presence or absence of yellow pigment in the tissues. Nicoll (1909), who reported *Z. rubellus* as consistently abundant in *Anarhichas lupus* from St. Andrews Bay, described the worms as pale yellow in color, 0.75–1 mm. in length and about one-half

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as wide as long. Referring to the descriptions of Looss, Odhner and Goldschmidt, Nicoll stated, "My specimens agree best with Goldschmidt's description." It is significant that the worms studied by Nicoll and Odhner came from the same region, whereas Goldschmidt's material was collected in the Mediterranean and presumably was identical with that of Looss. Concerning specific determination, Nicoll expressed the opinion that, "Looss's *Zoögonus mirus* is in all probability identical with Odhner's *Z. rubellus* . . . at any rate, features sufficient to distinguish them are not at present apparent."

In a systematic review of the family Zoögonidae, Odhner (1911) maintained that the specimens of *Zoögonus* from the Mediterranean and North Sea are specifically distinct. After collecting material from both locations, he distinguished the two species on differences in size and color, size of suckers and shape of sucker cavities, location of the acetabulum, length of the digestive ceca, position of the cirrus sac, and number of eggs in the uterus. Odhner stated that extended specimens of *Z. mirus* never exceed 0.6 mm. in length and 0.2 mm. in width, whereas similar specimens of *Z. rubellus* measure 0.9–1.2 mm. in length and about 0.25 mm. in width. He noted that the pharynx and the miracidia are approximately the same size in the two species, but appear to be larger in the smaller specimens of *Z. mirus*.

The specimens of *Z. mirus* studied by Odhner were apparently contracted and probably not entirely mature. His statement that extended worms never exceed 0.6 mm. in length cannot be accepted, since the type specimens described by Looss measured 1.55 mm. in length. The number of eggs in the uterus obviously is correlated with the degree of sexual development, and the other features employed by Odhner to differentiate the species manifest so much variation that subsequent authors have disagreed on the identity or distinctness of the European species of *Zoögonus*. It is apparent that morphology of the adult stages is inadequate for a final solution of the problem.

Although a beginning has been made on the life history of *Zoögonus*, information from this source is still too fragmentary to permit final specific determination. In a series of papers, Timon-David (1933, 1934, 1936, 1938) described encysted metacercariae, identified as larvae of *Z. mirus*, in sea-urchins collected in the Gulf of Marseilles and along the coast of Roussillon. The metacercariae were found only in the muscles of the lantern of Aristotle. The degree of infection was variable and from one to sixty cysts were recovered from individual urchins. The incidence of infection in *Paracentrotus lividus* reached 50 to 60 per cent, a somewhat lighter infection was common in *Sphaerechinus granularis*, only a few cysts were recovered from *Arbacia acquituberculata*,

whereas no infection was observed in *Echinus acutus* or *Psammechinus microtuberculatus*. All the parasites apparently belonged to a single species. The cysts increased in size with the development of the metacercariae, and measured from 0.15 to 0.25 mm. in diameter. A cyst (1934, Fig. 1), fixed in picro-formol-alcohol solution under moderate compression, measured about 0.4 mm. in diameter, according to the scale accompanying the drawing. The cyst wall measured 0.003 mm. in thickness and was not surrounded by a connective tissue capsule. The worm was bent upon itself, with the dorsal surface applied to the wall of the cyst. Released from their cysts, the mature larvae averaged 0.6 mm. in length and 0.2 mm. in width. Specimens from *P. lividus, A. aequituberculata* and *S. granularis* (1934, Figs. 2, 3, 4), fixed in extended condition, measured 0.83, 0.93 and 0.66 mm. respectively (length calculated from scales accompanying the figures). Timon-David (1936) reported that metacercariae fed to *Blennius gattoruginae* excysted and persisted in the intestine for 45 days. Such a specimen, figured in the report, measured 0.64 mm. in length and was little if any farther advanced in development than larvae freshly removed from their cysts. The observation of Timon-David, that remains of sea-urchins were frequently present in the intestine of *Labrus merula,* supports his opinion that the metacercariae from the urchins are actually larval stages of *Z. mirus*. In his (1934) paper he recalled that the development of the miracidia has been known since the accounts by Looss (1901), Goldschmidt (1902) and Wassermann (1913), but that the first intermediate host remains as yet unknown. He expressed the belief that the cercarial stages are to be sought among the gastropods of the region.

In a report on larval trematodes from the region about Roscoff, Finistère, Stunkard (1932) described a tailless cercaria, *C. reticulatum* from *Nassa reticulata,* which shows such remarkable morphological agreement with the metacercariae described by Timon-David that the two must be closely related and may possibly belong to the same species. One item in the description of Stunkard requires correction. In the figure, the pharynx is represented as only a short distance in front of the acetabulum, whereas notes made at the time state that the pharynx is situated about midway between the suckers.

A single species of *Zoögonus* has been recorded from the Atlantic coast of North America. It was first described in the cercarial stage by Leidy (1891), who named it *Distomum lasium*. The larvae develop in *Nassa obsoleta*. Subsequent studies on the cercaria were reviewed by Stunkard (1938), who completed the life cycle. The cercariae encyst in polychaete annelids, principally *Nereis virens*. Natural infections were found in eels and sexually mature specimens were recovered after ex-

perimental infection of the eel and toadfish. Comparison of adult specimens with descriptions of *Z. rubellus* and *Z. mirus* provided no positive basis for specific distinction and so notwithstanding certain differences in hosts, life cycle, and morphological details, Stunkard regarded the American specimens as specifically identical with *Z. rubellus* and *Z. mirus*.

Subsequent studies of the European species carried on at the Station Zoologique de Wimereux in 1939 and of the American species at the Marine Biological Laboratory in Woods Hole during 1940, have yielded such discordant results that the question of specific identity must be reconsidered. The results of these observations were reported in abstract (Stunkard, 1940).

A sojourn at Wimereux, France, from July 8 to August 14, 1939, provided an opportunity to reexamine European phases of the *Zoögonus* problem. Metacercariae were found in *Psammechinus miliaris*, the common sea-urchin of the region. Urchins were collected at different locations from the Port de Boulogne to Ambleteuse, a stretch of some ten kilometers. The heaviest infection appeared in specimens from the Port de Boulogne where encysted larvae were recovered from more than 50 per cent of the urchins dissected. Different individuals harbored from one to thirty-six metacercariae. Lighter incidence and degree of infection were found in urchins taken between Boulogne and Ambleteuse. All of the larvae appeared to belong to a single species.

The metacercariae were encysted in the muscles and connective tissue of the lantern of Aristotle. The cysts were transparent, with no obvious reaction of the host to the parasite. The cyst wall was thin, colorless and very tough. The cysts measured from 0.2 to 0.28 mm. in diameter. The larvae, freed from their cysts, measured 0.42 to 0.65 mm. in length and 0.15 to 0.22 mm. in width. The cuticula was spined, although the spines were reduced in size and number behind the level of the testes. Larvae fixed under cover-glass pressure measure up to 0.95 mm. in length and a small one (Fig. 3), apparently equally mature but fixed without pressure, is only 0.37 mm. in length. When the worms are fixed without compression, the preacetabular portion bends ventrad, so that in mounted specimens the oral sucker may appear above or below and immediately in front of the acetabulum (Figs. 1, 2, 3). A representative specimen, fixed under slight pressure and shown in Fig. 4 is 0.75 mm. in length. The acetabulum, situated near the middle of the body, measures 0.08 by 0.086 mm. in diameter. The pharynx is located about midway between the suckers and measures approximately 0.06 mm. in diameter. When the specimen was extended the pharynx tended to be longer than wide. Its anterior end is dentate and the nuclei of the organ are concentrated

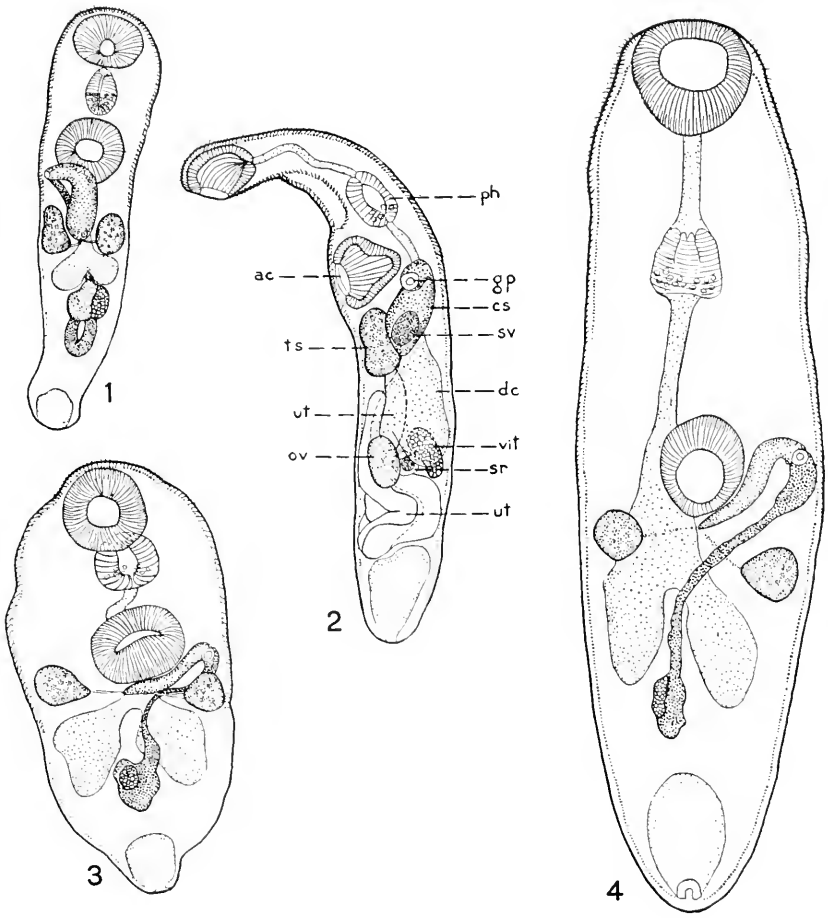

PLATE I

Abbreviations

ac	acetabulum	*sr*	seminal receptacle
cs	cirrus sac	*sv*	seminal vesicle
dc	digestive cecum	*ts*	testis
gp	genital pore	*ut*	uterus
ov	ovary	*vit*	vitellaria
ph	pharynx		

All figures are drawn to the same scale from fixed, stained and mounted specimens.

Fig. 1. Mature metacercaria, developed six weeks in *Nereis virens*, fed to a toadfish and removed two days later; Woods Hole. One of the largest specimens, fixed without compression, anterior end bent ventrad; dorsal view.

Fig. 2. Sexually immature specimen, developed six weeks in *N. virens* and six weeks in an eel; Woods Hole. Shows preacetabular ventral bending of body in specimens fixed without compression; lateral view from left side.

Fig. 3. Mature metacercaria from *Psammechinus miliaris;* Wimereux. One of the smallest specimens, fixed without compression, anterior end bent ventrad; ventral view.

Fig. 4. Mature metacercaria from *P. miliaris;* Wimereux. An average size specimen, fixed under cover-glass pressure, 0.75 mm. long; ventral view.

in its posterior half. The excretory system was worked out completely and agrees in detail with that of *Cercariaeum reticulatum* and with that of the American species of *Zoögonus*.

Measurements of the metacercariae from Wimereux do not differ greatly from those given by Timon-David for metacercariae from sea-urchins in the Mediterranean. The suckers in my specimens are slightly smaller than those measured by Timon-David, although his figures show the apertures of the suckers to be wide open and the specimens may have been more flattened. He figured the acetabulum slightly behind the middle of the body, whereas in my material it tends to lie slightly in front of the middle, although this feature is variable and changes with the extension or retraction of anterior and posterior regions of the body. Consequently, it appears likely that the specimens from Wimereux are specifically identical with those from the Mediterranean.

After discovery of the metacercaria, attempts were made to find the other stages in the life cycle of the European species of *Zoögonus*. Examination of many fishes, including several specimens of *Labrus sp.*, were fruitless. In view of the possibility, expressed previously, that the tailless larva, *C. reticulatum*, may represent a stage in the life history, wide search was made for it. The host, *Nassa reticulata*, is abundant in the region but the examination of more than 1200 specimens did not yield a single infection with tailless cercariae. Over 800 of these snails were collected from mud between rocks of the breakwater in the Port de Boulogne, and sea-urchins taken from these rocks were heavily infected with metacercariae. Other mollusks examined for *Zoögonus* larvae, with negative results, included 240 *Mytilus edulis*, 146 *Barnea candida*, 28 *Tapes pullastra*, 10 *Ensis sp.*, 240 *Patella vulgaris*, 35 *Purpura lapillus*, 34 *Littorina obtusa*, 86 *L. rudis*, 56 *L. littorea*, and 45 *Gibbula cineraria*.

To determine whether annelids as well as echinoderms harbor metacercariae of the European species of *Zoögonus*, worms were carefully dissected under a binocular microscope. The examination of 14 *Eunereis longissima*, 12 *Nereis errorata* and representatives of other unidentified polychaetes from the Port de Boulogne did not disclose any metacercariae.

In view of the failure to discover other stages in the life cycle of *Zoögonus* in the Wimereux area, the origin of the infection in the sea-urchins and subsequent fate of the larvae are entirely problematical. The completion of the life history by trematode parasites would be difficult in this region since the tides have an amplitude of eight to ten meters and the collecting grounds, exposed at low tide, are covered by an enormous volume of water six hours later.

Comparison of the larval stages of *Zoögonus* found along the north coast of Europe and the eastern coast of North America shows slight but apparently significant differences. Cercariae from *Nassa reticulata* at Roscoff average slightly larger than those from *N. obsoleta* at Woods Hole and the suckers are also larger. In the European form the ranges of size are: acetabulum, 0.068–0.076; oral sucker, 0.076–0.085; and pharynx, 0.03–0.04 mm., whereas measurements of the corresponding structures in the American form are: 0.062–0.075; 0.043–0.055; and 0.022–0.028 mm. Moreover, in the European form the prepharynx is relatively shorter and the pharynx is about midway between the suckers, whereas in the American form the pharynx is farther posteriad and frequently overlaps the acetabulum. The metacercariae from Wimereux and from Woods Hole show the same differences as the cercariae from the two regions. Cysts of the European form are slightly larger, the metacercariae are larger (compare Figs. 1, 2, and 3) and the relative sizes of suckers persists. Average measurements of ten specimens from the two localities give the following sizes (dimensions of Wimereux specimens first, of Woods Hole specimens second): acetabulum 0.085 *vs.* 0.075; oral sucker 0.09 *vs.* 0.065; pharynx, 0.06 *vs.* 0.042 mm.

To determine whether the American species of *Zoögonus* may occur in sea-urchins as well as polychaete annelids, attempts were made at Woods Hole in the summer of 1940 to infect urchins with *Distomum lasium* (= *C. linfoni*). Many freshly dredged urchins, both *Arbacia punctulata* and *Strongylocentrotus drobachiensis*, were dissected with negative results. Since enormous numbers of these animals have been used during the past forty years for embryological and other studies without the reported finding of metacercariae, natural infection with trematode larvae must be absent or very slight. Portions of dissected urchins, including the denticles and attached tissues, were placed in dishes of sea water with scores of naturally emerged cercariae of *Zoögonus*. The larvae crawled about over the tissues but did not penetrate or encyst. They were not attracted toward intact sea-urchins or dissected portions of them. Single urchins were exposed for several hours during the day in finger bowls to hundreds of cercariae and maintained during the intervening time in large aquaria. Dissection of the urchins later did not disclose any metacercariae.

Although the factors concerned with infection of the secondary intermediate host are virtually unknown, it is apparent that experiments devised to secure experimental infection in the laboratory must approximate natural conditions as closely as possible. Accordingly, on August 16, 1940, twenty specimens of *N. obsoleta* from which cercariae were emerging in large numbers were placed in each of two aquaria. Fifteen speci-

mens of *A. punctulata* were added to one aquarium; five specimens of *A. punctulata* and five specimens of *S. drobachiensis* to the other. After an interval of a week, dissection of the urchins was begun. No infection was found in *S. drobachiensis* but *Zoögonus* larvae were recovered from eleven specimens of *A. punctulata*. These urchins, examined in the period from August 23 to September 11, yielded 79 cysts in which the larvae were dead, 60 cysts containing living larvae, 32 unencysted, dead larvae, and 16 unencysted, living larvae. The dead, encysted larvae were often partly disintegrated. Live larvae in cysts had extruded their stylets, but showed no evidence of development. The gonads were no larger than those of the cercaria and the ducts of the penetration glands were still visible. It is evident from these results that cercariae of the American species of *Zoögonus* will enter and encyst in sea-urchins and that they may live there for a time. But no development was observed and the finding of so many dead larvae, both free and encysted, indicates that *A. punctulata* is not a suitable host. It is probable, therefore, that these sea-urchins are not involved in the life cycle of the parasite.

The problem of specificity in host-parasite relations can be solved only by the experimental methods developed in studies on the life cycles of parasites. Formerly it was believed that different species of hosts harbored different parasites. In the case of trematodes, it is now known that a single parasitic species may infect a wide variety of hosts. *Allassostoma parvum* may infect frogs and turtles; *Zygocotyle lunata* may infect birds, rodents and ruminants; *Cryptocotyle lingua* may infect birds, rodents and carnivores; *Notocotylus urbanensis* may infect ducks and muskrats; *Fasciola hepatica* may infect cattle, pigs, rodents, the elephant, kangaroo and man; *Echinostoma revolutum* may infect various species of birds and mammals; *Psilostomum ondatrae* may infect the muskrat, duck, pigeon and canary. These examples, selected from a large list, represent five families and show that the possibility of multiple hosts is general. Furthermore, as a result of development in widely separated hosts, representatives of a single trematode species may manifest morphological differences which under other conditions might reasonably be regarded as specific. Specimens of *F. hepatica* from a guinea pig and others from a cow would hardly be assigned to the same species on the basis of morphology.

The digenetic trematodes manifest a comparable lack of specificity in their intermediate hosts. This is true particularly in cases involving a second intermediate host, often nothing more than a "transfer host" in which no development occurs. The condition is similar to that in *Fasciola* and *Zygocotyle*, where cercariae encyst on vegetation or other objects which are eaten by the final host. Even in the first intermediate

host, specificity may be far from rigid. For *Fasciola hepatica,* which has become cosmopolitan in distribution, snails belonging to the following genera may serve as first intermediate hosts: *Lymnaea, Galba, Bulinus, Physopsis, Physa, Stagnicola, Fossaria, Pseudosuccinea* and *Ampullaria.* In any particular region, one variety of snail is selected, but in different regions the species is different.

The data on *Zoögonus* are hard to interpret. In view of the lack of specificity in the life cycles of other trematodes, it is not impossible that a single species of *Zoögonus* employs different primary, secondary and definitive hosts on the two sides of the Atlantic ocean. In such event, the morphological variations are readily explained. On the other hand, the bionomic and morphological differences may represent valid specific criteria. This opinion is supported by cytological observations. According to Goldschmidt (1905), *Z. mirus* has 10 chromosomes, while Brooks (1930) found 12 chromosomes in the American form. At present there is no basis for a positive distinction between species of *Zoögonus* from the North Sea and the Mediterranean, but it appears probable that the European and American forms are specifically distinct. If this proves to be true, the American species is *Z. lasius* (Leidy, 1891) Stunkard, 1940.

Summary

Encysted metacercariae of *Zoögonus* are reported from the sea-urchin, *Psammechinus miliaris,* at Wimereux, France. Comparison with descriptions of other larval stages found at Roscoff and Marseilles indicates that all belong to the same species. Attempts to infect sea-urchins at Woods Hole with the American form of *Zoögonus* were only partially successful. Bionomic and morphological differences between the European and American representatives of *Zoögonus* are discussed. It appears probable that they belong to different species.

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FACTORS INFLUENCING MOULTING IN THE CRUSTACEAN, CRANGON ARMILLATUS

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Introduction

According to Darby (1938), *Crangon armillatus* exhibits diurnal moulting in which most moultings occur during the early afternoon. Although this is the warmest period of the day, he did not consider that there was any correlation with temperature. This investigation was undertaken in order to determine whether light or temperature, or both of these factors, are concerned with the diurnal moulting of this animal.

Crangon of different sizes were collected and placed in individual finger bowl culture dishes in the laboratory. The animals were fed abundantly on algae and the flesh of the spiny lobster, *Panulirus argus*. Some were kept on the table in the laboratory while others were placed in constant temperature incubators.

Diurnal Moulting

One hundred and thirty-six specimens of *Crangon armillatus*, varying in length from 10 to 39.5 mm., were selected so that there were approximately the same number in each size group, as shown in Table III. They were kept in individual finger bowls on the laboratory table where at midday the average light intensity was approximately 75 foot-candles. The experiment was begun on June 19 and continued until each animal had moulted twice with the exception of eight fatalities, for which other specimens were substituted. The data obtained in this study are shown in Table I.

This study confirms the existence of diurnal moulting in this animal under the conditions of this experiment. The great majority of the animals moulted between the hours of 10:00 A.M. and 5:00 P.M., with the largest number moulting between the hours of 1:00 and 2:00 P.M. No animals moulted between the hours of 9:00 P.M. and 7:00 A.M.

There is a diurnal rise and fall of air temperature at Tortugas which corresponds very closely with the diurnal moulting of *Crangon armillatus*. During the period of this experiment the average temperature in the

laboratory at 8:00 A.M. was 28.6° C., at 1:00 P.M. it was 31.9° C. and at 5:00 P.M. it was 29.4° C. The temperature in the laboratory at night fell to an average of approximately 27.8° C. Since the animals that were used in this experiment were kept in small dishes with a comparatively small amount of water they were, to a very large degree, subject to these temperature changes.

Under the conditions of this experiment the animals did not begin moulting until the temperature had risen to approximately 29° C. in the morning and ceased moulting when it fell to approximately this same temperature in the late afternoon or evening.

Crangon armillatus lives near low tide in small bays at Tortugas that are protected from strong wave action. At night and at high tide the temperature at this season is approximately 28° C. At low tide, at midday, the temperature in these bays often rises to 39° C., although the

TABLE I

Number of *Crangon armillatus* that moulted between the hours indicated when kept in individual culture dishes on the laboratory table.

7–8 A.M.	8–9 A.M.	9–10 A.M.	10–11 A.M.	11–12 A.M.	12–1 P.M.	1–2 P.M.	2–3 P.M.	3–4 P.M.	4–5 P.M.	5–6 P.M.	6–7 P.M.	7–8 P.M.	8–9 P.M.
1	6	6	21	26	27	55	33	31	25	18	16	5	2

average is four or five degrees lower. Thus in nature these animals are subject to much the same temperature changes as in the laboratory.

Effect of Keeping Animals at a Constant Temperature

This study was undertaken to ascertain if possible the effect of reversing the effect of daylight from daytime to night. Since it was discovered that the midday heat affected the incubators by raising the temperature above 30° C., when they were set at this temperature, a higher temperature was used.

Two incubators with thermostat control, which were set at 33° C., were used for this study. The inside of one of the incubators was illuminated from 8:00 P.M. to 8:00 A.M. by a Mazda lamp which cast a light of approximately 75 foot-candles on the animals, thereby reversing the relation between light and daytime. The inside of a second incubator was kept in total darkness both day and night in order to determine whether the light used in the first incubator had any effect on moulting.

Sixty-two *Crangon*, each in separate culture dishes, were kept in the illuminated incubator, and 57 were kept in the non-illuminated incubator.

TABLE II

Percentage of *Crangon armillatus* that moulted from 8:00 A.M. to 8:00 P.M., and from 8:00 P.M. to 8:00 A.M. when kept at 33° C. One group was kept in total darkness while the other was illuminated only at night.

	In total darkness	Illuminated with 75 f.c. at night
Moulted from 8:00 A.M. to 8:00 P.M.	54.4	51.6
Moulted from 8:00 P.M. to 8:00 A.M.	45.6	48.4

The number of moulted individuals were counted twice daily; at 8:00 A.M. to determine the number of individuals that moulted during the night and at 8:00 P.M. to determine the number that moulted during the daytime. The results obtained are shown in Table II.

Although the results above show that a few more animals moulted during the daytime than at night, moulting is not restricted to the daytime when *Crangon* are kept at a constant temperature. It further shows

TABLE III

Comparison of the average number of days between moults of *Crangon* that were kept in the laboratory at an average daily temperature of approximately 29.5° C. with those that were kept at a constant temperature of 33° C.

Size	10–15 mm.	15–20 mm.	20–25 mm.	25–30 mm.	30–35 mm.	35–40 mm.	Average days
At lab. temp. of approx. 29.5° C.	7	9.7	11.1	12.5	14.7	18	12.2
At 33° C.	5	7.8	9.2	9.6	11.5	14.1	9.5

that light of 75 foot-candles has little if any effect on the moulting of these animals. *Crangon* that are kept in culture dishes on the laboratory table moult only in the daytime or early evening whereas at a constant temperature they moult practically as often at night as in the daytime.

EFFECT OF AGE AND TEMPERATURE ON MOULTING RATE

Animals which were used in the preceding studies included 136 that were kept in the laboratory at laboratory temperature, and 119 which were kept at a constant temperature of 33° C. These were selected so that there were approximately the same number of animals in each of the six age groups shown in Table III. The average number of days between moulting periods for each size group is shown in the table. In this study, size was taken as a general criterion of age.

As shown in Table III the length of the period between moults was shortened, on an average, 2.7 days. By raising the average temperature

3.5° C. the moulting interval was decreased by 22.1 per cent. This is in agreement with Smith (1940), who showed that the length of the intermoult in young crayfish is directly dependent on temperature.

Moulting Rate of Non-seeded Females, Seeded Females and Seeded Females from Which Embryos Were Removed

For this study, 30 non-seeded females, 30 seeded females, and 30 seeded females from which the embryos were removed were placed in separate finger bowls on the laboratory table. All of the seeded females selected including those from which the embryos were removed were carrying very young embryos. Numbers were equally distributed among the three size groups shown in Table IV.

Table IV

Comparison of the average moulting interval in days of non-seeded females of different sizes with the interval between time of collecting and the next moulting period of seeded females bearing very young embryos, and with that of seeded females from which very young embryos were removed.

Condition of females	Size		
	20–25 mm.	25–30 mm.	30–35 mm.
Non-seeded females	11.4	12.1	14.3
Seeded females	16.1	15.7	18.4
Seeded females from which embryos were removed	11.2	10.3	12.9

In the above study it is impossible to state how much time elapsed between the last moulting period and ovulation in the case of the seeded females. The embryos that were attached to the seeded females were from one to three days old when the experiment began. If these seeded females moulted three or more days before ovulation, which seems probable, the period between moults of the seeded females would be approximately twice as long as that of non-seeded females. Moreover, when the embryos were removed from the seeded females the period between moults of these females was materially shortened. This indicates that there is something which inhibits moulting in seeded females.

In no instance did a seeded female moult while she was carrying embryos. However, all seeded females moulted within five days after shedding their embryos and twelve moulted within one day.

Discussion

The data presented in this paper indicate quite clearly that light of the intensity of 75 foot-candles has very little if any effect on moulting in *Crangon armillatus* and hence cannot be considered as an important factor in causing the diurnal moulting. On the other hand, the daily rise and fall in temperature is a very important factor in causing the diurnal moulting. Increase in temperature sets in operation the factors causing moulting, while a fall in temperature checks them.

From the above data it seems probable that at least two factors are concerned with moulting in *Crangon armillatus*. One, which causes moulting, manifests itself when the temperature in which the animal lives rises to or above approximately 29° C. The other, which inhibits moulting in seeded females, is apparently dependent upon the attachment of the embryos to the female. Moulting in insects, as shown by Wigglesworth (1934) and others is apparently caused by hormones. Brown and Cunningham (1939), Hanström (1939), Abramowitz and Abramowitz (1940), and Smith (1940) have shown the importance of a moult-inhibiting substance produced in the eye-stalk of crayfish and certain other crustacea.

Conclusions

1. *Crangon armillatus* exhibits diurnal moulting which begins in mid-forenoon, reaches its height at about 1:30 P.M. and ceases in later afternoon or early evening.

2. Light of 75 foot-candles has very little if any effect on moulting in these animals.

3. Temperature changes are very important in causing the diurnal moulting. Increase in temperature sets in operation the factors causing moulting while a fall in temperature checks them.

4. Animals kept at a constant temperature fail to exhibit diurnal moulting.

5. By raising the average daily temperature approximately 3.5° C. the moulting interval was decreased by 22.1 per cent.

6. Females carrying embryos do not moult even though the period of carrying embryos exceeds the normal period between moults.

7. At least two factors appear to be concerned with moulting. One, which is greatly influenced by temperature changes, causes moulting. The other, which inhibits moulting in seeded females, appears to be dependent upon the attachment of the embryos to the female.

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FEEDING MECHANISMS AND NUTRITION IN THREE SPECIES OF BRESSLAUA

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The question of food taking by protozoa has attracted considerable attention in the past and there have appeared numerous accounts of the various mechanisms employed, the type of food taken and the conditions of acidity and alkalinity during the digestive process. Regarding the last-mentioned observations there seems to be general agreement that, in the bacteria-feeding species at least, there is an acid-alkaline cycle from the time the food is ingested until the residue is defecated. Practically all of the observers employed some type or combination of types of indicator dyes, watching for the color changes which occur as the food is ingested, digested and the residue defecated. The most frequently used indicator has been neutral red because of the ease with which most protozoa take up this dye. Unfortunately, this indicator is useful only to detect shifts in hydrogen ion concentrations through a relatively small range.

We have examined the problem of feeding and acid-alkaline reactions in three species of the genus *Bresslaua*. These ciliates are carnivorous members of the family Colpodidae and one species, due to its peculiar feeding habits, offers exceptional opportunities for direct observations for long, uninterrupted periods of time.

In the ensuing account we will give a short description of the experimental organisms, an account of their feeding habits, some evidence for an acid-alkaline cycle during digestion and a brief account of food selectivity.

Material and Methods

The carnivorous ciliates were obtained from dry hay collected in Stuart, Florida. The same procedures of excystation and isolation were employed as were previously used in the case of most of the *Colpoda* material reported from this laboratory (Kidder and Claff, 1938; Kidder and Stuart, 1939; Burt, 1940).

For studies on the hydrogen ion concentration within the vacuoles

and the protoplasm various indicator dyes were used. These will be described in a later section. It was found expedient first to stain the food organisms (usually *Colpoda steinii*) and then to add a few *Bresslaua* to the culture. The culture was then placed in a moist chamber until the food organisms had all been ingested. As in the case of *Woodruffia metabolica* (Johnson and Evans, 1939; 1940), these carnivores formed resistant cysts after the food had become depleted. These were caused to excyst by the addition of fresh hay infusion. Food organisms were then added and the feeding process studied under a water immersion lens ($\times 40$). The dye brought into the protoplasm of the carnivores during the previous period of feeding was sufficient to allow us to gain an idea of the changes in acidity and alkalinity which took place during feeding, digestion and the subsequent defecation of residue.

For the study of food selectivity bacteria-free ciliates were necessary. The *Bresslaua* were freed of their associated microörganisms by the employment of our modification of the Parpart method of direct washing, using Syracuse watch glasses enclosed in cellophane bags (Kidder, Lilly and Claff, 1940). Because of the structural peculiarities of these ciliates it was found necessary to allow them to encyst and divide after the tenth wash in sterile hay infusion. Close watch was kept of the dividing ciliate so that the washing could be continued immediately after the emergence of the daughter organisms. Each of the two, four or more daughters was then washed individually through five or more changes of sterile medium and placed in tubes containing the food organism to be tested. Adequate bacteriological tests showed that the majority of the carnivores so treated were free of bacteria.

The food organisms tested will be discussed in a later section. They have all been mentioned in previous accounts from this laboratory.

Description of Bresslaua Kahl

The three members of this genus which we have studied resemble the various species of *Colpoda* in their general structure, mode of division within a cyst and permanent cyst formation. They all possess a macronucleus of the *Colpoda cucullus* type (Kidder and Claff, 1938; Burt, 1940) and a single micronucleus. The chief differences are found in the structure of the mouth, which has become modified and extended for the carnivorous mode of life. The following brief descriptions are given to add to the account of Kahl (1931) of *B. vorax* and to establish two new species.

Bresslaua vorax Kahl (*Fig. 1, A*).—This species is evenly rounded posteriorly, but the anterior end is compressed laterally. The left an-

terior side is depressed in such a way that the whole anterior end is twisted. This twisted appearance is seen best in an organism immediately after excystment. The size varies greatly depending upon the amount and kind of food taken. Freshly excysted ciliates range in length from 40 μ to 90 μ and in width from 25 μ to 50 μ. Ciliates which have fed on relatively large prey (such as *Glaucoma scintillans* or *Colpoda cucullus*) attain a size of 180 $\mu \times 100\ \mu$ or even larger.

The ciliary pattern, as seen after the silver technique of Klein or when treated with opal blue or nigrosin, resembles that of other members of the family. The peripheral cilia arise in pairs, as is true of most of the cilia among the members of the genus *Colpoda* (Taylor and Furgason, 1938; Burt, 1940). This is in contrast to the condition in *Woodruffiia metabolica* (Johnson and Larson, 1938) where the cilia are single. The cilia are relatively short and delicate. The ciliary rows originate from a short keel and extend over the general body surface as well as the right interior of the cytostomal cavity, converging in a field at the posterior end of the body.

The mouth is a large, cilia-lined cavity, open toward the ventral surface and the left side. On the roof of the mouth are folds or "rugae," roughly resembling those on the hard palate of mammals. On the floor of the mouth, which is somewhat raised, there is a row of membranelle-like structures, 40 to 45 in number. These beat in such a way as to create a strong current *out* of the mouth. At the back of the mouth there is a rather short, broad gullet directed posteriorly. It is on the brink of this gullet that the membranelles are located.

Bresslaua vorax exhibits activity when not actually feeding. It tends to remain on or near the bottom of the culture and to move in small circles. It comes in contact with the bottom so that the left side of the body, and therefore the mouth-opening, is up. Prey are swept into the mouth by strong currents. During the time the live prey is in the mouth until it has entered the food vacuole at the base of the gullet the movement of the carnivore is much reduced. This is due to a change in the beating of all the peripheral cilia and will be described in greater detail in the case of one of the other species. After the prey has been successfully trapped in the posterior food vacuole, movement is resumed.

Bresslaua insidiatrix sp. nov. (*Fig. 1, B*).—The general departures from the *Colpoda*-like structure which were described for *B. vorax* are accentuated in this species. The mouth opening is more extensive in relation to the size of the body and the twisting of the anterior end is somewhat greater. No "rugae" are present in the mouth. This species varies in size from 40 $\mu \times 25\ \mu$ when starved to 120 $\mu \times 90\ \mu$ when

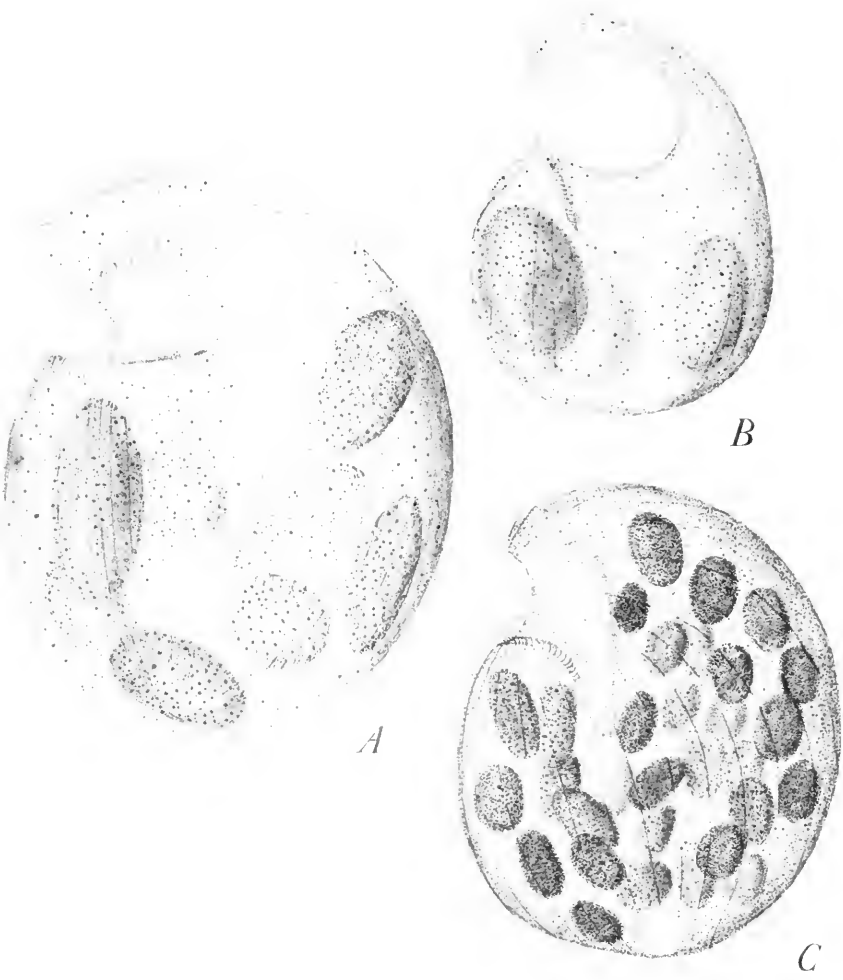

FIG. 1. All drawings were taken from life. × 460. *A. Bresslaua vorax.* The food inclusions are *Glaucoma scintillans. B. Bresslaua insidiatrix* sp. nov. during early stages of feeding on *Glaucoma scintillans. C. Bresslaua sicaria* sp. nov. after ingesting a number of *Colpoda steinii.*

ready to divide after active feeding. The general pattern of the peripheral ciliary lines is similar to that in *B. vorax*. The cilia originate in pairs and are very long and stiff. They are easily visible in life and stand out at nearly right angles to the body while the organism is at rest. There are 10 to 15 membranelle-like structures in the mouth located in the same relative position as those in *B. vorax*.

One of the most characteristic things about *Bresslaua insidiatrix* is its mode of feeding. It normally rests on the bottom of the culture dish with its right anterior end in contact with the substratum. It will remain for two to three hours in one spot, only occasionally pivoting slightly. During this time there is a strong current being directed into the very large mouth-opening and all small objects are drawn in. Inanimate objects are rapidly whirled toward the posterior border of the mouth and shot out by means of the out-going current created by the membranelles. Moving ciliates or flagellates, on the other hand, receive different treatment. Some mechanism within the mouth seems to be stimulated by ciliated or flagellated organisms and this appears to affect the whole neuromotor system. The peripheral cilia immediately lose their stiff, vibratile appearance and move slowly in waves (Fig. 2, *B, C*). The current going into the mouth slackens or disappears as does the outgoing current along the posterior border. The mouth-opening is contracted, forming an efficient barrier against the escape of the prey. The prey moves about freely in the mouth for from one to two minutes and gradually the posterior border of the mouth begins to form the prospective food vacuole. This vacuole forms well ahead of the prey and not under direct impact of it. The prey may partially enter the forming food vacuole and draw back into the mouth a number of times before it is finally trapped. Once well within the vacuole it begins to rotate and the vacuole closes off. The closure is effected by what appears to be a thin sheet of protoplasm originating from the region just posterior to the zone of membranelles and flowing across the vacuole opening from ventral to dorsal. At the instant the sheet of protoplasm fuses with the opposite side the prey is killed. This phenomenon will be discussed later in the section on hydrogen ion concentrations. The closure of the vacuole also sets off another reaction which immediately causes the peripheral cilia to resume their stiff, vibratile condition (Fig. 2, *D*).

Bresslaua insidiatrix appears to be the most highly specialized for a carnivorous habit of the three species observed by us. It feeds only on living ciliates and flagellates. Other bodies (cysts, amoebae, algae, yeast and detritus) do not evoke the "swallowing" response. That this evocation is largely physical is indicated by the following fact. In an excysting culture of *Colpoda steinii* it is common to see these small

ciliates rotating rapidly within the thin endocyst. These ciliates may be drawn into and swept out of the mouth of *B. insidiatrix* a number of times while the endocyst is still intact, but immediately the *Colpoda* escapes its cyst wall and is drawn into the mouth, it evokes the general responses noted above. In contrast to this, both *Bresslaua vorax* and the third species, yet to be described, are able to ingest certain types of non-moving microörganisms, but not all organisms ingested are adequate as food.

Bresslaua sicaria sp. nov. (*Fig. 1, C*).—This species shows a closer resemblance to the typical *Colpoda*-form than either of the above-mentioned species. The mouth opening is confined to the ventral surface and does not extend to the left side. The interior of the mouth cavity is similar in structure and relative size to that of *B. vorax,* but lacks "rugae." The zone of membranelle-like structures is composed of from 20 to 25 components and occupies the same general position as that in the preceding species. A well-formed gullet is present running posteriorly a short distance into the cell.

Bresslaua sicaria varies from 35 μ to 110 μ in length and from 23 μ to 92 μ in width depending upon its state of nutrition. The peripheral ciliary lines are less numerous than those of the other two species, but the general patterns are very similar. The cilia are long and wavy and originate in pairs.

Bresslaua sicaria, unlike the other two species, rarely comes to rest. It swims in a characteristic looping fashion and draws its prey into the mouth while swimming. There is a change in the ciliary motion during the act of swallowing resulting in general and violent movement of the whole organism. Immediately a food organism is caught the *Bresslaua* starts rotating rapidly on its lateral axis and continues the rotation until the prey enters the vacuole, when it resumes its swimming motion. The feeding reactions of this species are very difficult to observe because of its extreme activity.

The feeding habits of the three species described above are so characteristic that it is possible to identify each of them under very low magnifications. *Bresslaua vorax* and *B. insidiatrix* take their prey while they are in contact with the solid substratum, while *B. sicaria* feeds while swimming free in the medium. Of the first two, only *Bresslaua insidiatrix* remains motionless while waiting for its prey. Because of this characteristic, *B. insidiatrix* is an ideal carnivore to use in experiments and observations on feeding mechanisms.

The establishment of two new species of the genus *Bresslaua* seems to us to be justified because of the characteristics noted above (number of

ciliary rows, length and characteristics of cilia, shape and extent of cytostomal opening, feeding habits and food selectivity).

Food Vacuoles and Hydrogen Ion Concentration

After *Bresslaua insidiatrix* has fed on *Colpoda steinii* previously stained with a 1:12 million dilution of neutral red, it becomes highly colored by virtue of its food inclusions. After the food has been exhausted the carnivores form protective cysts. Many red food balls are still present in the encysted organisms. These food balls are defecated during or shortly following excystment (Fig. 2, *A*, *B*), leaving the ciliates nearly colorless. Under the water immersion lens it is possible to detect a number of neutral red stained granules in the endoplasm. Excystment with alkaline hay infusion imparts a slight yellowish tinge to the medium, but does not change the color of the endoplasmic granules. The small freshly excysted ciliates settle to the bottom of the culture dish and immediately begin feeding when numbers of *Colpoda steinii* are added with the excysting fluid (Fig. 2, *B*). The clearest observations are made during the capturing and killing of the first several ciliates.

As the prospective food vacuole forms its fluid contents become slightly pink (Fig. 2, *C*). This coloration deepens as the prey enters, but there appears to be no change in the motions of the prey at this time. At the instant that the food vacuole is closed off by the protoplasmic sheet there suddenly appear a large number of brilliant red granules or droplets in the protoplasm surrounding the vacuole (Fig. 2, *D*). The fluid surrounding the prey then becomes more deeply colored and simultaneously the prey is killed. The prey becomes motionless and the cilia stand out from the body. The fluid rapidly disappears from the vacuole and its lining comes to lie very close to the prey. The red granules in the cytoplasm rapidly fade out. There appears to be no indication that they enter the vacuole as has been described by Nirenstein (1905) for *Paramecium*. This is the first color-change to be noted. The reaction with neutral red shows that an acid condition is suddenly attained and that the hydrogen ion concentration is equal to or less than a pH of 6.8.

The above observations were repeated a number of times using a number of indicator dyes. None of them was quite as spectacular as the neutral red, either because they did not penetrate or because the colors were more difficult to see. Methyl red, methyl orange, brom cresol green, brom phenol blue, brom phenol purple, chlor phenol red, para-dimethyl-amino-azobenzene (Töpfer's reagent), Congo red and benzene-azo-alpha-naphthylamine were used and of these methyl red was by far the best. Although not as brilliant as the neutral red reaction, all of

the phases appeared with this dye. The appearance of bright red granules with methyl red indicates that their acidity must be in the neighborhood of pH 4.2 or lower. Failure of blue coloration with Congo red indicates that the hydrogen ion concentration is probably not higher than pH 3.0.

It appears likely that the sudden death of the prey is the result of the release of an acid from the protoplasm of the carnivore into the vacuole. Töpfer's reagent failed to give positive results in this organism, although the dye penetrated well. Nirenstein (1905; 1925) had reported using this dye to detect the presence of mineral acid in the vacuole of *Paramecium*, as indicated by the appearance of a red color. No red coloration was obtained in *Bresslaua*. Just what type of acid is released is obscure.

Separate experiments show that the acidity indicated by the color changes with methyl red are compatible with the death points of the various types of prey. Thus, *Colpoda steinii* is killed almost instantly in a phosphate buffer of pH 3.8, while the *Bresslaua* is still alive after one hour at pH 3.4. *C. steinii* died only after long periods at pH 4.5 and above this value no death was observed. This experiment simply shows that *Bresslaua* is more resistant to high acidity than is *Colpoda* and lends support to the idea that the killing within the vacuole is a result of the release of acid. A similar conclusion regarding the function of acid was reached recently by Mast and Bowen (1940) in the case of *Vorticella*. Other food organisms which were tested were more resistant than *Colpoda*. *Euglena gracilis and Astasia klebsii* survived for a long time at pH 3.8 and this checks with the reactions of these two flagellates within the food vacuole of *Bresslaua*. After the protoplasmic sheet has closed over either of these organisms there elapses from two to ten minutes before euglenoid movement ceases.

Following the killing process the body of the *Colpoda* begins to move anteriorly due to the general cyclosis of the protoplasm of the *Bresslaua*. When the prey contains an indicator dye, such as neutral red, it is possible to follow the color changes occurring during the hour or two required for digestion. At first the prey is nearly colorless, but it rapidly becomes yellowish. This indicates a faintly alkaline reaction and corresponds to the situation found in *Paramecium* (Nirenstein, 1925) except that the vacuole has never been observed to swell. The yellow color remains for from 15 to 20 minutes and then gradually changes through orange to a bright cherry red (Fig. 2, *E*). By the time the prey has reached the red condition its general outline is lost and it has become a compact ball. A number of these balls later fuse and form the fecal mass which is extruded during or following the next encystment, either

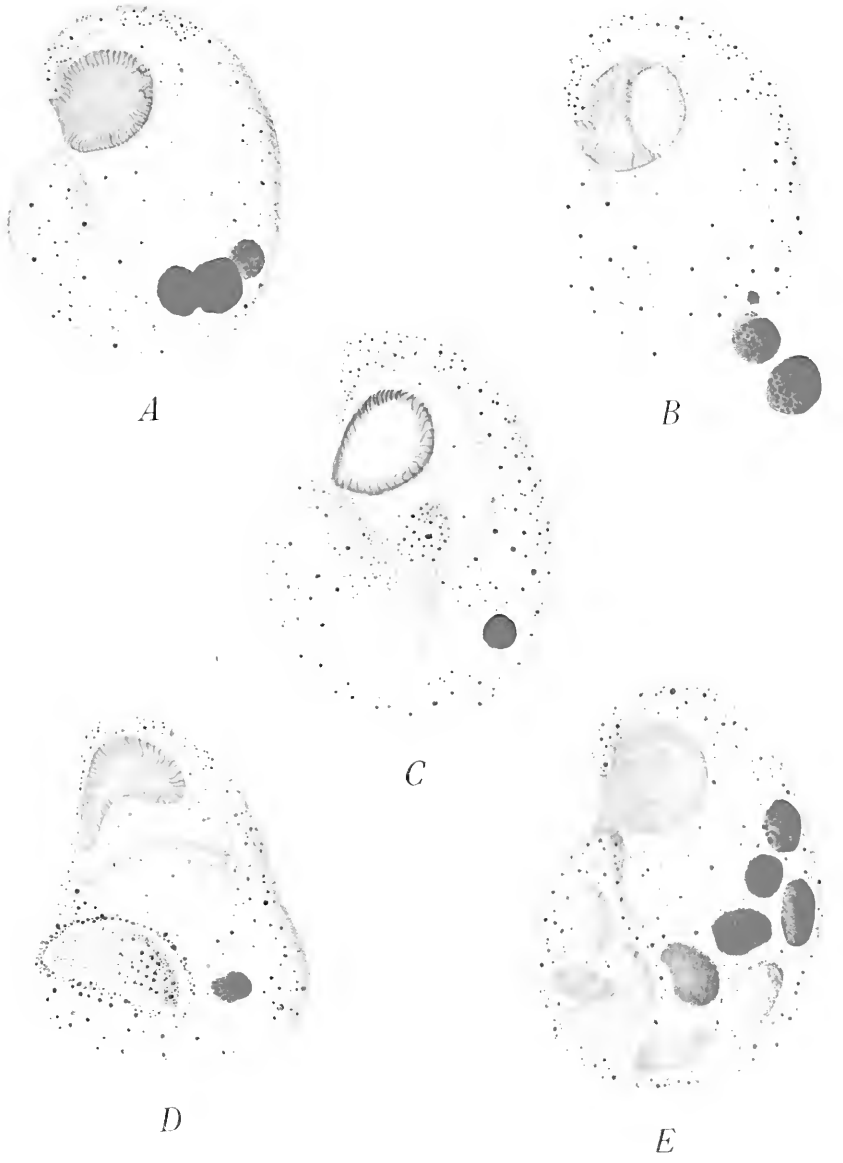

FIG. 2. *Bresslaua insidiatrix* showing the color changes with neutral red during feeding. *A*. Freshly excysted ciliate with old residue. Note the position of the cilia. *B*. Trapping of prey, *Colpoda steinii*, and defecation of residue. The cilia are bent and move in slow waves during this stage. *C*. Prey entering the prospective food vacuole, the fluid content of which is a faint pink. *D*. Food vacuole closed off from the mouth. At this stage the prey is instantly killed. Note the appearance of the cherry red granules in the cytoplasm surrounding the vacuole. The peripheral cilia of the carnivore have again assumed a stiff appearance. *E*. Carnivore after having ingested a number of ciliates. Note the color changes in the bodies of the prey as digestion proceeds.

from a division cyst or a protective cyst. Once released into the surrounding medium the fecal masses rapidly lose their red color, become pale yellow and disintegrate.

As mentioned previously, these observations are best made with *Bresslaua insidiatrix,* because of its feeding habits. As far as could be detected, the same general phenomena take place in the other two species. Certainly the color changes during digestion and defecation are the same, but the color changes accompanying killing, being of such short duration, could never be definitely established due to the constant movement of the carnivores.

Food Selectivity

The following account of the food selectivity is based on our observations of the three species of *Bresslaua* in the presence of a mixed flora of bacteria and in bacteria-free culture. While these observations do not represent a complete survey of the possibilities, they are presented in order to indicate the differences between the species and the possibilities for future work. In Table I we have listed the various food organisms which were used and have summarized the pertinent observations. It will be noted that ingestion does not invariably mean that the organism in question represents adequate food for growth. The various species of *Colpoda* supported growth in all three species of *Bresslaua* and these ciliates probably represent their natural food. The very nature of their protective cyst formation makes this assumption plausible. When dry hay is placed in spring water the various species of *Colpoda* excyst first, feed and multiply before the *Bresslaua* excyst. This means that in nature there would usually be a source of *Colpoda* at the right time.

Glaucoma scintillans was ingested by *Bresslaua vorax* and *B. insidiatrix,* while in the case of *B. sicaria* this was never observed. Thriving bacteria-free cultures of *B. vorax* and *B. insidiatrix* were maintained for a number of months with *G. scintillans* as food. In neither case, however, were normal protective cysts formed. After the food organisms had all been ingested the carnivores continued in the trophic condition for many days, getting smaller and smaller. Occasionally, in the case of *B. vorax*, they would round up and form temporary cysts (Johnson and Evans, 1940) from which they would spontaneously excyst within a few hours. This process might be repeated for days until finally all of the carnivores were dead. Serial transplants were always made while some food was still present. Eventually the various series declined in division rate and failed in transfer. The causes associated with this decline must receive further investigation.

TABLE I

Hay = sterilized hay infusion; *V* = *Bresslaua vorax*, *I* = *B. insidiatrix* sp. nov.; *S* = *B. sicaria* sp. nov.; (*N*) = non-sterile; (*S*) = sterile; (*S**) = no bacteria present but *Colpoda* were growing on *Stichococcus;* 1, 2, 3, 4, = relative growth where 4 is maximum observed. Where blanks occur in columns labeled "Ingestion" and "Growth" those organisms were not used.

Food Organism	Medium	Ingestion			Growth			Remarks		
		V	*I*	*S*	*V*	*I*	*S*	*V*	*I*	*S*
Colpoda cucullus	Hay, H_2O	+	+	+	1	2	2	Large, normal (*N*)	Very large, normal (*N*)	Large, normal (*N*)
C. steinii	"	+	+	+	4	4	4	Medium, normal (*S*N*)	Medium, normal (*S*N*)	Medium, normal (*S*N*)
C. maupasi	"	+	+	+	4	2	3	Medium, normal (*N*)	Medium, normal (*N*)	Medium, normal (*N*)
C. aspera	"	+	+	+	1	4	1	Small, cysts with low viability (*N*)	Small, normal (*N*)	Small, cysts with low viability (*N*)
C. inflata	"	+	+	+	1	3	1	Small, cysts with low viability (*N*)	Small, normal (*N*)	Small, cysts with low viability (*N*)
Glaucoma scintillans	Yeast-Harris, H_2O	+	+	−	3	2	0	Very large, normal (*SN*)	Large, normal (*SN*)	(*SN*)
Tetrahymena geleii	Hay, H_2O	+	+	+	1	0	0	Very large, abnormal. Cysts non-viable (*SN*)	Toxic (*SN*)	Toxic (*SN*)
Stichococcus bacillaris	"	+	−	+	3	0	0	Small, normal (*SN*)	(*SN*)	Encystment without division. (*SN*) Non-viable
Chlorella	"	+	−	+	2	0	0	Small, normal (*SN*)	(*SN*)	Encystment without division. (*N*) Non-viable
Euglena gracilis	"	+	+		0	0		Encystment without division. Non-viable (*N*)	Encystment without division. (*N*) Non-viable	
Astasia klebsii	"	+	+		0	0		Encystment without division. Non-viable (*N*)	Encystment without division. (*N*) Non-viable	
Chilomonas paramecium	"	+	+		0	0		Encystment without division. (*SN*) Non-viable	Encystment without division. (*SN*) Non-viable	
Yeast (live)	H_2O	+	−	+	3	0	3	Small, normal (*SN*)	(*SN*)	Small, normal (*N*)
Aerobacter	"	+	−	−	1	0	0	Small, normal (*N*)	(*N*)	(*N*)
Unknown bacteria	"	+			3			Small, normal (*S*)		

Tetrahymena geleii was ingested by all three species but did not support continued growth in any case, although a few divisions of *Bresslaua vorax* usually resulted. *Tetrahymena* appears to be toxic to *B. insidiatrix,* for after a single organism had been ingested the carnivore would leave the bottom of the dish, swim rapidly for a few minutes and then round up and encyst. These cysts were never viable. *Bresslaua sicaria* behaved in a similar manner.

One other item worthy of note in these investigations on nutrition is the case of *Stichococcus bacillaris. Bresslaua vorax* readily ingests this alga and flourishing cultures result. Normal protective cysts are formed and may be collected, dried and stored for future use. By use of the glass plunger-sponge method (Kidder, 1941) any number of sterile carnivores may be kept on hand.

Discussion

While the work on the bacteria-free cultures has not progressed to a point where the nutritional requirements of the members of the genus *Bresslaua* can be stated definitely, a number of points of interest have come to light. One of the most interesting observations is the great difference in the food organisms as evidenced by the differences in nutritional quality between *Glaucoma scintillans, Tetrahymena geleii* and the various species of *Colpoda* for *Bresslaua. Colpoda* was utilized by all three species of *Bresslaua. Glaucoma* was utilized by *Bresslaua vorax* and *B. insidiatrix,* while *Tetrahymena* was utilized by only *B. vorax* and then the growth was poor and not transplantable. This is exactly the reverse of the situation with the carnivorous hypotrichs, *Stylonychia* and *Pleurotricha.* Lilly (1942) has shown that these ciliates will feed and reproduce on *Tetrahymena* but not on *Glaucoma* and *Colpoda.* It is becoming apparent that the exact nutritional requirements of carnivorous ciliates are delicately adjusted and this fact may be of use in the future for comparisons between food organisms.

Regarding our observations on the hydrogen ion changes during swallowing, killing, digestion and defecation, a few comparisons with reports of other workers may be noted. Prowazek (1898) described neutral red granules around the periphery of food vacuoles of *Paramecium.* He supposed that these granules might be the carriers of the digestive enzymes. An alkaline stage during the digestive process was not described. The work of Nirenstein (1905; 1925) was the most complete on this subject. He describes an initial acid phase in the newly-formed food vacuoles of *Paramecium,* the pH being equal to that of a 0.3 per cent solution of HCl. These vacuoles were much more acid than com-

parable ones in a number of other ciliates. After the initial acid stage the food vacuoles increased in volume and became alkaline. Nirenstein believed that digestion occurs only at this stage, the digestive enzymes being trypsin-like in nature. It had earlier been proposed by Hemmeter (1896) that the appearance of an acid phase was the response to living prey and that the acid served as a killing agent. This contention was denied by Métalnikow (1912) because he was unable to demonstrate any regularity in the acid production even in the event that living prey were ingested. In our work on *Bresslaua insidiatrix* the conclusion was reached that the initial acid production around the vacuole was stimulated by the closure of the vacuole and that it was probably this acid which caused the death of the prey. The prey did not become acid in its reaction, however, which may have been due to the combination of the acid with its proteins. Later, enough alkaline material was taken up to cause the protoplasm of the prey to give an alkaline reaction. This alkaline phase appears to be the phase of active digestion, indicating, therefore, that the enzymes involved are catheptic in nature. Before defecation the residue becomes acid, possibly due to the acidic properties of some of the products of digestion. The appearance of a final acid stage in the food vacuole in *Bresslaua* seems to differ from the condition in *Paramecium*. In the latter organism the residue remains alkaline (Shapiro, 1927).

Up to the present time most of the observations on the hydrogen ion concentration of food vacuoles have been confined to bacteria-feeding ciliates. It will be interesting to see if the conditions described above will be found in other carnivorous types.

Summary

1. Three species of *Bresslaua, B. vorax* Kahl, *B. insidiatrix* sp. nov. and *B. sicaria* sp. nov. are described.

2. These ciliates are carnivorous and feed on other small ciliates, members of the genus *Colpoda* being especially favorable as food.

3. Using indicator dyes it was found that the prey is killed simultaneously with a sudden release of an acid into the newly-formed food vacuole. The hydrogen ion concentration of the vacuole fluid was estimated to be between pH 3.0 and pH 4.2. This range includes the death point of various species of *Colpoda*. During digestion the protoplasm of the prey becomes alkaline. The undigested residue becomes acid before defecation.

4. Bacteria-free *Bresslaua* were tested with a number of food organisms and a preliminary survey of their food requirements recorded.

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SECRETION[1] OF INULIN, XYLOSE AND DYES AND ITS BEARING ON THE MANNER OF URINE-FORMATION BY THE KIDNEY OF THE CRAYFISH

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Introduction

The following data are the outcome of an attempt to find whether filtration occurs through the nephron of the crayfish. While they do not conclusively exclude filtration, they are sufficiently interesting to warrant presentation.

In contrast to the glomerular kidney of vertebrates, the aglomerular vertebrate kidney cannot eliminate glucose, even during hyperglycaemia and phloridzination (Marshall, 1930), and cannot eliminate xylose (Jolliffe, 1930), or inulin (Richards, Westfall, and Bott, 1934). Furthermore, there is little or no doubt that inulin is not secreted by nor passively resorbed through the vertebrate nephron (see Smith, 1937). Accordingly, it was presumed that the presence or absence of inulin in the urine of the crayfish (a classical freshwater invertebrate), after its injection into the haemocoele, would demonstrate whether filtration occurs.

It is here shown that although inulin and xylose do appear in the urine of the crayfish, they are, at least in part, actually secreted. It is therefore unnecessary to invoke filtration to explain the excretion of these carbohydrates. The ability of all parts of the nephron of this animal to secrete or accumulate one dye or another (see below) and of the coelomosac to secrete calcium (Maluf, 1941*a*) indicates that this nephron is mainly, if not entirely, a secretory organ.

The subject is *Cambarus clarkii*, which frequents the freshwater swamps of southern Louisiana.

[1] Throughout this paper, *secretion* implies the transport of a substance from a region of lower to one of higher diffusion potential for that substance. *Excretion* refers merely to the outward elimination of undesirable material, regardless of whether the latter is secreted or filtered.

This work was begun in the Department of Zoology, The Johns Hopkins University, while the author was Johnston Research Scholar. Many thanks are due to Prof. S. O. Mast for numerous kindnesses and appreciative criticism.

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Methods

Inulin

Analysis.—The concentration of inulin in the blood and urine was measured, after acid-hydrolysis, by the Shaffer-Hartmann-Somogyi method (see Shaffer and Somogyi, 1933) using Shaffer-Hartmann reagent " 50 " and Somogyi's (1931) procedure for deproteinization of the serum. The technique was adapted to the small quantities used in this work as follows. Blood was taken by amputating a leg at the femur and allowing about 0.15 cc. to run into a small test-tube (9×75 mm.). Bleeding was instantly and permanently stopped by compressing the stump with the hot tips of a blunt forceps. The test-tube was stoppered and heated at 80° C. for about a minute or until the blood became opaque and was then cooled rapidly. The resulting solid was broken up with a fine glass-rod and the tube centrifuged for a few minutes. A sample of the supernatant liquid, usually about 75 cu.mm. was drawn into a fine calibrated pipette of the constricted type (Fig. 1, *A*). It was deproteinized by adding an equal volume of 7 per cent $CuSO_4 \cdot 5H_2O$ and another equal volume of 10 per cent $Na_2WO_4 \cdot 2H_2O$. Distilled water was added according to the required dilution (5 to 16 times), the same pipette being used in adding the water and reagents as that in taking the sample. After stoppering, shaking, and permitting to stand for at least 20 minutes, the tubes were centrifuged and 80 cu.mm. aliquots drawn for analysis. About 150 cu.mm. distilled water were added to increase the volume and then about 70 cu.mm. N H_2SO_4 for hydrolysis. The tubes were capped with glass-bulbs and heated in rapidly boiling water for 15 minutes. After cooling, a small drop of phenolphthalein was added. The solutions were neutralized with N KOH. If the color became too intense it was brought to pink with 0.1 N H_2SO_4; 0.161 cu.mm. of the Shaffer-Hartmann reagent was added and then a few drops of distilled water to augment the volume to about 1 cc. The test-tubes were shaken, capped with glass-bulbs, and heated without agitation in rapidly boiling water for 15 minutes. After cooling, the cap was removed only just before the contents of that tube were to be titrated and about 250 cu.mm. N H_2SO_4 introduced. The solid was completely dissolved with a glass-rod without undue agitation and the contents titrated with 0.01 N $Na_2S_2O_3$ until the color, due to the free I_2, became a very light yellow. About 35 cu.mm. of a 1 per cent aqueous solution of starch were added and the titration continued to the end-point. Titration was from a Linderstrøm-Lang-Keys microburette of 250 cu.mm. capacity, divided into cubic millimeters, and of uniform bore as shown by measurements of the length of a drop of mercury at all levels. The concentration of

inulin was ascertained by interpolation in a graph established from aqueous solutions containing a known quantity of the inulin (Pfanstiehl inulin, c.p.). Blanks and standards were run frequently. To obtain values with respect to the plasma, 5 per cent was deducted from the ascertained value, this being the approximate quantity of total solids in the whole blood of the crayfish and presumably close to the quantity which fell out by heating the blood at 80° C. The accuracy was within 5 per cent of the amount present.

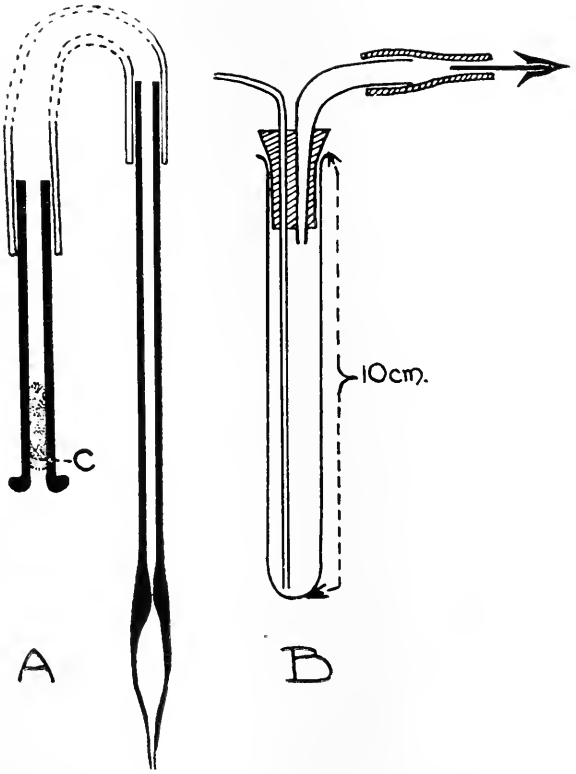

FIG. 1. *A*. Volumetric micro-pipette of the constricted type with mouth-piece and rubber tubing. *c*, cotton-plug. *B*. Apparatus for the collection of urine and emptying of bladders. Arrow indicates direction of suction.

The urine was treated in the same manner as the blood with the exceptions that heating and deproteinization were unnecessary, the urine being protein-free, and that, in the first 26 experiments, a correction for evaporation had to be applied (see below).

The preparation of inulin contained some impurity which, without being hydrolyzed, reduced Benedict's qualitative and which could not be

removed by yeast. The Pfanstiehl Company advised us that they had not been able to eliminate the impurity by repeated crystallization. The impurity was, however, negligible because within less than an hour after the injection of inulin, in the quantities used in this work (see Table II), the blood (whole or protein-free) was non-reducing unless subjected to acid-hydrolysis. Evidently the tissues removed the reducing substance rapidly. At no time could the urine reduce Benedict's reagent without preliminary acid-hydrolysis. Yeast-adsorption was therefore unnecessary in the analysis of inulin. Possibly because the animals were starved for a few days, the blood (whole or protein-free) of unsubjected animals was non-reducing even with preliminary acid-hydrolysis; the urine of these animals was invariably non-reducing. Crayfish can endure, without appreciable injury, starvation for four months at least (Brunow, 1911).

Inulin-clearance.—The renal clearance of a substance has a definite physiological meaning, being the virtual volume of blood cleared of that substance per unit time by the kidneys. It is expressed by $C = UV/P$, in which C is the clearance, P the concentration of the substance in the plasma, and V the rate of urinary flow. It is necessary to know the average concentration of that substance in the blood throughout the time that the urine to be analyzed is being formed.

Before an experiment, the crayfish was kept overnight fully submerged in running aerated freshwater. In measuring the inulin-clearance, the integument and branchial chambers were drained of moisture, the animal was weighed, and a fraction of a cc. of crayfish-saline [3] containing a given quantity of inulin injected slowly through the proximal abdominal venter and the wound cauterized. The amount of inulin injected was adjusted mainly by altering the concentration of the dissolved inulin in the saline because it was not desired to augment the blood-pressure by injecting a relatively large quantity of liquid (Table II). By thorough bleeding, the total quantity of blood in an average-sized *Cambarus clarkii* was found to be 6.6 per cent of the wet weight, which corresponds closely to the 6.7 of Herrmann (1931), who used the same method with *Potamobius astacus*.

After about 45 minutes the animal, including its branchial chambers, was drained of moisture and the anterior margins of the latter plugged with cotton-wool. The bladders were emptied by suction (about 12 mm. Hg) applied at the nephropores through the arrangement in Fig. 1, *B*.

[3] The saline was based on the most acceptable data on the concentration of inorganic electrolytes in the blood of the crayfish (see Maluf, 1940, for references) and was as follows (g./l.): NaCl, 7.81; $CaCl_2$, 1.31; $MgCl_2$, 0.82; KCl, 0.70; buffered at pH 7.6 by 0.5 cc. M/5 Na_2HPO_4/NaH_2PO_4. This assumes a $\triangle$ of about 0.66° C. (see Lienemann, 1938, and Schlatter, 1941).

When no urine could be obtained, firm bilateral digital pressure was applied to the integument lateral to the bladders (Maluf, 1941*a*) and suction again used until no further urine issued. The first sample of blood was taken immediately after and at what was considered zero time. The concentrations of inulin in blood from the pericardial sinus and from a leg were practically identical at that time, thus showing uniform distribution of the foreign material. The nephropores were cauterized to ensure a dry surface. "Ames Temporary [dental] Cement:

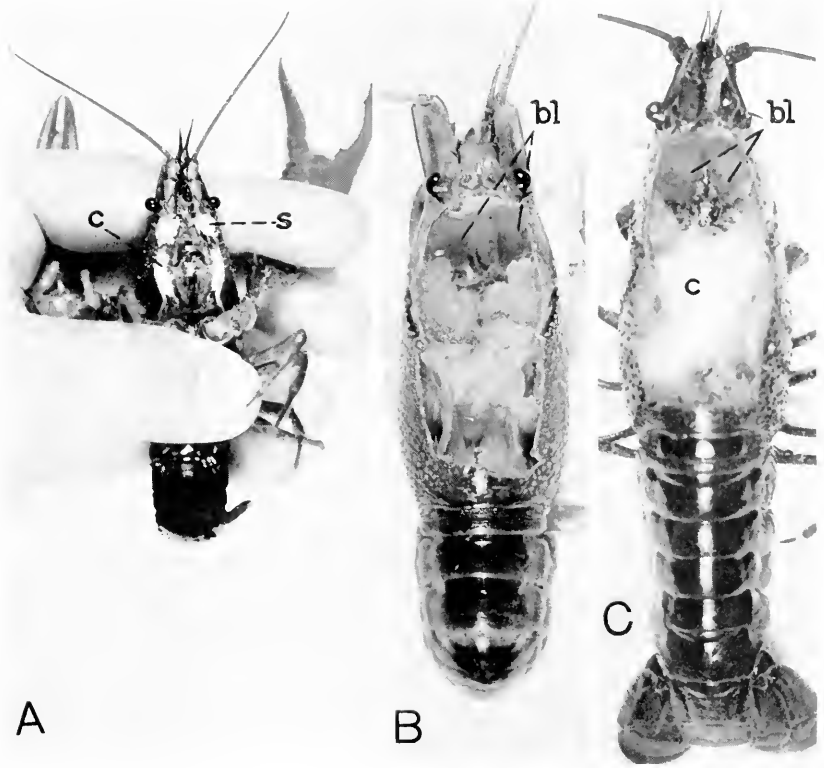

FIG. 2. *A*. Manner of handling the crayfish while the bladders are emptied and the nephropores are being sealed. *c*, cotton-plug in anterior margin of right branchial chamber; *s*, seal on the basal segment of the left antenna. *B*. Dorsal aspect of a crayfish with dorsal part of carapace and crop-gizzard removed, showing both bladders, *bl*, distended. *C*. Same as *B* but with viscera moved posteriorly and with wad of cotton, *c*, between viscera and bladders, *bl*.

hydraulic, non-irritant" was applied to the excretory eminence and basal segment of the antenna in two layers, under a magnification of 10.5 ×, by means of a forceps. The animal was handled as shown in Fig. 2, *A*. The cement should not extend beyond the basal antennal segment because movement of the antenna would be likely to crack the dried seal. Hard-

ening, which is due to the formation of zinc phosphate from zinc oxide and phosphoric acid, is rapid. Other cements were tried but were incomparably inferior. Ten minutes after completing the application the animal was fully immersed in freshwater and kept undisturbed.

Three or four blood-samples were taken through the experiment, which lasted 8 to 15 hours. Immediately after the last sample, the ventral nerve-cord was transected at the proximal level of the abdomen so as to prevent abrupt abdominal flexion, the chelipeds were amputated basally, and the dorsal surface of the carapace and the crop-gizzard, which is wedged over and between the bladders, were carefully removed. The distended bladders presented themselves conspicuously (Figs. 2, *B* and *C*, *bl*). The urine contained in the translucent bladders was crystal-clear and the kidneys could be seen beneath (Fig. 2, *C*). The viscera were then pushed back and a wad of absorbent cotton (Fig. 2, *C*, *c*) was applied over them to keep any fluid from flowing near the bladders. The urine was rapidly and completely collected by applying suction (about 12 cm. Hg) through the orifice of the arrangement shown in Fig. 1, *B* to the surface of each bladder. The animal was tipped on the side of collection with the head downward while this was done. The rate of urinary flow was thus accurately measured. The rapid collection obviated a correction for loss by evaporation. In the very few instances in which both bladders were not equally distended, the kidney corresponding to the lower rate of urinary flow was diminutive in size.

In the first 26 experiments on inulin-clearance the nephropores were not sealed because it was assumed that undisturbed animals with emptied bladders would not urinate appreciably during the interval. Urine, in these instances, was collected by suction from the nephropores and a correction applied for the fraction of water lost by evaporation. This is quite appreciable, and because previous investigators have not taken it into account the writer has no doubt that their values for the concentration of solutes in the urine of the crayfish are higher than the actual. The necessary corrections were obtained by aspirating, with the same pressure, a known quantity of distilled water, from the tip of a fine pipette of the constricted type (Fig. 1, *A*), into a test-tube of the same dimensions as that used for the collection of urine (Fig. 1, *B*). The resultant quantity of water, after light centrifugation of the test-tube for a few seconds, was measured by drawing it into a calibrated glass-tube. The loss in collecting 0.148 cc. in two minutes was 16.4 per cent and that in collecting 0.0739 cc. in five minutes was 35 per cent. The first correction was the one generally applied, as it was the writer's policy to collect the maximal quantity of urine in the minimum time with the above pressure. Generally an amount of 0.15 to 0.2 cc. could be

readily collected within two minutes. A successful and rapid collection depends to a great extent upon the aspirating tip. This should not have sharp edges but should be blunt and regular; its diameter should not be so large as to cover the entire excretory eminence. There is no doubt that the water lost was due entirely to evaporation. Scrupulous care was taken to prevent water-contamination of the urine, by draining the animal thoroughly, sucking water from the branchial chambers and rostral region, and plugging the anterior margins of the chambers with cotton-wool. The urine was not contaminated with blood, as was shown by negative biuret-, heat-, and H_2SO_4-tests, by an uninjured operculum, and by the fact that the concentration of inulin and dyes in the urine was considerably greater than in the blood (see below).

The rate of urinary flow was not measured in the first 26 experiments. It was assumed to be constant from one individual to another per unit weight, being determined, in a fully submerged animal, by the rate of diffusion of water into the body (Herrmann, 1931). Otherwise the rate of flow was measured by the above technique which necessitates sacrificing the animal at the end of the experiment. In ten experiments with inulin (Table I), the rate averaged 5.0 cc. per 100 grams per 24 hours. This is quite close to the average ($=5.2$) of Lienemann (1938), who collected the urine by aspiration from nephropores which had been sealed, and was taken as the rate of flow for the animals in the first 26 experiments. The rate of urinary flow in the crayfish is low as compared with the frog (Forster, 1940) and freshwater turtle (Friedlich, Holman, and Forster, 1940), and even relative to that in birds and a terrestrial reptile (Marshall, 1932). This emphasizes the low permeability of the gills of the crayfish to water.

The inulin-clearances and U/P's obtained through the use of direct measurements of urinary flow with sealed nephropores (Figs. 6 and 7, *inulin;* solid circles) and from an average rate of flow with unsealed nephropores (Figs. 6 and 7, *inulin;* open circles) are quite comparable.

The average concentration of inulin in the blood through the experimental period was secured by averaging the interpolated values at the mid-period of each hour (see curves representing concentration-time, Fig. 3). Three or four blood-samples were sufficient to establish the shape of the curves. Furthermore, it was undesirable to take more blood than necessary.

Xylose

The analysis of xylose was identical with that of inulin except that acid-hydrolysis was omitted.

The nephropores were sealed and the rate of urinary flow measured directly (see above and Table I).

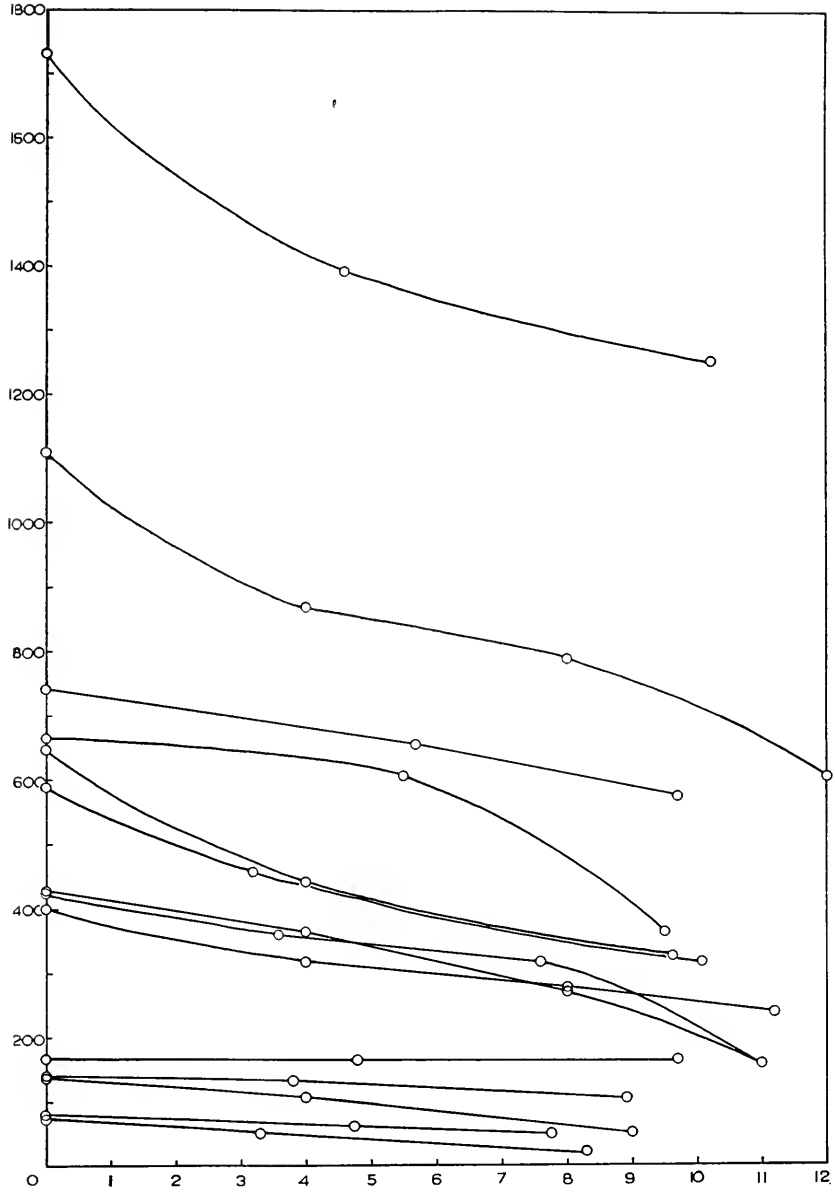

FIG. 3. The concentration of inulin in the blood in mg. per cent (ordinate) as a function of the time in hours (abscissa) during measurements of inulin-clearance.

Three blood-samples were sufficient (Fig. 4). The average concentration of xylose in the blood through the experimental period was calculated in the same way as for inulin.

Creatinine

Deproteinization of the serum was unnecessary because of the large dilution (about 26 ×). To 80 cu.mm. of serum or urine were added 2 cc. of distilled water. The tubes were capped and shaken and 1 cc. of the alkaline picrate was added to each. A Dubosque-type colorimeter with

TABLE I

Urinary flow in cc. per 100 grams per 24 hours.

No.	Male	Female	Material injected
1		3.4	0.3 cc. 10% inulin in crayfish-saline.
2		4.5	0.4 cc. 10% " " " "
3		3.3	1 cc. 20% " " " "
4		4.4	0.4 cc. 5% " " " "
5		6.1	0.4 cc. 5% " " " "
6	5.4		0.6 cc. 10% " " " "
7	6.4		0.6 cc. 10% " " " "
8		8.8	0.2 cc. 5% " " " "
9		4.4	0.2 cc. 5% " " " "
10	3.7		0.2 cc. 5% " " " "
11		5.4	1 cc. 30% xylose in ⅔ crayfish-saline.
12	7.2		1 cc. 30% " " " " "
13	7.9		0.3 cc. 10% xylose in dist. water.
14		8.85	0.2 cc. 10% " " " "
15	7.1		0.5 cc. 10% " " " "
16	4.6		0.5 cc. 10% xylose in crayfish-saline.
17		2.9	0.5 cc. 5% creatinine in crayfish-saline.
18	4.6		0.5 cc. 5% " " " "
19		5.9	0.2 cc. 5% " " " "
20		5.7	0.2 cc. 5% " " " "
21	7.6		0.5 cc. 10% creatinine in dist. water.
22		8.9	0.5 cc. 10% " " " "
23	2.9		0.5 cc. 15% creatinine (somewhat toxic) in dist. water.
24		3.1	0.5 cc. 15% " " " " " "

1-cc. cups was used. The light was passed through a green filter. Standards were made each time as expected. There was never as much as a 50 per cent difference between the samples and the standards. The blood of unsubjected animals did not give a positive Jaffé reaction.

The blood-curves were almost straight lines (Fig. 5). The average concentration of creatinine in the blood through the experimental period was calculated in the same way as for inulin.

The nephropores were sealed and the rate of urinary flow measured directly (see above and Table I).

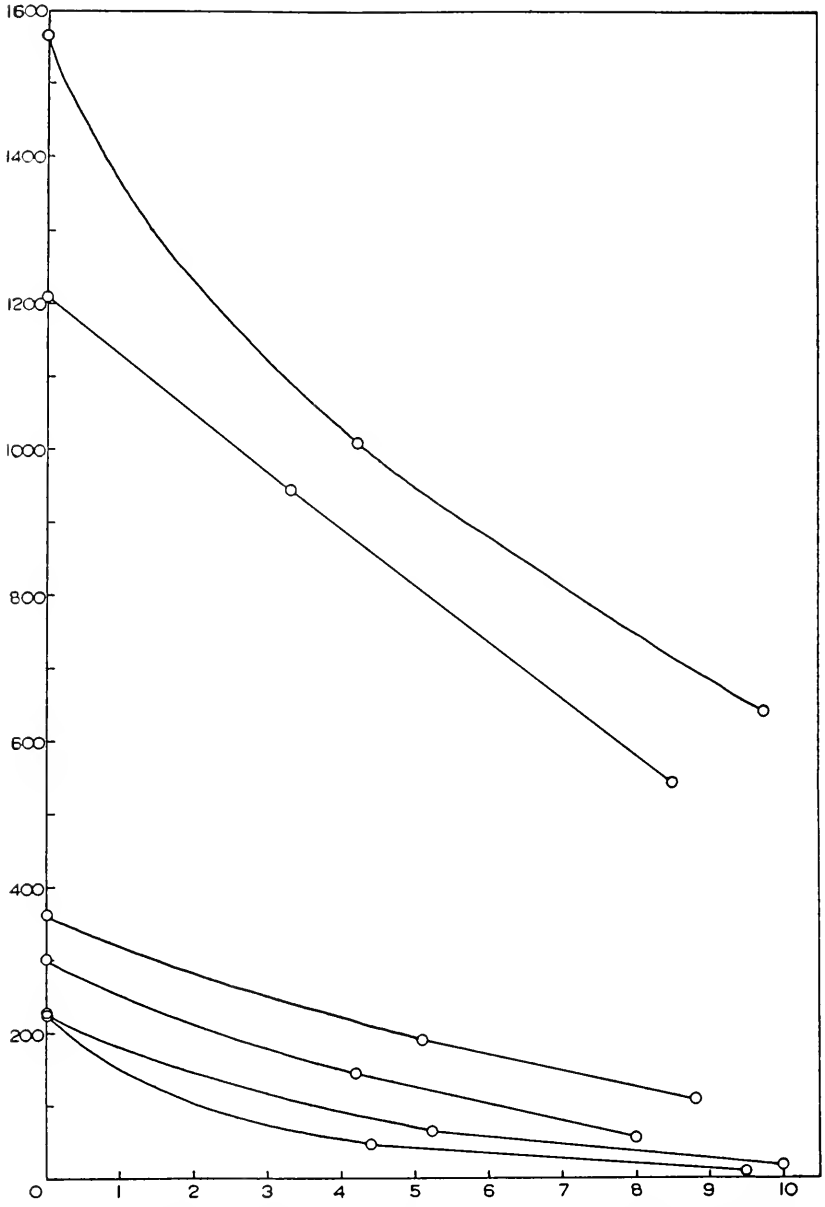

FIG. 4. The concentration of xylose in the blood in mg. per cent (ordinate) as a function of the time in hours (abscissa) during the measurements of xylose-clearance.

Results

Excretion of Inulin

In all the preliminary experiments, the injection of 0.2 cc. 5 per cent inulin in crayfish-saline resulted in a renal output of inulin. Thus, the urine, which was collected by suction from the nephropores, gave a positive result with Benedict's qualitative only after acid-hydrolysis. Before the introduction of inulin the urine was non-reducing even with acid-hydrolysis.

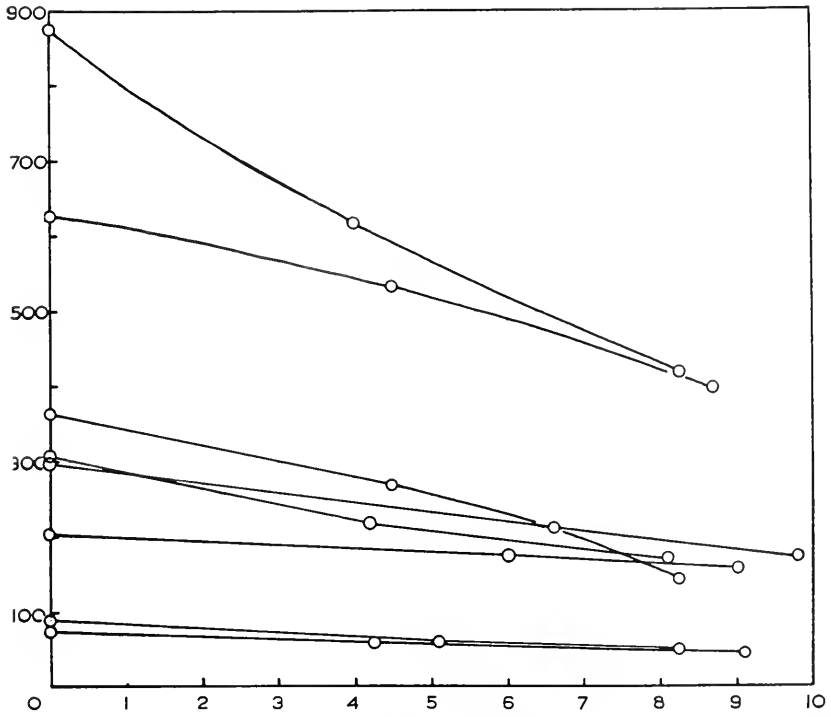

Fig. 5. The concentration of creatinine in the blood in mg. per cent (ordinate) as a function of the time in hours (abscissa) during the measurements of creatinine-clearance.

Similar results followed the injection of xylose or glucose. To guard against possible glucose-contamination in the sample of xylose, the urine was shaken for a few minutes with an equal quantity of a 20 per cent suspension of washed yeast in distilled water and centrifuged. Blanks, with only the yeast-centrifugate, were non-reducing. The glucose was given in high concentration (0.6 cc. 70 per cent per 40 grams) because the tissues tended to remove it from the blood.

Because glucose, xylose, and inulin are excreted by the kidney of the crayfish and not by the vertebrate aglomerular kidney, it at first seemed that filtration occurs in the former. This might imply that the hypotonicity of crayfish-urine is produced as in the Amphibia, namely, by the formation of a protein-free filtrate at the proximal end of the nephron and by subsequent resorption of relatively more salts than water by the tubule. On the other hand, other important data (see Discussion) contra-indicate filtration.

Because inulin is neither secreted by nor passively resorbed through the vertebrate nephron, the inulin-clearance in this phylum is an unvarying function of the concentration of inulin in the plasma. This is true

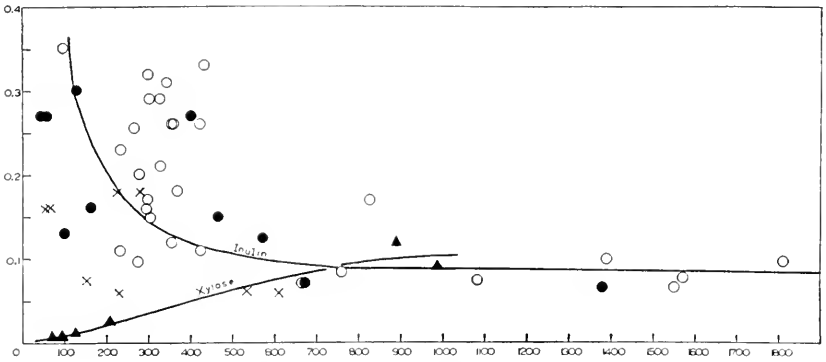

FIG. 6. The renal clearance of inulin, xylose, and creatinine in cc. per hour (ordinate) as a function of the concentration of these compounds in the plasma in mg. per cent (abscissa). *Solid circles,* inulin-clearances with direct measurement of urinary flow and nephropores sealed; *open circles,* inulin-clearances without direct measurement of urinary flow and nephropores not sealed; *triangles,* xylose-clearances with direct measurement of urinary flow and nephropores sealed; *crosses,* creatinine-clearances with direct measurement of urinary flow and nephropores sealed. Each point stands for a single separate animal.

even at the low plasma-concentrations (Miller, Alving, and Rubin, 1940). To find whether secretion can account for the marked occurrence of inulin in the urine of the crayfish, a study was made of the inulin-clearance at various levels of inulin in the plasma. Variation of the renal clearance with the plasma-concentration would demonstrate secretion. Parenthetically, even if the renal clearance of a substance does not vary with its plasma-concentration, secretion is not theoretically excluded (Shannon, 1938, 1939).

The actual inulin-clearance: plasma-inulin relationship (Fig. 6, *inulin*) demonstrates an outward secretion of inulin. The U/P: plasma-inulin curve (Fig. 7, *inulin*) is similar and the U/P's were above unity. Because the renal clearance is a product of the U/P and rate of urinary

flow, the approximate identity in the curves of Figs. 6 and 7 is equivalent to stating that the rate of urinary flow tends to be constant among different individuals.

It should be pointed out that the wet weight of both kidneys is normally a direct rectilinear function of the wet weight of the crayfish, at least in animals weighing between 10 and 50 grams. The relationship is expressed by $y = 0.0026x$, in which y is the mass of both kidneys and x the mass of the entire animal. Because the inulin-secreting mass of the kidney is probably a direct function of the total mass of the kidney, all animals should be approximately the same weight in an ideal set of experiments. In this investigation, because the lower plasma-concentrations were by no means confined to the larger animals (Table II), size, within the experimental range, cannot have been a determining factor in the inulin-clearance : plasma-inulin relationship. This is further brought out by the fact that the variation in the inulin-clearance with the concentration of inulin in the plasma, in crayfish which range between average and large size, is determined practically entirely by the U/P (Figs. 6 and 7, *inulin*) and not by the volume of urine excreted, which is greater in the larger animals although fairly constant per unit weight. In other words, the hourly differences in the absolute rate of urinary flow among individuals of somewhat different size are relatively small and inconsistent as compared with the variation of the U/P with the concentration of inulin in the plasma. Assuming a constant concentration of inulin in the plasma, the inulin-clearance ($= UV/P$) would doubtless vary with the mass of the kidney, but the U/P probably would not because the inulin-secreting mass of the kidney may bear a constant value with respect to the water-secreting mass. This implies that while the large kidney would secrete more inulin than the small one, it would also secrete proportionally more water.

The shape of the U/P : plasma-inulin curve indicates that the renal cells asymptotically become functionally saturated with inulin as the plasma-level of this compound rises. If filtration does not occur one would expect that, at *extremely* low concentrations of inulin in the plasma, the U/P's would be less than unity because there would be very little inulin available to the renal cells within a given interval of time. Apparently because of the relatively high avidity of the renal cells for inulin, it was not practicable to measure inulin-clearances at extremely low plasma-levels; sufficient urine was not formed before the blood was freed from inulin. With xylose, U/P's below unity occur even at moderate plasma-levels. It is possible that, at moderate concentrations, the kidneys secrete relatively more inulin than water and that the reverse is true for xylose.

Attempts were made to locate the site of inulin-secretion in the nephron by the colorimetric method of Alving, Rubin, and Miller (1939). About 0.8 cc. 10 per cent inulin in crayfish-saline were injected into

TABLE II

Excretion of Inulin

No.	Wgt. in g. and sex	Inulin		Urinary flow	Amt. of crayfish saline and conc. of inulin injected	Duration of experiment
		Plasma	Urine			
		mg. per cent	*mg. per cent*	*cc./hr.*		*hrs.*
1	42.7 ♀	433	1,590	0.088	0.6 cc. 10%	12.5
2	30.0 ♀	345	1,716	0.062	0.25 cc. 10%	11.5
3	27.8 ♀	355	1,600	0.058	0.27 cc. 10%	13.75
4	42.5 ♀	1,810	1,970	0.088	1 cc. 20%	14.25
5	34.2 ♂	1,390	1,974	0.072	1 cc. 20%	14.75
6	31.0 ♂	99	540	0.064	0.2 cc. 5%	13.5
7	25.7 ♂	234	468	0.054	0.2 cc. 5%	13.7
8	33.0 ♀	1,086	1,246	0.068	0.75 cc. 10%	12.5
9	42.7 ♂	235	578	0.090	0.4 cc. 5%	13
10	44.8 ♂	296	494	0.094	0.4 cc. 5%	12.6
11	33.4 ♂	758	883	0.070	0.5 cc. 10%	11.3
12	23.1 ♀	276	550	0.048	0.17 cc. 10%	11.5
13	51.1 ♂	304	825	0.106	0.38 cc. 10%	11
14	37.3 ♂	280	742	0.076	0.28 cc. 10%	13
15	51.7 ♂	356	841	0.108	0.45 cc. 10%	12
16	26.0 ♀	359	825	0.054	0.26 cc. 10%	12.3
17	28.0 ♂	299	858	0.058	0.28 cc. 10%	12.4
18	25.0 ♀	1,568	2,350	0.052	0.5 cc. 20%	11.6
19	23.0 ♀	1,549	2,130	0.048	0.5 cc. 20%	12
20	46.5 ♂	307	494	0.096	0.65 cc. 10%	14
21	48.5 ♂	425	462	0.10	0.68 cc. 10%	12.5
22	27.6 ♀	830	1,155	0.12	0.7 cc. 10%	12
23	21.7 ♀	330	718	0.094	0.2 cc. 10%	11
24	25.0 ♂	330	882	0.108	0.3 cc. 10%	10.6
25	20.4 ♀	300	1,090	0.088	0.4 cc. 10%	11.2
26	21.4 ♂	370	1,156	0.092	0.4 cc. 10%	11.2
27	28.0 ♀	670	1,180	0.040	0.3 cc. 10%	9.7
28	33.5 ♀	574	1,160	0.06	0.4 cc. 10%	9.5
29	30.3 ♀	1,380	2,220	0.042	1 cc. 20%	10.2
30	26.5 ♀	166	530	0.049	0.4 cc. 5%	9.8
31	29.5 ♂	131	520	0.075	0.4 cc. 5%	8.9
32	47.3 ♂	467	720	0.106	0.6 cc. 10%	9.7
33	32.0 ♂	424	1,250	0.089	0.6 cc. 10%	10.6
34	23.8 ♀	60	192	0.088	0.2 cc. 5%	7.75
35	29.5 ♀	102	240	0.054	0.2 cc. 5%	9
36	42.0 ♂	45	180	0.065	0.2 cc. 5%	8.3

medium-sized animals. After about three hours the kidneys were removed, rinsed in saline, and the coelomosac, tubule, and labyrinth teased apart. Approximately equal amounts of coelomosac, labyrinth, and tubule were put into separate small test-tubes. To each was added 1

cc. of the freshly prepared diphenylamine reagent. The tubes were capped and put into a boiling water bath for six minutes. The color which developed at the end of this time was evidently maximal. Unaided visual examination of the intensity of color did not indicate any differences in the amount of inulin present in the tubes. The intensity was determined solely by the mass of tissue used.

Regardless of whether a substance is removed from the blood by extrarenal tissues, the *renal* clearance of the substance will be a function of the concentration of that substance in the blood. It was nevertheless of interest to find if inulin can be hydrolyzed by the tissues of the cray-

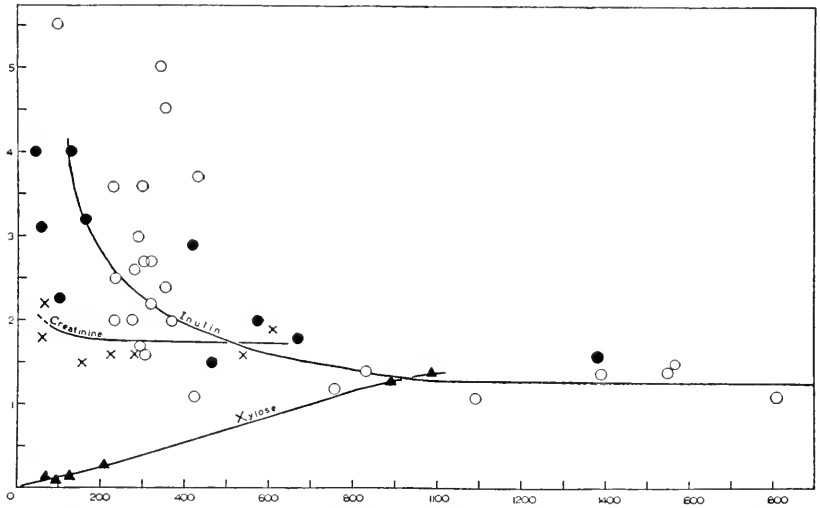

FIG. 7. The *U/P* ratio of inulin, xylose, and creatinine (ordinate) as a function of the concentration of these compounds in the plasma (abscissa). Notations identical with those in Fig. 6. Each point stands for a single separate animal; the same as those in Fig. 6.

fish. The kidneys and samples of the hepatopancreas, somatic muscles, and blood were frozen in solid carbon dioxide, thoroughly macerated, and extracted in a known quantity of saline. To aliquots of the centrifugates were added a solution of inulin and a small drop of xylol. The mixtures were analyzed for inulin immediately and after 13 hours at room temperature. The controls contained only a solution of inulin and the preservative. There was no change in the concentration of reducing carbohydrate, with or without acid-hydrolysis, in any tube. This indicates that, under the conditions of the experiments at least, inulin is not hydrolyzed by the tissues of the crayfish. Similar experiments showed a destruction of d-xylose in the following descending

order: hepatopancreas, kidneys, somatic muscles, blood. This may explain how the concentration of xylose in the blood falls more rapidly than that of inulin (Figs. 3 and 4) even though the renal xylose-clearances (see below) are lower than the inulin-clearances. Xylose may also diffuse out through the gills.

Excretion of d-Xylose

The xylose-clearance varies directly with the concentration of xylose in the plasma (Fig. 6, *xylose*) and at moderately low plasma-levels the *U/P*'s are well below unity (Fig. 7, *xylose*). Assuming the occurrence of filtration and resorption, this relationship may be explained by an incapacity of the nephron to resorb xylose beyond a maximal rate; as a

TABLE III

Excretion of d-Xylose

No.	Wgt. in g. and sex	Xylose		Urinary flow	Remarks
		Plasma	Urine		
		mg. per cent	*mg. per cent*	*cc./hr.*	
1	29.6	988	1,370	0.067	1 cc. 30% xylose in ⅔ crayfish-saline; 9.75 hr. duration.
2	38.1	890	1,120	0.115	1 cc. 30% xylose in ⅔ crayfish-saline; 8.5 hr. duration.
3	31.5	89	10	0.10	0.3 cc. 10% xylose in dist. water; 10 hr. duration.
4	22.5	64	10	0.083	0.2 cc. 10% xylose in dist. water; 9.5 hr. duration.
5	33.6	210	61	0.10	0.5 cc. 10% xylose in dist. water; 8.8 hr. duration.
6	34.5	127	19	0.066	0.5 cc. 10% xylose in crayfish-saline; 8 hr. duration.

consequence, an increasing amount would "spill over" as the plasma-level is raised. Because at low plasma-concentrations the *U/P* is below unity (Fig. 7, *xylose*) the resorption would presumably be active, i.e. xylose would be inwardly secreted. There is a resemblance to the handling of glucose and other threshold-substances by the mammalian kidney.

On the other hand, the process can be readily explained, without resort to filtration, by assuming that both xylose and water are outwardly secreted and that, at low plasma-levels of xylose, the rate of secretion of water is relatively large compared with the secretion of xylose. At moderately high plasma-levels the xylose-clearance is nearly identical with the inulin-clearance (Fig. 6). In the experiments which necessitated the introduction of sufficient xylose to raise the average

plasma-concentration to about 1000 mg. per cent (see Table III), the animals became torpid soon after the injection but recovered completely within several minutes. It was therefore not considered within the scope of a physiological experiment to measure xylose-clearances at still higher plasma-levels. The injurious effects are probably osmotic. Inulin was not toxic even at the high concentrations.

For the same reason as with inulin, the xylose-clearance : plasma-xylose curve is practically identical with the U/P : plasma-xylose curve (Figs. 6 and 7, *xylose*).

TABLE IV

Excretion of Creatinine

No.	Wgt. in g. and sex	Creatinine		Urinary flow	Remarks
		Plasma	Urine		
		mg. per cent	*mg. per cent*	*cc./hr.*	
1	25.5 ♀	230	460	0.031	0.5 cc. 5% creatinine in crayfish-saline; 9.8 hr. duration.
2	26.5 ♂	155	240	0.051	0.5 cc. 5% creatinine in crayfish-saline; 9 hr. duration.
3	30.0 ♀	65	140	0.074	0.2 cc. 5% creatinine in crayfish-saline; 9.1 hr. duration.
4	39.0 ♀	60	105	0.093	0.2 cc. 5% creatinine in crayfish-saline; 8.25 hr. duration.
5	34.7 ♂	280	450	0.112	0.5 cc. 10% creatinine in dist. water; 8.25 hr. duration.
6	32.0 ♀	225	350	0.118	0.5 cc. 10% creatinine in dist. water; 8.1 hr. duration.
7	33.8 ♂	537	850	0.040	0.5 cc. 15% creatinine in dist. water; 8.7 hr. duration. (Somewhat toxic.)
8	25.5 ♀	610	1,150	0.033	0.5 cc. 15% creatinine in dist. water; 8.25 hr. duration. (Somewhat toxic.)

Excretion of Creatinine

Because the inulin- and creatinine-clearances are identical in certain vertebrates at all plasma-levels, it was desirable to compare the same clearances in the crayfish. The results were not elucidating and are presented here merely for record because it is believed that they are accurate (Figs. 6 and 7, *creatinine;* Table IV). Plasma-concentrations higher than 900 mg. per cent were definitely injurious if not fatal. The maximal ones on record are just within the threshold of toxicity, judging from the activity of the animal.

Excretion of Dyes

The initial objective of the experiments under this heading was to find if the nephron of the crayfish is capable of eliminating dyes which

the vertebrate aglomerular kidney is incapable of excreting. It was also desirable to study the capacities of the different parts of the nephron to secrete or accumulate various kinds of dyes.

The dyes were dissolved in crayfish-saline immediately before use. A description of the chemical composition of most of the dyes can be found in Conn's (1925) monograph.

Cyanol (DuPont).[4]—This is an aniline dye giving an intense blue in solution even when very dilute. Cyanol is not eliminated by the aglomerular vertebrate kidney if given in doses of the order of several mg. per kg. (Höber, 1930) but is slightly excreted when in quantities of 125–300 mg./kg. (Marshall and Grafflin, 1932).

Immediately after emptying the bladders, a fraction of a cc., containing a dose of about 1.7 mg./kg., was injected through the proximal abdominal venter. This colored the blood a vivid blue. Urine was collected after five hours and had to be diluted about tenfold to bring the intensity of color down to that of blood taken only one hour after the injection. Within five hours the blood lost all trace of blue. The experiment was repeated with similar results. As stated above, the concentration of foreign material, one hour after injection, is about equal in blood taken from the legs as in that from the pericardial sinus.

Other subjects were opened one to two hours after the injection. The viscera were rinsed with saline. Cyanol was not found in any organ other than the labyrinthic epithelium. The intensity of blue in the labyrinth not only greatly exceeded that of blood at the time but even that of blood taken only twenty minutes after the injection. The dye did not stain the bladder nor diffuse out from the contained urine even at a time, five or six hours after the injection, when it was absent from the blood.

The accumulation of cyanol in the labyrinth cannot be considered due to a resorption of water by the labyrinth, from a filtrate conceivably formed at the coelomosac, because: (1) The dye is greatly concentrated in the labyrinthic cells and yet not appreciably apparent in the more distal parts of the nephron; (2) the labyrinthic cells, even in "living" hanging-drop preparations, indicate a marked outwardly secretory activity as shown by the frequent presence of globules apparently being pinched off toward the lumen: the labyrinth therefore can scarcely be considered as a water-resorbing organ from a cytological standpoint; (3) the data indicate that the coelomosac is a secretory organelle (Maluf, 1941*a*, and below).

Ferrocyanide.—Iron salts, such as ferric ammonium citrate and sodium ferrocyanide, are not excreted by the aglomerular vertebrate ne-

[4] Kindly supplied me by Professor E. K. Marshall, Jr.

phron (Marshall and Grafflin, 1932) but are filtered through the glomerular nephron of vertebrates (see Smith, 1937).

Both bladders were emptied and 0.5 to 1.2 cc. of 2.4 per cent sodium ferrocyanide injected into animals weighing from 27 to 48 grams. The Prussian blue color was developed by adding a known quantity of Folin's (1929) ferric sulfate reagent to the Na_2WO_4–H_2SO_4 protein-free blood-centrifugate. At the end of five hours a scarcely appreciable quantity of urine could be collected, which gave a Prussian blue test. The ferric sulfate reagent produced an intense blue throughout the teased nephron; the color was more intense than that of the blood taken only 0.5 hour after the injection and seen through the same or greater depth. This experiment indicates that the kidney is capable of accumulating ferrocyanide but that the cells apparently become too poisoned to secrete urine. The hepatopancreas, muscles, and alimentary tract, rinsed free from blood and teased apart, gave no reaction.

Phenol Red.—Phenol red is secreted by the aglomerular teleost kidney (Marshall and Grafflin, 1932). The bladders of the crayfish were emptied immediately before the injection of the dye. The dose was 1 cc. of 34 mg. per cent phenol red into animals weighing about 30 grams. To develop the maximal intensity of color, both urine and blood were either exposed to NH_3 or received a known quantity of NH_4OH. The urine, collected five hours after the injection, had to be diluted over ten-fold to reduce its intensity to that of blood taken 20 minutes after. On examining the kidneys in situ five hours after the injection, only the posterior part of the labyrinth had a reddish tinge. On adding a drop of 0.1 N NaOH to the nephron in crayfish-saline, the whole labyrinth became an intense red which was even deeper than that of blood taken as early as 0.5 hour after the injection. The labyrinth is thus capable of secreting phenol red and the pH of its cells is evidently about 7.0. Other tissues, including the coelomosac and nephric tubule, after being briefly rinsed from blood, showed no trace of phenol red.

The urine, as it issued from the nephropore, was a clear orange-red, not the purple-red of maximal intensity, and therefore has a pH of about 7.5.

Neutral Red.—In the three animals studied (dose: 1.2–1.8 cc. 80 mg. per cent per 30 grams) there was no indication of a concentration of this dye in the urine. The dye penetrated the labyrinth and tubule but the coelomosac did not show a trace of it. There seemed to be some accumulation in the hepatopancreas as the color was more intense in this organ (on adding a drop of acetic acid) than in the blood of equal depth. As the urine issues from the nephropore it is a light yellow and turns pink on the addition of acid. This shows that its pH is greater than 7.4.

It has already been noted that the phenol red experiments indicate a pH of about 7.5.

"Indigo Carmine."—Indigo carmine is composed of carmine blue and indigo disulfonate. The sample used had been passed by The Commission on Standardization of Biological Stains. It is long-known that indigo disulfonate is outwardly secreted by the vertebrate tubule. The dose was 0.7 cc. 80 mg. per cent per ca. 30 grams. Four hours after the injection the dye was markedly more concentrated in the bladder-contained urine than in blood even when collected only 25 minutes after the injection. On examination of the kidneys, no dye was found in the coelomosac or distal portion of the tubule. In one example concentrated dye was seen to leave the lumen of the proximal portion of the tubule upon application of pressure to the labyrinth, but there was no indication that the cells of the tubule take up the stain. The dye was concentrated in irregular patches in the labyrinth especially at the posterior end. There was no trace of it in the hepatopancreas and other tissues.

Congo Red.—Six-tenths of a cc. of 160 mg. per cent Congo red was injected into a 31-gram animal. Blood taken forty minutes later was a very light pink. The kidney was examined four hours after the injection; the coelomosac was a deep pink but the dye was absent from the rest of the nephron, and from the hepatopancreas, muscles, and gut.

"Basic Fuchsin" (aniline red; diamond fuchsin R.F.N.; magenta; passed by the C.S.B.S.).—The dose was 0.75 cc. 80 mg. per cent 30 grams. The animal was opened four hours after the injection. The stain had penetrated the muscles, hepatopancreas, coelomosac, nephric tubule, and other tissues. As compared with the blood, it was concentrated only in the labyrinth where it was a very intense purple. Soon after the injection the animals lay on their side in semi-torpor but recovered completely and removed all traces of dye from the blood.

Acid Fuchsin.—Eight-tenths of a cc. of 80 mg. per cent dye was injected into a 40-gram animal. The kidneys were examined after about 4.5 hours, at which time the dye was more concentrated in the bladders than in blood taken even 35 minutes after the injection. The labyrinth was a more intense pink than the blood seen through the same depth. The nephric tubule, coelomosac, hepatopancreas, muscles, and gut were not stained.

Methylene Blue (Passed by the C.S.B.S.).—Five-tenths of a cc. 50 mg. per cent were injected into a 22-gram animal. A blood-sample, taken after about two hours, was a very light blue. The animal was opened after about five hours. Methylene blue was concentrated only in the proximal portion of the tubule, where it existed as intracellular granules. After fixing the fresh kidney in 20 per cent formalin, the blue

concretions disappeared and the proximal portion of the tubule became a uniform blue. Evidently the intracellular granular condition depends upon an active process. The hepatopancreas and gills, but not the muscles, were merely stained. The experiment was repeated with identical results.

Colloidal Carbon.—Five-tenths of a cc. of "Higgins American India Ink: waterproof, black," diluted 6 × with crayfish-saline, was injected into a 20-gram animal. This was sufficient to give a very dark brown color to the blood. The animal was opened after four hours and the organs rinsed in situ with saline. The colloid had not penetrated any tissue. This is a functional demonstration of the absence of a nephrostome.

Discussion

The primary question is whether filtration occurs through the nephron of the crayfish. The paper of Bethe, von Holst, and Huf (1935), which appears to furnish positive evidence for filtration, should be read with care, especially as certain investigators have taken their results at face-value. Bethe et al. augmented the internal hydrostatic pressure of the crab, *Carcinus maenas,* by a vertical column of saline which communicated with the haemocoele. The aqueous column then sank in abrupt steps, indicating a fall in the internal pressure. They stated that this is evidently a physiological event because raising the hydrostatic pressure after death resulted in only a slight fall of the column which they attributed to an expansion of the soft membranes of the integument. They also pointed out that if the crab dies during the experiment the column of saline either does not fall or sinks very slowly. The animals, which were observed in air, were stated to have shown a loss of fluid from three sites: (1) the gill-chambers; (2) the mouth; and (3) the nephropores. These investigators noted that the fluid from the gill-chambers contained protein but was cell-free; the writer thinks that this fluid may have issued partly from the mucus-secreting glands. Above all, the authors explicitly remarked that, during the fall of the aqueous column, generally no loss of liquid by way of the nephropores could be observed. Their suggestion that the kidneys of the crab regulate the internal hydrostatic pressure per se, i.e. even when the osmotic pressure does not vary, is therefore unfounded. As noted above, augmentation of the blood-volume by about one-third apparently does not increase the rate of urinary flow.

By measuring the oncotic pressure of the blood and the haemocoelic pressure of crayfish, Picken (1936) indicated that filtration is apparently possible.

The writer is not aware of any facts which can be taken as positive evidence for the filtration-resorption theory or against the absence of filtration and the outward secretion of liquid. Analogy with the vertebrate nephron is inadequate. Furthermore, outward secretion of liquid is known to occur in aglomerular fish (Marshall, 1930; Bieter, 1931). The urine of the latter is, like that of the crayfish, hypotonic to the blood. It is unknown, however, whether the hypotonicity of the urine of aglomerular forms is due to an outward secretion of a hypotonic liquid or to the elimination of an iso- or even hypertonic liquid, in the proximal part of the nephron, followed by a resorption of salts. Owing to phylogenetical reasons (see Marshall, 1934), the latter method does not appear probable.

There are several facts which indicate that the nephron of the crayfish is primarily if not entirely an organ of outward secretion:

1. There is no tenuous syncytium such as the glomerular capsule of the vertebrate nephron (Maluf, 1939, 1941*a*).

2. Large calcareous concretions sometimes occur in the lumen of the coelomosac, the most proximal organelle of the nephron, thus indicating that the coelomosac can secrete calcium (Maluf, 1941*a*). The coelomosac is also capable of accumulating Congo red (see above).

3. Experimental cytological evidence indicates an outward secretion of water by the distal half of the tubule (Maluf, 1941*b*).

4. Histologically there is no doubt that the labyrinth secretes material outwardly (Maluf, 1939). The labyrinthic cells are capable of accumulating and outwardly secreting cyanol, phenol red, indigo carmine, basic fuchsin, and acid fuchsin (see above).

5. The cells of the proximal portion of the tubule can accumulate methylene blue (see above). All parts of the nephron are therefore capable of secreting or accumulating one dye or another.

6. Inulin is outwardly secreted (see above).

7. From a teleological viewpoint the coelomosac is evidently not a filtration-organelle (Maluf, 1941*a*).

8. Injecting into a moderate-sized crayfish 1 cc. of crayfish-saline, i.e. a volume about one-third that of the initial blood-volume, and thus very probably increasing the internal hydrostatic pressure, does not augment the rate of urinary flow (Table I).

The Malpighian tubule of insects, as a result of physiological and cytological evidence, probably should be considered as an entirely secretory nephron. The beautiful live preparations of Wigglesworth (1931*a*, *b*, *c*) show that the Malpighian tubule can excrete fluid, in an apparently normal way, even under conditions when the hydrostatic pressure is zero.

The ingenious experiments of Patton and Craig (1939) show that the Malpighian tubule can absorb various isotonic salines isosmotically even when the hydrostatic pressure must be zero (the saline rose into the capillary gauge up to 10 to 15 mm. admittedly by capillarity). They also state that hydrostatic pressure does not cause an increase in "filtration" rate. It is not apparent to the writer why Patton and Craig assumed that the isosmotic uptake of solution by the Malpighian tubules is due to filtration. It is known that the alimentary epithelium of vertebrates absorbs solutions isosmotically and, at the same time, absorbs, selectively, ions of a particular species.

Kowalevsky (1889), Cuénot (1895), and Bruntz (1904) studied the affinity of the crustacean nephron, in situ, for ammonium carminate, indigo carmine, and certain other dyes. They did not indicate, however, whether the dyes were concentrated by the nephron because they made no statements as to the relative intensity of dye in the blood and urine. Kowalevsky and Bruntz noted that ammonium carminate and litmus stain the coelomosac but not the rest of the nephron while indigo carmine stains the tubule and labyrinth. Because the coelomosac stained red with litmus, Kowalevsky concluded that this organelle has an acid reaction. He also observed that the coelomosac, and not the labyrinth, has an affinity for Congo red and methylene blue. Cuénot believed that the labyrinth of the crayfish, lobster, and crabs has a *strongly alkaline* reaction (italics his) because it "energetically decolorised acid fuchsin"; the color reappeared on macerating the kidney in acetic acid. He also noted that alizarin violet (an alkaline dye) retains its color instead of going into the orange-red phase. I have found, on the other hand, that the labyrinth is capable of concentrating acid fuchsin and that treating the nephron with acetic acid does not augment the intensity of color. The dye was more concentrated in the urine than in the blood.

The present observations with the pH indicators, phenol red and neutral red, show that the cytoplasmic pH of the labyrinthic cells is about 7.0 and that the pH of the bladder-contained urine is about 7.5. Because the labyrinth will take up an acid dye, such as indigo carmine, is no reason to believe that its cells are basic. The uptake of dyes during life is not equivalent to the affinity of fixed dead tissues for dyes. This is a distinction which Kowalevsky and Cuénot did not make.

The statements of Kowalevsky and Cuénot that the labyrinth is alkaline led the writer (1938) to suggest that the nitrogenous products of protein-catabolism are outwardly secreted by the labyrinth. The facts that the labyrinth is not alkaline and that the concentration of the N–P–N is markedly lower in the urine than in the blood (see Delaunay, 1927 and 1931, for the crabs *Maia squinado* and *Cancer pagurus;* the crayfish has

not been studied with this regard) have greatly weakened that supposition. It should be borne in mind that *M. squinado* and *C. pagurus* are marine crabs without nephric tubules and eliminate a urine isotonic with their blood. It is therefore unlikely that the N–P–N is subjected to dilution by an outward secretion of water.

Because the main nitrogenous excretory product of the Crustacea is a highly diffusible substance,—ammonia (Delaunay, 1927, 1931), it seems probable that this escapes largely through the gills. Although we possess data on the over-all rate of ammonia-output by the crayfish (*Potamobius astacus;* see Brunow, 1911), there is no statement in the literature concerning the concentration of ammonia in the urine; consequently the rate of output of ammonia by the renal route is unknown. Partly because the concentration of ammonia is practically the same in the urine as in the blood of the above-mentioned crabs, it is possible that the existence of ammonia in the urine is merely due to diffusion.

Summary

1. The techniques of measuring the rate of urinary flow and of collecting urine are described. The collection of urine from the nephropores by suction is a satisfactory procedure provided a correction is applied for the water lost by evaporation.

2. The techniques of collecting blood and of measuring renal clearances in the crayfish are described.

3. Raising the internal volume by one-third and therefore, presumably, augmenting the internal hydrostatic pressure, by the injection of 1 cc. of crayfish-saline, does not increase the rate of urinary flow.

4. Inulin and xylose will appear in the urine after being injected into the haemocoele. Glucose will occur in the urine provided enough is injected to permit its existence in the blood for a sufficient period.

5. The inulin-clearance and the U/P ratio of inulin vary inversely with the concentration of inulin in the blood. This demonstrates that inulin is secreted.

6. Inulin is not hydrolyzed by the hepatopancreas, kidneys, somatic muscles, or blood.

7. At low plasma-levels, the U/P ratios of xylose are very much below unity but rise above unity at high plasma-levels. This shows that xylose is either actively resorbed from a filtrate or is outwardly secreted but, with the low plasma-levels, at a relatively low rate compared with the secretion of water. The xylose-clearance : plasma-xylose curve is practically identical in shape with the U/P : plasma-xylose curve.

8. Although the renal clearances of xylose are much lower than the renal clearances of inulin, the plasma-concentration of the monosaccha-

ride falls more rapidly than that of the polysaccharide. This may be partly because the tissues can destroy xylose.

9. Only the labyrinthic cells can accumulate and outwardly secrete cyanol, phenol red, indigo carmine, basic fuchsin, and acid fuchsin. The coelomosac, but not the labyrinth or tubule, can accumulate Congo red. These dyes cannot accumulate in, and apparently do not penetrate into, other tissues of the body.

10. Only the cells of the proximal half of the tubule accumulate methylene blue.

11. Colloidal carbon does not enter the kidney; this is functional proof of the absence of a nephrostome.

12. The cytoplasmic pH of the labyrinthic cells is about 7; the pH of the bladder-contained urine is about 7.5.

13. The available facts (histological, chemical, physiological, and phylogenetical) indicate that the nephron of the crayfish is primarily if not entirely an organ of outward secretion.

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ACTIVATION OF CUMINGIA AND ARBACIA EGGS BY BIVALENT CATIONS [1]

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The activation of unfertilized eggs by isotonic salt solutions was first described by R. S. Lillie (1910, 1911*a*, 1911*b*). Since then, the activation by isotonic salt solutions of the eggs of seven marine invertebrates, belonging to three different orders, has been reported; *Pomatoceros* by Hörstadius (1923), *Asterias* by Dalcq (1924*a*, 1924*b*), *Phascolion* by Pasteels (1935), *Hydroides* by Pasteels (1935), *Barnea* by Dalcq (1928), *Thalassema* by Hobson (1928) and *Nereis* by Spek (1930).

The present work is a study of the effects of isotonic solutions of $CaCl_2$, $BaCl_2$, $SrCl_2$, $MgCl_2$, NaCl, KCl and LiCl, singly and in varying binary mixtures and proportions, on the eggs of *Cumingia tellinoides* and *Arbacia punctulata;* of the relative effectiveness of $CaCl_2$ in the activation of ovary eggs and shed eggs and of shed eggs that have been washed and shed eggs that have not been washed; and of the relative effectiveness of solutions of isotonic $CaCl_2$ which vary in pH. While its principal contribution is an extension of our knowledge of the effects of various isotonic salt solutions in the activation of eggs, it is hoped that it may illuminate further our understanding of the fundamental reaction or series of reactions which underlie the vital response of the cell.

Many careful investigators have shown that various types of stimulation cause an increase in permeability of the plasma membrane of various kinds of living material. However, Heilbrunn (1937) points out "There is one type of stimulation which can scarcely be conceived of as producing an increase in permeability. This is the stimulation produced by calcium salts. Students of permeability are quite unanimous in regarding the calcium ion as a permeability lowerer rather than a permeability increaser. Hence the action of calcium in producing stimulation cannot be explained on the basis of the permeability theory." Heilbrunn and his students have developed a colloid chemical theory of stimulation in which calcium plays the dominant rôle (see Heilbrunn, 1928; Heil-

[1] A thesis in zoölogy presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

brunn and R. A. Young, 1930; Heilbrunn and Daugherty, 1933; Heilbrunn and Mazia, 1936; Angerer, 1936; Mazia and Clark, 1936; Heilbrunn and Wilbur, 1937; Donnellon, 1938). This theory postulates the following series of changes: calcium is released from the cortex resulting in a liquefaction of the cortex; free calcium enters the interior of the cell; as the concentration of free calcium increases in the cell interior, a series of reactions is initiated which includes an initial decrease in viscosity followed by a characteristic clotting reaction. This series of reactions constitutes the vital response of the cell. My observations on the activating effect of bivalent cations appear to support the colloid chemical theory of stimulation as developed by Heilbrunn and his students.[2]

Material and Methods

In most of the experiments the same general procedure was employed. In any given comparison the eggs from one female were used. The eggs were shed into sea water, the supernatant fluid withdrawn and two drops of a dense suspension of eggs were quickly pipetted into dishes of experimental solutions previously prepared. No attempt was made to control the temperature of the experimental solutions. The temperature of the air was no higher than 26° C. at any time and was usually between 21° and 25°. In any given experiment the range was rarely more than 2° C.

Merck's C.P. chemicals were used in making the solutions. NaCl, KCl and LiCl were made up in 0.53 M concentration and $MgCl_2$, $BaCl_2$, $CaCl_2$ and $SrCl_2$ in 0.3 M concentration. These solutions are isotonic with sea water and the eggs do not shrink or swell in them. In studying the effects of various mixtures of isotonic salt solutions in the activation of eggs, the pH of the various solutions was adjusted, by the addition of 0.1 N HCl and 0.1 N NaOH, so as to lie in the range found experimentally to be most favorable for activation, i.e. pH 6.2 to 8.6 for *Cumingia* eggs and pH 8.8 to 9.0 for *Arbacia* eggs.

Eggs were exposed for varying periods of time to isotonic solutions of a single chloride or to mixtures of chlorides in varying proportions. The eggs were not transferred to sea water as is the usual procedure in experiments of this kind, inasmuch as a high percentage of cleavage could be obtained in the experimental solutions. In each experiment, hundreds of eggs were examined and 100 eggs were counted. The time factor is very important in determining the percentage of cleavage. At the end of

[2] This work was done at the Marine Biological Laboratory at Woods Hole during the summers of 1935, 1936 and 1937.

The problem was suggested by Dr. L. V. Heilbrunn. I wish to express my appreciation for his invaluable guidance and kind criticism during the course of this investigation.

The complete data are on file in the Library of the University of Pennsylvania.

a certain period, which is roughly three hours in the case of *Cumingia* eggs and five hours in the case of *Arbacia* eggs, there is no further increase in the percentage of cleavage and cell injury occurs a little later. It is desirable to count the percentage of activation at the end of this optimum period which varies with the solution, the pH and the temperature.

Conclusions concerning the effectiveness of a reagent in activating eggs are based on the percentage of cleavage. Although the first visible sign of activation of *Cumingia* eggs is the extrusion of polar bodies, it is difficult to make an accurate count of the percentage of eggs with polar bodies, for if the egg lies with the animal pole down, the polar bodies cannot be seen. While the first sign of activation when *Arbacia* eggs are inseminated is the elevation of the vitelline membrane, this reaction cannot be employed with isotonic solutions since they do not cause membrane elevation although they do cause the membrane to swell.

TABLE I

Experiments were performed to determine the relative effectiveness of barium, calcium and strontium solutions on the eggs of 33 individuals and tables were prepared of the percentage of cleavage and polar body formation and of the number of minutes elapsing before polar body formation.[4] The following results were obtained.

Activated by	Average time of pb formation	Average percentage cl	Average percentage pb
barium	11 min. 48 sec.	5.3	3.6
calcium	11 min. 27 sec.	35.4	26.1
strontium	10 min. 54 sec.	35.8	38.2
sperm	10 min. 6 sec.		

RESULTS

Cumingia

Effect of 0.3 M $CaCl_2$.—When unfertilized *Cumingia* eggs are placed in 0.3 M $CaCl_2$, at any pH between 6.0 and 8.6, the first polar body is extruded in from 5 to 12 minutes and the first cleavage is completed in from 40 to 60 minutes. This is approximately the same as the time of polar body formation and of cleavage in eggs activated by sperm. The percentage of cleavage at the end of several hours varies widely among the eggs of different individuals. In some individuals 100 per cent of the eggs undergo apparently normal activation. They continue to cleave for several hours, reach the 8–16 cell stage and appear to be healthy and normal. After several hours, however, the blastomeres pinch in and fall apart.[2] Polar body formation is extremely irregular, but there appears

to be an inverse relationship between the percentage of polar body formation and the percentage of cleavage (compare Morris, 1917).

The result of experiments in which eggs were exposed to solutions of $CaCl_2$ which vary in pH from 3.6 to 9.0 is shown in Table I. The various solutions from pH 6.0 to 8.6 are equally effective in inducing activation of the eggs of most individuals. The pH of the solutions appears to have more effect on the percentage of cleavage than on the percentage of polar body formation, polar body formation proceeding at pH 9.0 while the percentage of cleavage decreases above pH 8.6.[3] Both are almost completely inhibited at pH 4.1. (See Table II.) While the

TABLE II

Effect of pH on Activation of Cumingia Eggs by Isotonic Calcium Chloride

No. of exp.	1	2	3	4	5	6	7	8	9	10
Time of expos. Hrs.	4	4	4	6	6	7	4	4	6	7
Min.	20	55	15	20	20	15	20	55	55	15
Temp. °C.	21.3	21.3	21.3	20.0	20.0	20.0	21.3	21.3	20.0	20.0
	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl
pH										
4.1	0 0	3 1	0 0			0 0	0 0	0 0		
4.6				60 5	32 0	19 0	0 0		13 0	40 0
4.9	55 2	34 18	21 12				14 38	9 27		
5.8				13 70	40 13	60 2		7 70	18 1	35 2
6.1	13 45	16 51	20 19				16 50			
6.2				2 98	6 98	4 94			30 5	8 10
7.1				2 98	6 88	6 91			25 5	2 14
7.6	22 45	10 63	30 32	2 98	5 90	9 89	16 55	8 66	56 3	3 15
8.2	18 49	14 59	11 54	32 92			14 59	4 65		
8.3				2 96	6 92	6 90			52 0	6 26
8.6	19 60	18 50	20 55	13 87	11 88	10 77	19 60	10 50	35 1	12 27
9.0	17 33	24 22	6 52	34 22	27 67	27 67	17 33	12 55	20 3	13 18
sw	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0

strontium solution is somewhat more effective than the calcium solution, the former causes the blastomeres to separate in less time. It is difficult to make accurate counts of the percentage of cleavage when blastomeres separate.

Effect of Mixtures of Calcium Chloride and Some Monovalent Cations.—The result of experiments in which eggs were exposed to mixtures of potassium chloride, sodium chloride or sea water and calcium chloride in various proportions is shown in Fig. 1. The degree of activation decreases rapidly as the proportion of potassium increases up to a ratio of 1–16 and then remains fairly constant. It may be concluded that there is an antagonism of calcium by potassium between ratios 1–64

[3] This work is not a study in artificial parthenogenesis and no attempt was made to develop procedures for securing later stages of development.

and 1–16 since the percentage of activation decreases too rapidly to be due to the dilution of calcium ions by potassium ions. Sodium has only a slight inhibiting influence on the activating effect of calcium from ratio 1–64 to ratio 1–4, but inhibition increases markedly above an average ratio of 1–4. The slight decrease in the percentage of activation up to an average ratio of 1–4 is probably due to the dilution of the calcium

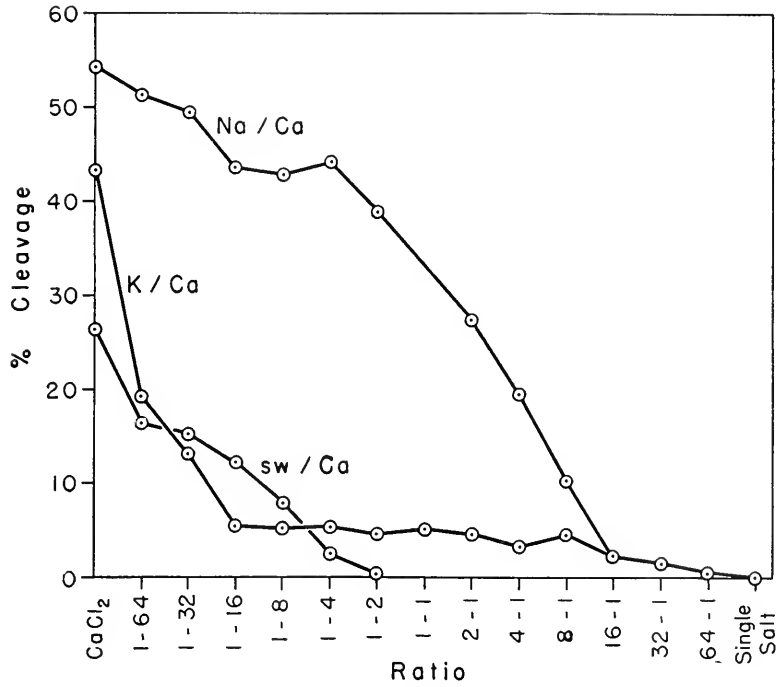

FIG. 1. Action on *Cumingia* eggs of isotonic solutions of some monovalent cations and of $CaCl_2$ singly and in various mixtures and proportions.

K/Ca—average of ten experiments.
Na/Ca—average of sixteen experiments.
sea water/Ca—average of five experiments.

solution, but there is an antagonism of calcium by sodium above this ratio. Sea water has an inhibiting effect on the activating effect of calcium beginning in a ratio of 1–64 and increasing as the proportion of sea water increases, with complete inhibition in most cases at a ratio of about 1–2. The inhibiting effect of sea water is no doubt due to the monovalent cations.

Effect of Mixtures of Calcium Chloride and of Some Bivalent Cations.—The result of experiments in which eggs were exposed to mixtures of strontium, barium or magnesium and calcium in various propor-

tions is shown in Fig. 2. Strontium is somewhat more effective than calcium in inducing cleavage in *Cumingia* eggs but causes the polar bodies to be extruded far from the cell surface and the blastomeres to separate in a short time. Strontium inhibits slightly the activating effect of calcium while calcium inhibits somewhat the activating effect of strontium. Solutions in which the Sr/Ca ratios are from 1–1 to about 64–1 are more injurious than the single salt solutions and it is difficult to make an accu-

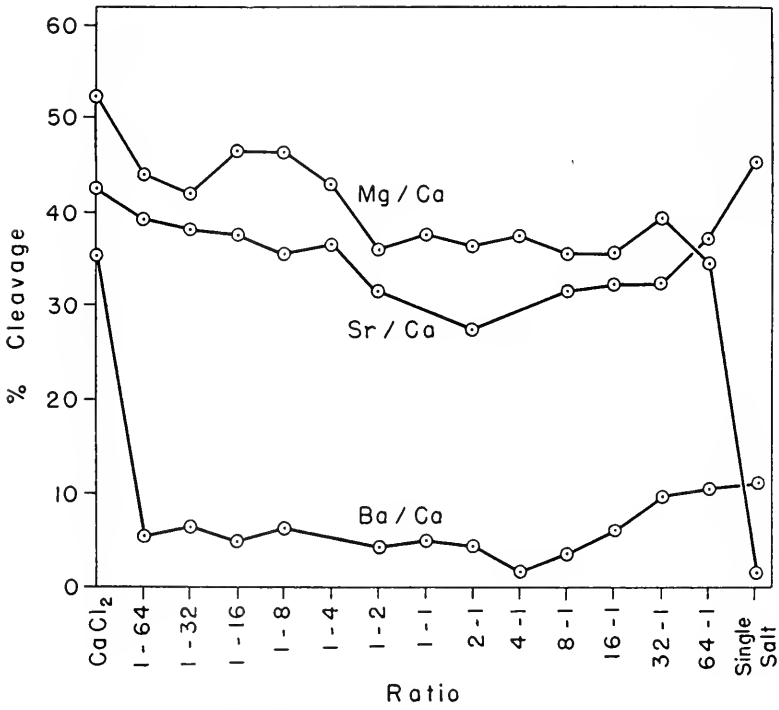

FIG. 2. Action on *Cumingia* eggs of isotonic solutions of some bivalent cations singly and in various mixtures and proportions.

Sr/Ca—average of seventeen experiments.
Ba/Ca—average of twelve experiments.
Mg/Ca—average of thirteen experiments.

rate count of the percentage of cleavage between these ratios. Barium has a slight activating effect on *Cumingia* eggs. It may be concluded that there is an antagonism of calcium by barium as the latter produces a marked inhibition of the activating effect of calcium, beginning in a ratio of 1–64. The percentage of activation resulting from exposing eggs to isotonic $MgCl_2$ is negligible. Magnesium inhibits very slightly the activating effect of calcium even in a ratio of 64–1. Inasmuch as magnesium has no activating effect on *Cumingia* eggs, it might be ex-

pected that as the dilution of the calcium solution by magnesium increases, the percentage of activation would decrease. It is interesting and noteworthy that this is not the case. Even in an Mg/Ca ratio of 64–1 there is a high percentage of cleavage.

In summary, monovalent cations are not effective in activating the eggs of *Cumingia* and they inhibit the activating effect of calcium, the effect increasing as the K/Ca, Na/Ca, sea/Ca ratios increase. The bivalent cations, with the exception of magnesium, are able to activate *Cumingia* eggs and in certain combinations and proportions, mutually inhibit activation. There is considerable variation in the behavior of different lots of eggs, but it is a variation in magnitude rather than in kind. The results of experiments performed on the eggs of a single individual (see original manuscript) are more interesting than the average of the results of many experiments as presented in this paper, because the former presents a more characteristic picture of the behavior of marine eggs.

Arbacia

Effect of 0.3 M $CaCl_2$.—When unfertilized *Arbacia* eggs were placed in 0.3 M $CaCl_2$, at any pH between 8.0 and 8.5, a certain percentage (rarely more than 25 per cent) of the eggs of most individuals undergo cleavage. It is difficult to make reliable counts of the percentage of activation in *Arbacia* eggs inasmuch as cells that have undergone cleavage usually occur in groups. The time required for maximum percentage of cleavage is from 7 to 10 hours or considerably longer than the time of cleavage in eggs activated by sperm. In a study of the eggs of 40 individuals the percentage of cytolysis was found to be high (33.5 per cent) if the eggs were aged for about 11 hours before being placed in the calcium solution.

A comparative study was made of the percentage of cleavage when the eggs were obtained in various ways. In some instances a fragment of ovary was placed directly in the solution to be tested. The exuding eggs are called ovary eggs. If such exuded eggs were washed in sea water they are called washed eggs. Shed eggs were obtained in the usual manner. Experiments were performed on the eggs of about 60 individuals and tables were prepared on the comparative percentage of cleavage. These tables are elaborate and it was thought unwise to attempt their publication (see original paper). The following results were obtained:

	Average percentage of cleavage
Ovary eggs	11.8
Shed eggs	22.8
Shed eggs	23.8
Washed shed eggs	30.9

TABLE III

Effect of pH on Activation of Arbacia Eggs by Isotonic Calcium Chloride

No. of exp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Time of | Hrs.	7	7	7	7	8	8	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	6	7	7	7	5	5	5	5	5
expos. | Min.	21	21	21	21	45	15	15	15	15	15	15	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	5	20	20	20	7	7	7	7	7
Temp. °C.	22	22	22	22	21	21	22	22	22	22	22	22	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	25	25	25	25	25	25	25	25
	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl
pH																																			
3.8	0	0	0	0																															
3.9					0	0																													
4.2	0	0	0	0	0	0																													
6.0	0	0	0	0																															
6.0					0	0																													
6.5														0	0	0	0	0	0	0	0	0	0	0	0	0	0								
6.9							1	0	0	1	0	0	0																							
7.0	0	0	0	0	0	0																													
7.2																												1	0	0	0	0	0	0	0
7.3							0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
8.0					2	0	0	0	3	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	1	0	0	0							
8.1																															0	0	0	2	2
8.2																												1	1	0					
8.3					10	0																													
8.4							0	0	5	1	10	12	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0				5	7	8	6	0
8.6	35	2	10	19	0	0	5	2																				23	33	2					
8.8							6	18	6	21	30	8	15	34	0	0	0	0	0	0	0										3	7	9	2	2
9.0							5	20	25	7	18	15	36	70	21	33	12	90	15	51	39	55	39	47	12	46	24	23	5	4					
9.4																														13	18	10	22	15	18
9.6	18	30	45	25	15	10																													
sw	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

There is a higher percentage of cleavage in shed eggs than in ovary eggs and a slightly higher percentage of cleavage in washed shed eggs than in shed eggs.

Table III shows that when the unfertilized eggs of one individual are placed in solutions of $CaCl_2$ in which the pHs vary from 3.8 to 9.6, for from 5 to 9 hours, the percentage of cleavage is low below pH 8.0 and is only slightly higher at pH 8.5 while the highest percentage of cleavage takes place at about pH 9.0. Cleavage takes place in the short-

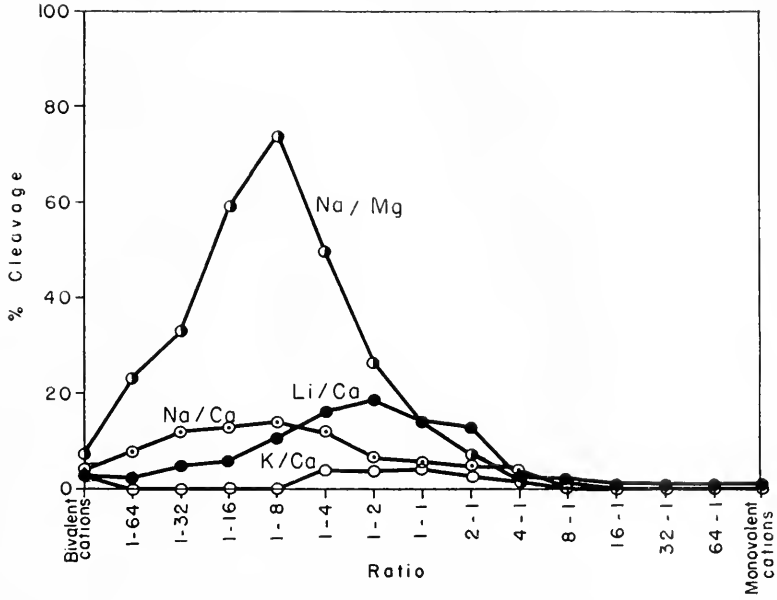

FIG. 3. Action on *Arbacia* eggs of isotonic solutions of some monovalent and bivalent cations singly and in various mixtures and proportions.

K/Ca—average of fourteen experiments.
Na/Ca—average of nine experiments.
Li/Ca—average of eight experiments.
Na/Mg—average of three experiments.

est time at pH 9.2 but the eggs soon undergo cytolysis. High alkalinity also seems to cause nuclear division without cytoplasmic cleavage in a large percentage of eggs. In many cases there is little and sometimes no cleavage in solutions of which the pH is about 8.8 while there may be a high percentage of cleavage in solutions of which the pH is about 9.0. *Arbacia* eggs are not activated by alkaline sea water.

Effect of Mixtures of Calcium Chloride and Some Monovalent Cations.—The result of experiments in which eggs were exposed to solutions of potassium, sodium or lithium and calcium chloride in various

proportions and of sodium and magnesium chloride in various proportions is shown in Fig. 3. There is a gradual increase in the percentage of activation as the proportion of potassium increases up to a ratio of about 1–2 while above this ratio there is a decrease with activation ceasing at a ratio of about 8–1. From ratios 16–1 to 64–1 there are many eggs in which the nucleus has undergone several divisions and in which

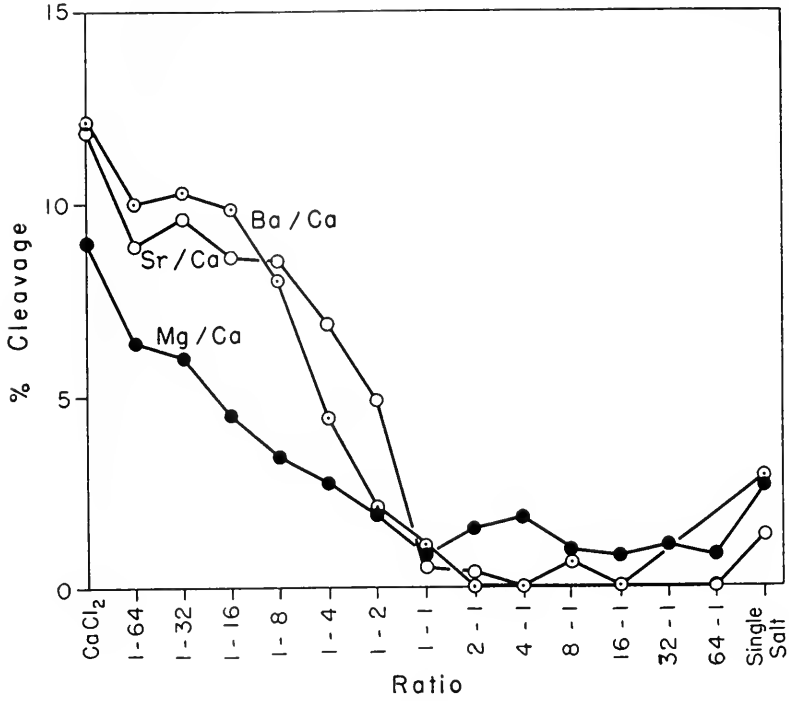

Fig. 4. Action on *Arbacia* eggs of isotonic solutions of some bivalent cations singly and in various mixtures and proportions.

Mg/Ca—average of eight experiments.
Sr/Ca—average of eight experiments.
Ba/Ca—average of eleven experiments.

there has been no cytoplasmic division. There is a marked increase in the percentage of activation as the proportion of sodium increases up to about ratio 1–4 followed by a marked decrease, with no activation in mixtures in which the ratio is above 16–1 or in the Na solution. Isotonic LiCl solution is able to activate a small percentage of *Arbacia* eggs. The degree of activation increases rapidly as the proportion of lithium increases beginning in a ratio of about 1–16, with the highest percentage at a ratio of about 1–2, followed by a sharp decline at a ratio of about

2–1. Isotonic $MgCl_2$ is able to activate a very small percentage of eggs. There is a very great increase in the percentage of activation as the proportion of sodium increases up to an average ratio of 1–8 followed by an equally sudden decrease with activation ceasing at a ratio of about 8–1. This result is of interest because there is a marked increase in the percentage of activation both in Na/Ca mixtures and Na/Mg mixtures between ratios of about 1–32 and 1–8.

Effect of Mixtures of Calcium Chloride and Some Bivalent Cations. —The result of experiments in which *Arbacia* eggs are exposed to solutions of magnesium, strontium or barium chloride and calcium chloride in various proportions is shown in Fig. 4. While magnesium has a slight activating effect, there is a mutual anatagonism between calcium and magnesium. Strontium is able to activate a small percentage of eggs but is much less effective than calcium. The degree of activation by calcium decreases gradually as the proportion of strontium increases up to a ratio of about 1–8 with a sharp decrease above a ratio of 1–8. It may be concluded that there is an antagonism of calcium by strontium. Barium alone is able to activate a small percentage of eggs. The degree of activation decreases as the proportion of barium increases with activation ceasing at a ratio of about 2–1. It may therefore be concluded that there is antagonism of calcium by barium. There are many eggs with nuclear divisions without cytoplasmic cleavage in mixtures where the Ba/Ca ratio is between 32–1 and 64–1.

In summary, Na and K are not effective in activating *Arbacia* eggs while Li activates a small percentage. The monovalent cations increase the percentage of activation by calcium when present in certain definite proportions. The bivalent cations, Sr, Ba and Mg each have an inhibiting effect on activation by Ca and the antagonism is mutual. There is even more variation in the behavior of *Arbacia* eggs than in *Cumingia* eggs but again it is a variation in magnitude rather than in kind (see original paper).

Discussion

The problem of the activation of unfertilized eggs by an alteration of their chemical environment has been vigorously attacked by a number of investigators. R. S. Lillie (1910, 1911*a*, 1911*b*) was the first to report activation of marine eggs by means of isotonic salt solutions. To date, the activation by isotonic salt solutions of the eggs of seven species of marine invertebrates has been reported. In each case, activation was accomplished by exposing eggs to varying combinations and proportions of the chlorides of the cations of sea water. In all, the presence of Ca appears to be essential while there is variation in the element which it is necessary to add.

R. S. Lillie (1910) reported the initiation of development in *Arbacia* eggs when exposed to isotonic NaCl for varying periods of time followed by return to sea water. He reported no activation of *Arbacia* eggs when exposed to isotonic solutions of $CaCl_2$, $SrCl_2$ and $MgCl_2$ followed by return to sea water. In the present work the opposite of these observations is reported. The difference in results obtained with the monovalent cations may be due to the fact that in the present work the eggs were not returned to sea water and the difference in results obtained with bivalent cations may be due to the fact that *Arbacia* eggs must be exposed to isotonic solutions of $CaCl_2$, $SrCl_2$ and $MgCl_2$ for hours in order to obtain a noteworthy percentage of activation.

The results of the experiments on the eggs of *Arbacia* reported in this paper are in agreement with the results of the work on marine eggs reported by Dalcq (1928) on *Barnea candida,* Hobson (1928) on *Thalassema neptuni* and Pasteels (1935) on *Phascolion* and *Hydroides* where alkalinity and the monovalent cations K, Na and Li increase markedly the percentage of activation of *Arbacia* eggs by Ca and are necessary in order to obtain a high percentage of activation of the eggs of most individuals and where cleavage appears to be more nearly normal in favorable binary mixtures than in isotonic $CaCl_2$ alone.

However, the results on activation of the eggs of *Cumingia* by isotonic salt solutions are not in agreement with the results of the work reported by Dalcq, Hobson and Pasteels on activation of marine eggs by isotonic salt solutions. The addition to the calcium solution of excess OH ions or of the monovalent cations Na or K does not increase the percentage of activation of *Cumingia* eggs but has the opposite effect. The segmenting eggs appear more nearly normal and more healthy in isotonic $CaCl_2$ alone than in any of the binary mixtures used. Activation by isotonic $CaCl_2$ with respect to time of polar body formation and percentage of activation compares favorably with activation of eggs by sperm. We may say that in the case of *Cumingia* eggs, Ca is the sole activating agent and that no other external agent or treatment is necessary.

There are several theories to explain the activation of unfertilized eggs. All these theories are aspects of more general theories of stimulation. The oxidation theory of activation was stated by J. Loeb (1913). It is now quite certain that not all activating agents increase the rate of oxidation. Heilbrunn (1915) pointed out that cyanide does not prevent the first stages of development in *Arbacia* eggs and (1920*a*) that maturation in *Cumingia* eggs is not dependent on an increase in oxygen consumption. Whitaker (1931, 1932) reported that in the eggs of *Nereis* and *Arbacia* there is an increase in the rate of respiration following fertilization whereas in the eggs of *Chaetopterus* and *Cumingia* there is

a decrease following fertilization. Activation of eggs by an isotonic solution of $CaCl_2$ can scarcely be due to an increase in the rate of oxidation for calcium is usually thought to decrease the rate of oxidation (see for example, Ahlgren, 1925; Holck, 1934; and Thunberg, 1937). It may therefore be concluded that rate of oxygen consumption is not the primary factor in the initiation of development of eggs.

The permeability theory of activation, founded by R. S. Lillie (1916, 1917, 1918) has been used to explain initiation of development in eggs. That there is an increase in permeability in some marine eggs following activation has been convincingly demonstrated by a number of careful investigators. The work of Lillie (1916, 1917, 1918) and McCutcheon and Lucké (1932) shows that the permeability of *Arbacia* eggs to water increases after fertilization and the work of Stewart and Jacobs (1932) shows that permeability of these eggs to ethylene glycol increases after fertilization. However, activation of eggs by isotonic $CaCl_2$ cannot be conceived of as due to an increase in permeability. It is universally agreed among students of permeability that bivalent cations such as magnesium and calcium cause a decrease in cellular permeability and antagonize those reagents known to increase it. Therefore the action of calcium in the activation of *Cumingia* eggs cannot be explained on the basis of the permeability theory.

Dalcq (1924*a*, 1924*b*) has developed a depolarization theory of activation. This theory depends upon the presence of charges of definite sign upon the cortex and constituents of the egg and upon the existence of a potential gradient on the cortex. He concluded that a disturbance of the intraovular cations results in depolarization, that Ca is the most effective agent in bringing about depolarization and that activation is the result of depolarization. However, the depolarization theory of Dalcq seems highly speculative and is difficult to understand from the electrochemical standpoint.

Heilbrunn (1915) favored the coagulation theory of activation. This theory, which is now termed the colloid chemical theory is, as the permeability theory, a broad theory of stimulation for all types of irritable systems. In a study of the chemical changes in the egg following activation, Heilbrunn and his students have shown that whenever a cell is stimulated, Ca is released from the cortex. Heilbrunn and his students have further shown that if Ca is first removed from egg cells by oxalate, stimulating agents are not effective but that upon the return to sea water the usual response may be obtained (see Heilbrunn and R. A. Young, 1930; Heilbrunn and K. Wilbur, 1937). For a full discussion of the colloid chemical theory of stimulation see Heilbrunn's "Outline of General Physiology," 1937. The results of the study of the activa-

tion of the eggs of *Arbacia* by favorable binary mixtures of bivalent and monovalent cations and of the study of the activation of the eggs of *Cumingia* by isotonic solutions of bivalent cations alone, where 100 per cent of the eggs of some individuals undergo apparently normal cleavage in a period of time which compares favorably with the time of activation of eggs activated by sperm, favor the colloid chemical theory of Heilbrunn and are directly opposed to any interpretation in terms of the oxidation or permeability theories.

Summary

1. When unfertilized *Cumingia* eggs are placed in 0.3 M $CaCl_2$, 100 per cent of the eggs of some individuals undergo apparently normal cleavage. The time of polar body formation and of the first cleavage in eggs activated by Ca is approximately the same as the time of polar body formation and of cleavage in eggs activated by sperm.

2. Polar body formation and cleavage in *Cumingia* eggs proceed normally in 0.3 M $CaCl_2$ at the various pHs between pH 6.2 and pH 8.6 but are inhibited above and below this range.

3. The bivalent cations Sr, Ca and Ba are able to activate *Cumingia* eggs and are named in the order of their effectiveness.

4. The time of polar body formation in *Cumingia* eggs activated by isotonic solutions of $SrCl_2$ and $BaCl_2$ is approximately the same as the time of polar body formation in eggs activated by sperm.

5. The monovalent cations K and Na and sea water inhibit activation of *Cumingia* eggs by Ca. The percentage of activation decreases as the K/Ca, Na/Ca and sw/Ca ratios increase. K has a greater inhibiting effect than Na.

6. Ba inhibits the activation of *Cumingia* eggs by Ca, Sr inhibits very slightly the activating effect of Ca, while Mg does not appear to have an inhibiting effect on activation of eggs by Ca.

7. When unfertilized *Arbacia* eggs are placed in 0.3 M $CaCl_2$, from 40 to 60 per cent of the eggs of most individuals undergo cleavage if the pH of the solution is between 8.8 and 9.2. No membrane is elevated in isotonic salt solutions.

8. Below pH 8.8 the percentage of cleavage is low and above pH 9.0 the percentage of cytolysis is high in *Arbacia* eggs activated by isotonic $CaCl_2$.

9. Isotonic solutions of $SrCl_2$, $BaCl_2$ and $MgCl_2$ are able to activate a certain percentage of *Arbacia* eggs, but these ions are not so effective as Ca and their action is somewhat variable.

10. The monovalent cations Na, Li and K in certain definite proportions increase the percentage of cleavage induced by Ca while in other

proportions they have the opposite effect. Similarly isotonic NaCl, in certain definite proportions increases markedly the percentage of activation by the Mg solution while in other proportions Na has the opposite effect.

11. Sr, Mg and Ba inhibit the activation of *Arbacia* eggs by Ca, the inhibiting effect increasing as the Sr/Ca, Mg/Ca and Ba/Ca ratios increase.

12. The results are brought into relation to the colloid chemical theory of stimulation.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1941

July 8

The source of pancreatic juice bicarbonate. Eric G. Ball.

(This paper has already appeared in full in the July, 1941 number of the *Journal of Biological Chemistry.*)

The permeability and the lipid content of the erythrocytes in experimental anemia. Arthur J. Dziemian.

In a series of albino rabbits the permeability of the erythrocytes to glycerol, diethylene glycol, ammonium propionate and ammonium salicylate was studied and the total lipid, cholesterol and phospholipid contents of the red cells, calculated per ml. of cells, per erythrocyte and per square micron of cell surface, were determined. The rabbits received subcutaneous injections of 50 mg. of phenylhydrazine hydrochloride and changes in the permeability of the red cells to the above substances were studied. Within about nine days after injection, practically an entirely new population of red cells was present in the animals, as shown by a study of changes in cell diameters. At this time the rate of penetration into the cells of diethylene glycol and glycerol had greatly increased, while the ammonium propionate and salicylate penetrated slower than normal. Thereafter the times of 50 per cent hemolysis of the red cells in all the solutions used returned slowly toward the original values. When lipid analyses on the red cells of the experimental animals were made, no correlation was found between the changes in permeability and the changes in the lipid content of the erythrocytes.

The rectifying property of the giant axon of the squid. Rita Guttman and Kenneth S. Cole.

Measurements of the resistance of the squid giant axon have been made by means of a direct current Wheatstone bridge between one injured end of the axon, placed in KCl, and the other end immersed in sea water, with the inter-electrode region hanging in oil. During the passage of small currents through the axon, the resistance of the fiber does not depend upon the magnitude and direction of the current. But when larger currents are used this is no longer true, i.e. if the uninjured end is the anode, the over-all resistance of the axon is greater than that found during the passage of small currents while at the cathode the reverse is true. The apparent resistance of the nerve fiber, then, depends upon the magnitude and direction of the current flowing through it. The nerve fiber thus does not obey Ohm's Law and is an electrical rectifier which permits current to pass more easily in one direction than the other, rather than a pure resistance.

Cocaine and veratrine cause progressive and reversible loss of rectification. As the axon dies, excitability is lost, and the rectification and the resting potential disappear. When the fiber is completely dead, there is no rectification and the fiber acts as a pure resistance.

Such a rectification is to be expected if the membrane conductance is a measure of ion permeability and this permeability is increased at a cathode and decreased at an anode. Also, rectification has been suggested as an explanation of some electrotonic and excitation phenomena.

Metabolism and fertilization in the starfish egg. Herbert Shapiro.

The respiration of immature, mature and fertilized eggs of the starfish, *Asterias forbesii,* was studied during May and June by means of Warburg manometers, over a temperature range 11.5 to 27.8° C. Oxygen uptake by unfertilized eggs may remain constant for periods as long as ten hours. Fertilized eggs show a relatively constant rate at first, and then a slowly increasing one, as embryological development advances. An average increase of approximately 30 to 50 per cent was found in the rate of fertilized eggs, as compared with unfertilized, the rise being slightly higher at the low temperatures.

Although some experiments yielded results in agreement with those of Loeb and Wasteneys (*Arch. entw.-mech. Organism.,* **35**: 555, 1912) and of Tang (*Biol. Bull.,* **61**: 468, 1931) in showing little or no change after fertilization, the more extended series reported here, done on eggs showing high percentages of cleavage, and during the optimal portion of the breeding season, demonstrate that the average starfish egg undergoes a significant acceleration of oxidations subsequent to fertilization.

JULY 15

Factors in the lunar cycle which may control reproduction in the Atlantic palolo. L. B. Clark.

Of all the physiological influences attributed to the lunar cycle, the coincidence of reproduction of certain marine polychaetes with specific phases of the moon has been best determined. Of such animals, the palolo worms are perhaps outstanding because of their size, striking reproductive behavior, and the apparent specific relation between the moon's phases and time of reproduction.

A number of experiments on artificially changing the light relations of the lunar cycle by illuminating or shading rocks containing worms were undertaken. The results of all the experiments are consistent in that if the average duration of light is increased, reproduction occurs before the controls, and if the average duration of moonlight is decreased, the time of swarming occurs after the controls or not at all. It is concluded, therefore, that this is a factor involved in reproduction and that the effectiveness of the various phases of the moon's cycle is correlated with the average duration of moonlight during the cycle.

If this were the only factor involved, the effectiveness of moonlight to induce swarming would increase to a maximum about three days after the full moon and then decrease. But the effectiveness of moonlight is bimodal, the modes centering about the first and last quarter moon, with the latter much more effective. Obviously there must be some other factor operating in moonlight. The only other factor varying in the desired manner is the daily difference in the rate of change of moonlight. This reaches a maximum at the new and full moons and a minimum at the first and third quarter. If it is postulated that the effectiveness of moonlight in determining the time of swarming bears some correlation to the reciprocal of the difference in the daily rate of change of moonlight, the resultant varies in a manner similar to the incidence of swarming during the lunar cycle.

Accumulation of manganese and the sexual cycle in Ostrea virginica. Paul S. Galtsoff.

Oysters of known age and origin, planted on an experimental bottom in Long Island Sound near Milford, Connecticut, showed a distinct annual cycle in the Mn-content which varied from 7.3 to 51.0 mg. per kilo d.w. During the twenty-nine months' period of observations the high Mn content (from 30 to 50 mg. p.k.d.w.) invariably coincided with the period of gonad development and sexual activity of the oysters (May–August) while low Mn content (from 7 to 11 mg.

p.k.d.w.) occurred in winter and early spring (November–April). Ovaries were found to be particularly rich in Mn (51.0–59.6 mg. p.k.d.w.) and testes contained only from 4.6 to 7.2 mg. p.k.d.w. Other tissues contained the following amounts: gills, 17 to 18 mg. p.k. in winter and from 35 to 38.6 in summer; mantle, 8.7 in January and from 14.2 to 17.0 in September; visceral mass, 8.9 to 18.4; and adductor muscle, 4.3 to 5.2 in July and 4.1 to 9.3 in January. These results indicate that the Mn cycle is associated with the development of a female phase of the oyster. The physiological role of the metal in the metabolism and its relation to the sex change in this species is not known.

Studies on the life history of Siphodera vinaledwardsii, a trematode parasite of the toadfish. R. M. Cable and A. V. Hunninen.

Experimental studies on the life history of *Siphodera vinaledwardsii* (Linton) have demonstrated that this trematode is related to the Heterophyidae as postulated by Manter, Price and Wilhelmi on the basis of morphological and serological investigations. The definitive host in the Woods Hole region is the toadfish, *Opsanus tau,* practically all of which are naturally infected. The small marine snail, *Bittium alternatum,* serves as the molluscan host in which the cercariae develop in simple, elongate radiae. The cercaria is a pleurolophocercous form of an unusual type since the tail is inserted ventrally and coiled when at rest, the fourteen penetration glands have two instead of the usual four bundles of ducts in the region of the oral sucker, and the excretory formula is $2[(2+2)+(2+2)]=16$ flame cells. The cercariae penetrate and encyst in various species of flounders, developing into apparently infective metacercariae in approximately two weeks. Cysts occur in the fins, body wall and even the myocardium of the flounder. Feeding experiments thus far completed indicate that the toadfish becomes infected by eating fish containing metacercariae. Three toadfish, isolated for four weeks, were fed fish containing 13-day metacercariae. Two of these have been examined to date and found to harbor large numbers of very young worms in addition to a few mature specimens from previous natural infection.

Pathology and immunity to infection with heterophyid trematodes. Horace W. Stunkard and Charles H. Willey.

The term heterophyid refers to a large family of digenetic trematodes which infect fish-eating birds and mammals. *Cryptocotyle lingua* is a common heterophyid species in the Woods Hole area; its life cycle was reported by Stunkard (1930). The larval stages are produced in *Littorina littorea* and *L. rudis,* while the cercariae encyst in the cunner and other fishes.

Stunkard and Willey (1929) studied the development of *C. lingua* in cats and rats. In these hosts, the worms developed to sexual maturity between the intestinal villi and no intramucosal invasion was observed. Since there is evidence to indicate that cats and rats are not favorable hosts, the studies were continued on terns and dogs.

Young terns developed a very severe infection from the sixth to the fourteenth day, when the number of eggs in the feces began to diminish. After the twentieth day the feces contained very few eggs and large numbers of young worms recently liberated from their cysts. After an initial heavy infection, gulls and terns develop a strong resistance to superinfection and the presence of a few worms serves to maintain a substantial immunity.

A dog, fed enormous numbers of cysts, began to pass eggs of the parasite on the fifth day. Large numbers of immature and mature worms were present on the surface of the mucosa and in the crypts between the villous folds. The villi showed acute inflammatory changes, desquamation, hyperemia, and excessive mucous secretion. There was no invasion of the intestinal glands or tunica propria. An-

other dog, similarly fed for fourteen days, was in a moribund condition and autopsy revealed the presence of thousands of sexually mature worms. Dogs were given moderate infections and allowed to recover. Eggs began to appear in the feces on the fifth day, were numerous for about four weeks, after which the number began to decline. At the end of three months very few eggs could be found and the feces were negative at the end of six months. After resistance had been established in dogs, the feeding of large numbers of metacercariae produced no visible ill effects and very few eggs appeared in the feces.

These experiments show that birds and dogs, if the latter survive an initial infection, effect a "self-cure" (as that term was defined by Stoll, 1929) and thereafter are resistant to any substantial reinfection.

July 22

The organization of the melanophore system in bony fishes. G. H. Parker.

Catfish color changes, which range from pale yellowish-green to coal-black, are mediated in the main by three receptors, the dorsal retina, the ventral retina, and the skin. The pale phase of this fish is excited through the dorsal retina, which is best illuminated when the fish is on a white background lighted from above. Chromatic nerve tracts extend from the dorsal retina through the central nervous organs and the autonomic system to the melanophores. The final fibers in these tracts are adrenergic in that they discharge adrenaline which blanches the fish by causing melanophore pigment concentration. The fiber tracts concerned with this response may be designated as the retino-adrenergic arc. The blood of a pale catfish is devoid of the darkening agent intermedine, a state which indicated the inhibition of the intermediate pituitary lobe. From the dorsal retina nerve tracts presumably extend through the central nervous organs to the pituitary gland and thus mediate pituitary inhibition. Such tracts may be called the retino-pituitary inhibition arc.

The dark phase of the catfish is induced through the ventral retina and the skin. When the fish is on a black background the ventral retina is excited by light from above. From it impulses pass through the central nervous organs and the autonomic system over whose cholinergic fibers they reach the melanophores. Here the resultant acetylcholine excites the melanophores to disperse their pigment and thus to darken the fish. The tracts concerned with these activities constitute the retino-cholinergic arc.

The second receptor concerned with the dark phase of the catfish is the skin, which can be studied best in blinded fishes. Such fishes, dark, intermediate, or pale, if put at once into darkness retain their original tint for days, but on being exposed to daylight quickly become coal-black. From the photoreceptors in the skin nerve-fibers pass in tracts to the pituitary gland which is thereby excited to discharge intermedine. This then passes by way of the blood to the melanophores whose dispersed pigment darkens the fish. These nerve tracts and blood courses may be called the dermo-pituitary arc. Other bony fishes whose melanophore systems are much like that in the catfish are the angelfish, eel, snakefish, Japanese catfish and the stickleback. The killifish and the flatfishes are similar but lack effective pituitary organs.

Some aspects of pigment deposition in feather germs of chick embryos. Ray L. Watterson.

A study of the developmental history of melanophores in the wing skin and feather germs of Barred Rock embryos indicates that pigment deposition is not a function of pigment cells alone, but is definitely controlled by recipient barbule cells. (1) Melanophores are packed with pigment granules early in development. Nevertheless, pigment is not distributed to epidermal cells until certain of them

become visibly differentiated as barbule cells. (2) Pigment granules accumulate at the tips of pigment cell processes and become pinched off, whereupon they lie freely among the epidermal cells. Pigment liberated in this manner is later taken up by barbule cells. (3) Pigment is deposited in each row of barbule cells in a definite sequence. The most peripheral barbule cells are the first to elongate and to form keratin; only when these visible differentiation processes begin can they receive pigment. As this wave of differentiation spreads toward the pulp, more axial cells become capable of receiving pigment. (4) A study of the development of down feathers with split barb-vanes indicates that pigment cell processes are specifically attracted toward barbule cells. In their development barbule cells differentiate in the center of a barb-vane ridge where they normally do not occur. Melanophore processes leave their normal paths, extend toward these centrally located cells and carry pigment to them. (5) Pigment deposition stimulates the melanophores involved to undergo proliferation. Melanophores undergoing mitotic division occur almost exclusively at those levels of feather germs where pigment deposition is in progress. (6) Feather germs elongate slowly until 10 days and 18 hours, whereupon they elongate rapidly, attaining their full growth by 13 days. The onset of pigment deposition coincides with the onset of rapid growth. Lillie and Juhn have estimated that 90 per cent of the axial growth of regenerating feathers is accomplished by cell elongation. Pigment deposition begins at that phase of development when barbule cells begin to elongate rapidly.

The influence of hormones on the differentiation of melanophores in birds. Howard L. Hamilton.

When explants of skin from embryos of birds which have red and black pigments in their feathers are grown in a tissue culture medium consisting of blood plasma and embryonic extract, black melanophores appear but red ones occur very infrequently. If sex hormones are added to the culture medium, then many red melanophores as well as black ones differentiate in the explant. The two kinds of pigment cells are recognized as discrete cell types because of color, shape, and solubility differences in the granules, viscosity differences in the cytoplasm as shown by more rapid granule movement in red melanophores, and differences in their reactions to various hormones. In general, sex hormones increase the number of red melanophores which differentiate in treated explants from red breeds. Sesame and olive oils also produce a stimulation (possibly due to traces of sterols). Black melanophores are inhibited by estradiol, but estrone and testosterone favor their differentiation.

Desoxycorticosterone, an adrenal cortical hormone, decreases the number of melanophores in the New Hampshire Red, White Leghorn, and Barred Rock breeds of fowl. Sex hormones produce a similar inhibition of black melanophores in the latter breed. Young tissue (5–6 days) yields few or no melanophores when grown in the presence of hormone; in older tissue (7–8 days) there is a reduction in number of melanophores and inhibition of feather germ formation as well. Crystalline hormones act apparently by slowing the process of melanin synthesis, so that the treated cells, although chronologically of the same age as the controls, are physiologically younger. It is concluded that genetic differences in the precursor cells must determine whether they become red or black melanophores, but that environmental factors (physiological differences in feather germs; hormones) may directly influence which of the two kinds will predominate.

The distribution and development of the melanophore hormone in the pituitary of the chick. Hermann Rahn.

In the chicken a structural pars intermedia is absent. The melanophore hormone, however, is present in considerable quantities and is found in greatest concen-

tration in the region of the pars anterior furthest removed from the infundibular process (Kleinholz and Rahn). Quantitative assays of the melanophore hormone were made on the pituitary of chicks throughout the whole development. The Anolis lizard test was used for these determinations. The first appearance of the hormone can be detected on the fifth day of incubation, i.e. five days before differentiation in the pituitary can be seen by ordinary cytological methods. During the last half of the incubation period the melanophore hormone per unit weight of pituitary tissue increases rapidly and reaches its greatest concentration at hatching time. All further apparent increase in hormone per total gland after hatching time can be accounted for by the increasing growth or weight of the pituitary gland.

July 29

Effect of sea water on the radiosensitivity of Arbacia sperm. T. C. Evans and J. C. Slaughter.

The percentage fertilization resulting from inseminations with sperm irradiated "dry" is much greater than that irradiated in sea water. The amount of injury to the sperm increases with dilution. For example, to reduce the fertilizations to 50 per cent, sperm diluted to 1 : 100 must receive 20,000 roentgens, whereas a suspension of 1 : 1000 needs only 3,000 r.

The sperm are more susceptible immediately after the addition of sea water, when the rate of oxygen consumption is high, than thirty minutes later when the rate of oxygen consumption is lower.

Concentrations of as low as 0.01 per cent egg albumen greatly increase the radioresistance of sperm in sea water. The resistance in sea water is also increased upon the addition of sufficient amounts of dead *Arbacia* sperm or living sperm of *Nereis*.

It therefore appears that the condition of the medium during irradiation markedly affects the radiosensitivity of the sperm as measured by the decrease in percentage fertilization.

The fractionation of cellular respiration by the use of narcotics. Kenneth C. Fisher.

An examination has been made of new data and of data from the literature concerning the effects of narcotics on oxygen consumption. The relation between concentration and effect seen in all these cases possesses a feature which suggests that the inhibitor affects independently two discrete parallel respiratory systems in each of the preparations. In general the concentration range over which the effects are graded is not identical for the two systems.

Inhibition of cell division in yeast, a protozoön, the sea-urchin egg and of light production in luminous bacteria, approaches completion at narcotic concentrations which affect oxygen consumption in the same preparation relatively much less. Actually, inhibition of these activities parallels closely inhibition of the more sensitive of the two respiratory systems.

One substance, benzoate, has been found for which the sensitivities of the two respiratory systems in yeast are just the reverse of those shown for narcotics, i.e., as the concentration of benzoate is gradually increased, the "second" system is inhibited before the "first." As would be expected ideally, this compound inhibits oxygen consumption approximately 55 per cent before beginning to affect the rate of cell division.

It is concluded that in many cells the oxygen consumption, at least at the site of action of narcotics and allied substances, is composed of two independent parallel respiratory systems. The metabolism for cell division and light production appears to be associated with only one of these two.

These observations have been made with the collaboration of Messrs. J. R. Stern, R. J. Henry and Richard Ormsbee.

Effect of azide on Cypridina luciferin. Aurin M. Chase.

There are a number of pieces of information now available which bear upon the chemical nature of *Cypridina luciferin*. Anderson (1936) has suggested a resemblance to certain naturally occurring hydroxy-benzene derivatives studied by Ball and Chen (1933), on the basis of the similarity in oxidation-reduction potential. Chase (1940) has described changes in the visible absorption spectrum during oxidation of luciferin which indicate a possible quinoid structure. Giese and Chase (1940) have postulated an aldehyde or keto group in the luciferin molecule on the basis of an irreversible combination with cyanide. Chakravorty and Ballentine (1941) have proposed as a partial structure for the luciferin molecule a hydroquinone nucleus with a keto-hydroxy side chain. The hydroquinone nucleus would explain the reversible, non-luminescent oxidation of luciferin and the keto group would be the point of combination with cyanide or with luciferase.

Giese and Fisher (unpublished data) have described inhibition of luminescence in luminous bacteria by sodium azide and this observation prompted the present study of the effects of azide on luminescence of purified *Cypridina luciferin* and luciferase. At pH 6.6 luminescence is found to be reversibly inhibited at azide concentrations from about 0.005 to about 0.1 M. At pH 5.4 these same azide concentrations are much more effective, indicating that the HN_3 may be the active agent. The effect appears to be chiefly upon the luciferin. Plotted in terms of the mass law equation, the data fall upon straight lines with slopes approximately equal to *one*.

It is tentatively suggested that HN_3 reacts with luciferin in the same way that it has been shown by Fieser and Hartwell (1935) to react with benzo- and naphthaquinones. Further evidence for a quinoid group in the luciferin molecule would therefore be indicated.

August 5

Aging phenomena, and factors influencing the longevity of Mactra eggs. Victor Schechter.

(This paper has appeared in the *Jour. Exper. Zoöl.*, Vol. 86, No. 3, for 1941.)

Comparison of the respiratory rates of different regions of the chick blastoderm during early stages of development. Frederick S. Philips.

As an introductory study of the chemical processes involved in the regional differentiation of the chick blastoderm (the area pellucida of the head-process embryo), the rates of oxygen consumption were studied of various isolated pieces. In addition, the respiratory rate of pieces containing most of the pellucid area was determined at various stages of development from the unincubated blastoderm to the 12-somite embryo. Oxygen consumption was measured in the Cartesian diver microrespirometer. The total-nitrogen of the tissues was estimated by a modification of the Conway micro-Kjeldahl procedure.

The pellucid area of head-process embryos was divided into pieces containing respectively the head-process, node and anterior streak, middle streak, posterior streak, and right and left lateral regions. The Qo_2' (m.μl. O_2 consumed/hour/γN) of all these regions is essentially similar. No major differences are apparent in the rate of oxygen consumption of the various regions studied which can be correlated with their marked regional differences in developmental potency.

The Qo_2' of the embryonic area increases from a value of about 33 in the unincubated blastoderm until it reaches a value of about 75 in the 17-hour embryo, the early definitive streak stage. The head-process, 5-6-somite, and 11-12-somite embryos have rates of oxygen consumption similar to that of the 17-hour embryo.

During this period of development from the unincubated to the head-process stage the total quantity of nitrogenous material in the whole area pellucida increases only slightly. However, the total amount of O_2 consumed/hour increases at least threefold. Apparently, therefore, the increase in respiratory rate during the first 17 hours of development depends on the large increase in cell-number coincident with the conversion of intracellular yolk material into active cellular constituents.

Further interpretations of the effects of CO and CN on oxidations in living cells. Mathilda M. Brooks.

In these experiments, the rate of O_2 consumption, of cleavage and length of life of eight different stages in the development of sea-urchin and starfish eggs, as affected by certain accelerators and inhibitors, was studied. The stages included unfertilized eggs, first cleavages, morula, blastula, early gastrula, late gastrula, early pluteus and late pluteus. The reagents were methylene blue (.00012 M); KCN (.00025 M) and CO as near 100 per cent as possible. It was found that methylene blue accelerated O_2 consumption in the early stages, decreased it or had no effect in the middle stages and increased it again in the late stages. The decrease produced by KCN varied, so that the rate varied from 63 per cent of the normal to about 6 per cent depending upon the stage of development; the decrease produced by CO varied causing a rate of 92 per cent of the normal in certain stages and a rate as low as 20 per cent in others. Methylene blue accelerated the rate produced by CO about 10 per cent, and either increased it when KCN was used or produced no effect.

In the case of cleavage, KCN produced multiple aster formation without cell division even in the presence of methylene blue at this concentration. Methylene blue prevented cytolysis produced by CO; doubled the life of the embryo; caused a faster rate of development; and increased the size of the pluteus stage of *Arbacia* from 280 μ (controls) to 420 μ.

These experiments suggest new aspects of the respiratory enzymes and associated redox systems. Since methylene blue poises the potential, it appears that the optimum redox potential at which these systems function changes with development. This can be interpreted either that the respiratory enzyme assumes a different rôle or that these enzyme systems are actually different at the various stages of development.

August 12

Studies on conditions affecting the survival in vitro of a malarial parasite (Plasmodium lophurae). William Trager.

The malarial protozoa are obligate intracellular parasites which have never been cultured *in vitro*. Indeed, little has thus far been discovered concerning even the most elementary conditions which might favor their survival outside of their living host. Accordingly, a series of experiments was conducted in which parasitized blood cells taken from a chicken infected with *P. lophurae* were placed in various media and their time of survival at 40–42° C. determined. The chief criterion of survival was the ability of the parasites to infect baby chicks under a set of standard conditions. It was found that survival *in vitro* was favored by aeration but not by a very high oxygen tension, by a balanced salt solution of high potassium content, by certain concentrations of glucose or glycogen, by glutathione, by red cell extract, by low concentrations of chick embryo extract and chicken liver extract, by daily renewal of the medium and by an optimal density of parasites per cu. mm. In the best preparations, as judged by infectivity, at least 40 per cent of the original parasites were alive on the third day, at least 20 per cent on the fourth day, about 1 per cent on the fifth day and about 0.05 per cent on the sixth day. In these preparations there was a small increase in parasite number during the first day of incubation.

The effect of dyes on the response to light in Peranema trichophorum. Charles C. Hassett.

When stimulated by a sudden increase in the intensity of light, the flagellate *Peranema trichophorum* responds by a shock reaction, i.e., it ceases forward motion and bends sharply, then moves off at an angle to its original direction of movement. The time required to produce this response was used as a measure of the photodynamic effect of a number of dyes. The average reaction-time of untreated peranemae was found to be 12.1 seconds; the optimum concentration of active dyes (ca. 5×10^{-4} M), decreased this to ca. 1.0 second; weaker solutions of these dyes and all solutions of less active dyes produced longer reaction-times, with 12 seconds as the approximate maximum. The order of effectiveness of the dyes was: rose bengal, eosin, neutral red, methylene blue, Nile blue sulfate, auramine O. Orange G had no effect. Brilliant green increased the reaction-time to 16.2 seconds in 5×10^{-5} M solution; weaker solutions produced shorter reaction-times down to 12.4 seconds at 1×10^{-7} M. This may have been due to the greater toxicity of brilliant green.

These results indicate that (1) the photodynamic action of dyes can affect the response of *Peranema* to light; (2) the fluorescence of a dye is not a measure of its photodynamic effect; (3) there is no correlation between the wave-length of the light absorbed by a dye and its effect on the response of *Peranema;* (4) dyes with very different molecular structures produce similar effects.

The utilization of ammonia by Chilomonas paramecium. John O. Hutchens.

Using a solution containing CH_3COONa, NH_4Cl, $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2$, $MgCl_2$, $FeCl_3$, and thiamin hydrochloride in which *Chilomonas* attains populations up to 10^6 cells/cc., sufficient quantities of cells have been obtained to permit accurate analyses of the composition of the cells. Also growth is accompanied by sufficient conversion of substrates to permit the compilation of balance sheets.

All data deal with cells and the conversions achieved by them during the logarithmic phase of the growth curve. The results of the experiments are as follows: 10^6 cells weigh 2.5 mg. wet and 0.61 mg. dry, i.e. they are 24 per cent solid material; 2.5 per cent of the wet weight is tungstic acid precipitable nitrogen, therefore the protein ($N \times 6.25$) accounts for 16 per cent of the wet weight or 67 per cent of the solids. Of this nitrogen 28 per cent is amide nitrogen, indicating a high percentage of dicarboxylic amino acids in the protein. Ammonia utilized by the chilomonads (up to 27 per cent of the original ammonia in the solution) is all recoverable by Kjeldahl digestion. No significant amounts of ammonia are oxidized to nitrate or nitrite. Therefore, while ammonia is a satisfactory source of nitrogen for *Chilomonas*, it is not utilized as a source of energy in the solution containing acetate.

The possibility of thiamin synthesis by ciliates. Virginia C. Dewey and G. W. Kidder.

The only ciliate so far investigated, *Tetrahymena geleii*, has been reported to require the whole thiamin molecule for growth. Using a vitamin-free casein basic medium, in which no growth of the ciliate occurred, it was found that the addition of thiamin permitted extremely slow, but transplantable growth. The addition of alfalfa or timothy hay extract to the casein permits optimum growth. In an attempt to make a quantitative estimate of the thiamin requirement, the alfalfa supplement was treated to destroy the thiamin by heating at 121° C. at pH 10 or higher for periods of 1–3 hours. The treated supplement, when added to casein, permitted optimum growth of *T. geleii* even in the absence of thiamin. This is

true also of *T. vorax* with regard to alfalfa, but not timothy hay. *Glaucoma scintillans,* however, requires both thiamin and the alfalfa supplement. The same experiment was repeated using proteose-peptone treated to destroy the thiamin as a basic medium with essentially the same results. Four possible explanations of this phenomenon are offered: (1) heat treatment does not destroy thiamin, which seems unlikely, since thiamin must be added to the medium besides the alfalfa in order to obtain growth of *Glaucoma;* (2) there is some substance in plant material which, acting catalytically, permits the resynthesis of thiamin from the fragments formed by the heat treatment; this seems most likely at present; (3) there are present in plant materials thiazole-like substances more resistant to heat than the thiazole of thiamin and which can take the place of the latter in the thiamin molecule; there is no evidence for or against this possibility; (4) there is some entirely different substance in plant materials which can substitute for thiamin. This last possibility seems to be excluded by the fact that *Glaucoma* will grow in the casein-heat treated alfalfa medium after it has supported a population of *Tetrahymena* which were killed by heat before inoculating the *Glaucoma.* This indicates synthesis of thiamin by *Tetrahymena.*

August 19

Electrical potential and activity of choline esterase in nerves. David Nachmansohn.

(Some of this material is scheduled to appear in the September, 1941 issue of the *Journal of General Physiology.* Part of it has already appeared in the *Journal of Neurophysiology* for July, 1941.)

Chemical composition of mitochondria and secretory granules. Albert Claude.

(This material is scheduled to appear in Symposia on Quantitative Biology, Cold Spring Harbor, Vol. 9, October, 1941.)

Native proteins and the structure of cytoplasm. Dorothy Wrinch.

General Scientific Meetings

August 26

Further studies of metamorphosis of ascidian larvae. Caswell Grave.

The mechanism of metamorphosis in the larva of *Cynthia partita* and in the larva of *Amaroucium constellatum* does not differ significantly from that in larvae of *Ascidia nigra* and of species of *Polyandrocarpa.*

Larvae of all of these species are induced to metamorphose very soon after hatching from the egg, or after they have been liberated from the brood pouch of the parent, by treatment with aqueous solutions of $CuCl_2$ in concentrations of the order of about 5×10^{-6} molar.

Observations of large numbers of larvae of *Cynthia* show that the normal duration of its free-swimming life varies between about 9 and 100 hours when segregated in vials containing 10 cc. of sea water, but that similar groups are induced to metamorphose within 2 hours when treated immediately after hatching with a 7×10^{-6} molar solution of $CuCl_2$.

The *Amaroucium* larva has a short free-swimming life of about 100 minutes maximum duration. This period is reduced to 40 minutes by treatment of larvae with a 1×10^{-4} molar solution of $CuCl_2$. Its mechanism of metamorphosis is even

more sensitive to an aqueous extract of the tissues of the adult *Amaroucium* zoöids. Groups of larvae treated with such an extract are induced to metamorphose within 20 minutes or about one-fifth of the normal period of larval life. Ascidian tissues are known to contain copper.

It is assumed that the copper salt may act as an enzyme poison, inhibiting an enzyme system of larval metabolism, thus ending larval life and liberating the adult action system, with its lower rate of metabolism, from the inhibition imposed upon it by the higher metabolic rate of the larval action system.

The "eye-spot" and light-responses of the larva of Cynthia partita. Caswell Grave.

The "eye-spot" of the *Cynthia* larva is degenerate and the responses the larva makes to light are correspondingly deficient.

The eye consists of a single defective, opaque lens and a small number of retinal cells that are wholly devoid of pigment granules and are not arranged to form an optic cup.

Cynthia larvae soon after hatching swim to the water surface in negative orientation to gravity but they show no orientation of their swimming movements to light. There is no persistent aggregation of the actively swimming larvae either at the most illuminated edge of the container or at the least illuminated edge. The larvae at all times tend to take an even distribution over the water surface.

However, if a lot of larvae are placed in a rectangular container (Leitz filter cell) enclosed in a box from which light is excluded except for a small opening at one end, the larvae after an interval of several minutes will be found to have moved to the least illuminated end of the cell. The same negative response to the directive beam will be found to have occurred as often as the cell is reversed in the box.

The mechanism by which larvae of the *Ammaroucium* type orient their swimming movements with reference to a source of light involves an eye with a relatively deep, pigmented optic cup into which light is concentrated intermittently at each rotation of the body of the actively swimming larva, by a system of three lenses. The *Cynthia larva*, lacking both optic cup and functional lenses, is deficient in an orientation mechanism. It may be partially compensated for by placing the larva in the path of a beam of directive rays of light.

Regeneration in the early zoöid of Amaroucium constellatum. Lloyd Birmingham.

Oözoids 1–8 days old were cut in one of three regions: (*a*) just below the pharynx, separating pharynx, atria, and hindgut in the anterior fragment and leaving stomach, gut, epicardium and heart in the posterior fragment, (*b*) through middle of pharynx leaving mainly pharyngeal and atrial tissues in the anterior fragment, and (*c*) just anterior to the heart leaving only the heart and part of the epicardium in the posterior fragment. The criterion of successful regeneration was the development of the beating heart.

Each fragment after operation (*a*) had about the same potentialities. Some 65 per cent of all fragments regenerated. About 70 per cent of fragments regenerating were members of the pair from one individual. The frequency of regeneration of anterior fragments was approximately the same as that of posterior.

Operation (*b*) showed a high frequency of regeneration in posterior fragments and intermediate frequency in anterior fragments. Only rarely did the posterior fragment from operation (*c*) regenerate; the anterior portion nearly always regenerated.

The time required for the development of a beating heart varied between 1 and 7 days. Average time was two days. The smaller the fragment relative to the total size of the individual the longer the time required.

The removal of the tail or adhesive papillae of the tadpole leads not to regeneration but to metamorphosis. Such treatment seems to hasten the breakdown of the larval action system allowing the adult action system to take over. Thus, regeneration of larval tissues has not been demonstrated; however, regeneration in the adult is of a complex type. Both tissue and organ regeneration take place. The latter requires the action of both totipotent cells and factors promoting proper differentiation.

Normal asexual reproduction of zoöids occurs 15–20 days after metamorphosis of the larva, yet the capacity for perfect regeneration is already present in such zoöids the day following metamorphosis.

Characteristics of the acceleration of Arbacia egg cleavage in hypotonic sea water. Ivor Cornman.

The acceleration, as previously reported, is a response of the egg to dilutions of sea water down to a concentration 88 per cent that of normal sea water. Acceleration of the first cleavage can be produced by beginning the treatment any time from a few minutes after insemination until at least as late as prophase of the first cleavage, and acceleration of the second cleavage by treating after the first cleavage. While the acceleration can result from exposure beginning during the mitotic cycle, a direct effect upon some phase of mitosis is not as yet demonstrated. Rather, evidence so far obtained favors the supposition of an indirect action, perhaps like a stimulus. (1) There is no clearly defined optimum when the acceleration is obtained by a range of dilutions. The effect resembles an all-or-none response, rising to a plateau in sea water diluted to 98 per cent, and continuing only slightly diminished to 90 per cent, where it begins to drop toward a retardation. (2) The first or second cleavage can be accelerated, but not both in the same egg. (3) Eggs from different urchins vary, some responding well, others not at all. This variation shows no correlation with the natural concentration of the sea water. Were the effect of hypotonicity a direct one, altering the cytoplasm so as to facilitate some phase of mitosis, it would be consistent with a more sharply defined optimum response, an acceleration of both cleavages, and possibly some correlation between the concentration of sea water and responsiveness of the eggs. On the contrary, the evidence, while not conclusive, points to a system within the egg which reacts to the full extent of its responsiveness to any dilution not great enough to interfere with cleavage, and which does not react in eggs which have been once stimulated, or for some natural reason lack the necessary energy or irritability.

Maternal inheritance in echinoderm hybrids. Ethel Browne Harvey.

Three different echinoderm hybrids have been studied, the California sea-urchins, *Strongylocentrotus purpuratus* ♀ × *S. franciscanus* ♂, the Woods Hole and Maine sea-urchins, *Arbacia punctulata* ♀ × *Strongylocentrotus dröbachiensis* ♂ and the Woods Hole sea-urchin and sand dollar, *Arbacia punctulata* ♀ × *Echinarachnius parma* ♂. In all three crosses, there is a marked maternal inheritance. The rate of development of the hybrid egg is that of the normal egg, and the size, shape, pigmentation and skeleton structure of the hybrid pluteus are like that of the mother with very little paternal influence. It has not been possible to obtain plutei from the reciprocal crosses, but Matsui, working at Woods Hole, found that "a cross between a female *Echinarachnius* and a male *Arbacia* . . . is in general purely maternal or nearly so, paternal characters in no case appearing" (1924, *Jour. Coll. Agr., Imperial Univ. Tokyo,* 7: 211–236). Early larval development in these echinoderms therefore seems to be controlled by the cytoplasm rather than the nucleus. These experiments are preliminary to crossing the non-nucleate half-egg of one species, obtained by centrifuging, with the sperm of the other species, whereby conclusive evidence will be obtained as to cytoplasmic versus nuclear inheritance.

Intermediary carbohydrate metabolism of eggs and sperm of Arbacia punctulata before and after fertilization. E. S. Guzman Barron and J. M. Goldinger.

In 1928, Perlzweig and Barron found that the eggs of *Arbacia punctulata* contained carbohydrates and produced lactic acid; the lactic acid formation was increased when the oxidation was inhibited by HCN. The eggs of *Arbacia* contain also pyruvic acid (about 850 micrograms per gram dry weight). When lithium pyruvate was added to a suspension of eggs, the unfertilized eggs metabolized pyruvate at a rate of about 70 micrograms per hour per gram dry weight. The utilization of pyruvate increased five-fold after fertilization (354 micrograms per hour). Pyruvate metabolism is presumably catalyzed by diphosphothiamine, as in mammalian tissues and bacteria, for it is present in both fertilized and unfertilized eggs. The increased pyruvate metabolism after fertilization is not due to increased concentration of diphosphothiamine, because its concentration is not altered by fertilization (2.72 micrograms per cc. packed cells). The metabolism of pyruvate in the eggs does not proceed through the Szent-Györgyi cycle, because neither α-ketoglutarate nor succinate is oxidized. Addition of pyruvate depressed the O_2 consumption of fertilized eggs. The sperm of *Arbacia* also utilized pyruvate. In addition, the sperm oxidized α-ketoglutarate, succinate, and *l*(+)-glutamate, thus possessing all the elements for the operation of Szent-Györgyi's cycle for carbohydrate oxidation. The diphosphothiamine content of sperm was about twice that of eggs, 5.15 micrograms per cc. Whether this fivefold increase in the metabolism of pyruvate after fertilization is responsible for the increased O_2 uptake of the eggs on fertilization cannot be demonstrated by these experiments.

On metabolism of the heart of Venus mercenaria. A. E. Navez, J. D. Crawford, D. Benedict and A. B. DuBois.

In the study of the substrate(s) underlying the contraction of the heart of *Venus mercenaria* investigated by us (1940),[1] the following preliminary observations were made. The excised heart will keep for a long period its characteristic contractions when it is placed in a small quantity of aerated sea water. It may be whole, or "cut" in 3–5 pieces or "chopped" up in a large number of small pieces: the tissue remains highly contractile. Also for long periods this tissue (in any form or even in completely "minced" state) will respire at a uniform rate. Compared to O_2 consumption of the whole heart (100 per cent) the "cut" heart exceeds it by about 10 per cent, while the "chopped" heart is lower by about 10 per cent and the "minced" heart by about 50 per cent. But all seem to carry on the O_2 fixation with an R.Q. around 1.0. This applies to the heart unwashed in sea water. Often the washing (repeated from 1–6 times) of the pieces with sea water lowers the rate of this reaction. Addition of the washings brings back the rate around its normal value (some times a little lower, occasionally a little higher). The "minced" pulp can be centrifuged in two components, whose respiration rates are here given as percentage of the rate of the original "minced" pulp (100 per cent): (1) supernatant fluid: 30–35 per cent and (2) granular part: 65–70 per cent. Acetonic extracts and residues when reunited in water are inactive or inactivated.

In the study of the respiratory system we used the cytochrome-cytochrome oxidase-dehydrogenase system as a working hypothesis, in view of the presence of cytochrome C and succinic dehydrogenase in the heart (Ball and Meyerhof, 1940,[2] confirmed by us also). The addition of p-phenylenediamine determines a large

[1] Navez, A. E., Crawford, J. D., and Benedict, D., *Biol. Bull.*, **79**: 358, 1940.

[2] Ball, E. G., and Meyerhof, B., *Jour. Biol. Chem.*, **134**: 791, 1940.

increase in Qo_2 (up to 350 per cent) depending on concentration. It persists for long periods. The poisoning of the heart by KCN (even at high concentration) determines an inhibition of 40 per cent at the most and the same in the presence or absence of p-phenylenediamine. The addition of succinate alone raises the Qo_2 by 10 per cent; succinate + methylene blue increase it to 150 per cent maximum, but this action is also elicited by M.B. alone and to the same extent. The inhibition of this reaction by KCN is as above 30–40 per cent. The other inhibitors tried gave markedly small or no effects: i.e. sodium fluoride, sodium azide, sodium iodoacetate, sodium selenite. Ethyl urethane alone has an enhancing effect (up to 20 per cent increase at concentration 0.1 per cent by weight). Definite indications (a strong lumiflavin reaction) point to the presence of a flavoprotein; a weak glutathione reaction is given also. In conclusion the simple working hypothesis does not fit the observational facts. Other experiments not reported here confirm this rejection. Additional observations are needed and are planned for the near future.

Coördination of ciliary movement in the Modiolus gill. Alfred M. Lucas and James Snedecor.

A study previously undertaken on this problem (*Jour. Morph.*, 1932, **53**: 243–276) employed moving pictures to record the waves of the lateral cilia of the *Modiolus* gill, but only wave-length could be satisfactorily determined and much time and film were used to obtain the data. The stroboscope employed in the present study has some advantages in that frequency and rate of wave propagation could be recorded: the wave-length was calculated. Even this method did not give sufficient number of records to allow critical analyses of data and some better procedure should be worked out for the problem.

A summary of results:

Temperature	Average frequency	Av. rate of wave propagation	Av. wave-length
° C.	*vib./sec.*	*μ/sec.*	μ
10	3.5	47	13.8
15	6.2	75	11.7
20	7.4	102	14.5
25	10.6	102	10.6
30	15.4	142	10.0
35	15.8	158	10.9
			Av. 11.9

The variation around the average was very great in every case so that the value of 11.9 μ for the wave-length is quite close to 13.1 μ obtained with moving pictures. Conduction of the coördination impulse in ciliated epithelium is similar to the conduction in nerves in that the wave-length is constant in both cases.

Preparing an animated diagram of somatic mitosis. Lorus J. Milne.

Although the factual basis for this study has been limited during the past year to the behavior of dividing epithelial cells from 25-mm. larvae of the salamander *Ambystoma tigrinum*, a much greater variation has been found than was anticipated. The timing of separation of daughter cells by formation of new cell membrane does not seem to be correlated with any given stage of the telophase transformations of the nucleus. It may be early before any alterations can be seen from the anaphasic condition of the chromosomes to very late, when the daughter nuclei are reorganized almost into interphase. Much variation is present in the dimensions of the spindle, the area of the metaphase plate and the volume changes

evident in the cytosome. Change in cell form has been followed in detail and observed to be polygonal in inter- and prophase, to become progressively more spherical in meta- and anaphase, and to recover the polygonal condition in late telophase or early interphase.

In technique a number of advances have been made, and an animation unit has been assembled from equipment provided by the Carnegie Corporation of N. Y. A very smooth S-curve was found to be the haversine relation such as given in tables of recent editions of Handbook of Chemistry and Physics (haversine $\theta = (1 - \cos\theta)/2$). This smooth curve was found excellent for transitions such as starts and stops of movements, rendering these completely free of "jump." Difficulty in applying ink and paint to cellophane was overcome by mixing the pigment solutions with 2 per cent honey and 8 per cent of 10 per cent Fotofoam, 90 per cent of water color. The honey keeps the pigment solution from drying out completely, hence it does not crack or peel off. Fotofoam, apparently a bile salt derivative, reduces the surface tension of the color and allows it to spread easily on the shiny cellophane. A dissolving shutter with both manual and automatic control has been developed, using the new Polaroid-H glass. The two uncrossed plates of this glass transmit about 50 per cent of incident light; crossing the axes to 85° cuts the transmission to about 1/400 of the 50 per cent value, while at 90° the transmitted light from bright sun through an F: 1.5 lens is photographically inactive to even the fastest films. The decrease in transmission is almost linear, and until crossed more than 86° seems uniform throughout the spectrum. Beyond that limit, the violet end is less restricted than the longer wave-lengths.

Stimulation by intense flashes of ultra-violet light. E. Newton Harvey.

Any effective stimulus must be of sufficient intensity and also change rapidly in intensity. In order to obtain high intensity ultra-violet light a three microfarad condenser discharge at 20,000 volts is passed through a quartz mercury vapor sterilamp, according to the method of Rentschler. A single discharge, lasting a few millionths of a second, is capable of immediately killing bacteria, disintegrating protozoa, stopping cyclosis, ciliary and amoeboid movement, contracting myonemes and suppressing bacterial luminescence. In *Nitella* the protoplasmic rotation is reversibly stopped, sometimes only on the side of the cell toward the ultra-violet or only in a portion of the cell covered with quartz, not in that region covered with glass. It was found that sometimes the ultra-violet light would start an action potential locally and sometimes the potential was propagated over the whole cell, showing that ultra-violet light can stimulate in the same manner as electrical stimuli. The stimulation of vertebrate muscle and nerve is not yet certain. None of the above effects ever occur when the cells are shielded from the discharge by glass.

The influence of the medium on the radiosensitivity of sperm. T. C. Evans, J. C. Slaughter, E. P. Little, and G. Failla.

The ability of sperm to fertilize eggs is affected by roentgen radiation. However, it has been found that: (1) Sperm irradiated in the seminal fluid in concentrated form are very radioresistant. (2) If the seminal fluid (containing sperm) is diluted with sea water and then irradiated, the sperm become progressively more radiosensitive with dilution. The increase of radiosensitivity in a certain range of dilution is inversely proportional to the concentration of the sperm. (3) Sperm in dilute suspensions can be made more resistant again by the addition, before irradiation, of small amounts of egg albumen, gelatin, *Arbacia* egg water, and glycylglycine. (4) Sperm, in sea water, made inactive (by centrifugation) are more radioresistant than those actively swimming about during the exposure to the roentgen radiation. (5) The actions possibly involve two stages: (1) some harmful agent is momentarily produced in the water, and (2) the activity of the sperm affects the amount of contact with the harmful agent.

The effect of roentgen radiation on the fertilizing capacity of the sperm may be regarded as an indirect effect which can be altered by changing the medium. A more direct action of the radiation (not easily modified by the medium) is the delay in cleavage observed when eggs are inseminated with irradiated sperm.

Comparative pharmacology of myogenic and neurogenic hearts. C. Ladd Prosser and George L. Zimmerman.

The hearts of mollusks and adult vertebrates are inhibited by acetylcholine. These are myogenic hearts. The hearts of decapod crustaceans of insects and of *Limulus* are accelerated by acetylcholine and are neurogenic.

Acetylcholine accelerates and raises the tonus of the hearts of *Arenicola cristata*. In high concentration it stops the heart in systole. The threshold is one part in 10^8 without eserine. Eserine potentiates and atropine antagonizes the action of acetylcholine. The dorsal vessel is accelerated by acetylcholine but higher concentrations are required. Small amounts of potassium added to sea water bathing the *Arenicola* heart accelerate and raise the tonus while small amounts of excess calcium slow the hearts. A pacemaker is located in the small vessel connecting the laterogastric vessel with the heart. Adrenalin accelerates the hearts and in high concentrations stops them in diastole. From the above results we postulate that the heart of *Arenicola* is neurogenic.

In the *Limulus* embryo the heart begins its beat myogenically on the twenty-first day. It becomes neurogenic at about the twenty-eighth to thirty-third day. During the myogenic period this heart is insensitive to acetylcholine (1 in 10^4) with or without eserine. Beginning from the thirty-first to thirty-fifth day the hearts are accelerated by acetylcholine.

The heart of *Daphnia* is inhibited by acetylcholine and in the heart of *Eubranchippus* there is no effect (as in early *Limulus* and in vertebrate embryos). The heart of *Talorchestia* is accelerated by acetylcholine.

It is suggested that those hearts which are accelerated by acetylcholine are neurogenic and that those which are inhibited or unaffected are myogenic.

Structures concerned with yolk absorption in Squalus acanthias. Lois E. TeWinkel.

In Balfour's monograph on the Development of Elasmobranch Fishes (1876) and in a paper by Beard (1896, *Anat. Anz.*, **12**: 334) it is stated that yolk, in the later embryonic stages of these fishes, passes bodily up the yolk stalk into an internal sac. This sac is an outgrowth of the stalk at its point of entrance to the intestine and yolk taken into it eventually reaches the alimentary canal, there to be digested.

Living *Squalus acanthias* embryos from 110–230 mm. in length and preserved specimens of 60 mm. have been studied. The internal yolk sac increases enormously in size between the 60 and 230 mm. stages. It is just clearly visible in gross dissections of the former, while, in the latter, the external sac has shrunk to an empty stub only 6 mm. in length and the internal sac is very large (approximately 45 mm. long and 18 mm. in diameter). The method by which yolk is transported to the internal sac has not yet been determined.

Sections show yolk platelets in the internal sac of a 60 mm. embryo but apparently not any are present in the intestine, whereas, in a 150 mm. specimen, the spiral valve region is filled with an emulsion of yolk in various stages of digestion. Cells of the simple, low columnar epithelium lining the external yolk sac contain scattered fat droplets and glycogen in the 110 and 150 mm. specimens studied, indicating that, so long as the external sac is large and well vascularized, it plays some part in embryonic nutrition.

The distribution of elastic tissue in the arterial pathway to the carotid bodies in the adult dog. William H. F. Addison.

In the examination of many series of sections through the region comprising the bifurcation of the common carotid artery, the carotid sinus and the carotid body, there is found great uniformity in the structural tissues constituting the walls of the arterial vessels supplying blood to the carotid body tissue. But, as is frequent in the vascular system, the arrangement of the vessels may present many variations. In the case here reported, from the right side of an adult dog, the most striking variation is that the carotid body is not aggregated into one large mass but is distributed along the usual arterial pathway as several separate masses.

The blood supply to the carotid body in the dog is from the occipital artery, which in this animal is the first branch of the external carotid artery above the bifurcation of the common carotid artery. The occipital artery arises at a variable distance from the bifurcation and sometimes from the bifurcation itself. In the present case there is an interval of 3 mm. between the bifurcation and the origin of the occipital artery. The structure of the walls of the beginning is different from that of the rest of the occipital artery, inasmuch as it is elastic-walled, non-muscular, similar in structure to the carotid sinus. Because of its structure and its wider diameter than the rest of the occipital artery, this beginning part of the occipital artery may be called the occipital sinus. The further course of the pathway to the carotid body is as follows. From the occipital sinus is given off a short branch which is at first elastic-walled. This branch soon divides into two sub-branches, of which one has muscular walls and the other has elastic-tissue walls. The latter gives off the blood supply to the carotid body and then becomes muscular in character.

In the present case this arrangement of the elastic-walled vessels is present, but the carotid tissue is distributed at intervals alongside the carotid sinus, its elastic-walled branch and the latter's elastic-walled sub-branch. From each of these parts of the arterial pathway little vessels come off to supply the separate masses of carotid tissue, and in each little vessel the wall is elastic in structure.

Thus, in this case where the carotid body tissue is divided into small portions, each portion is still provided with blood through an elastic-walled non-muscular series of channels, while the continuation of these vessels, except those terminating in the carotid tissue, is always muscular, and under the control of the vasomotor system.

Behavior of the arterioles in hypertensive rabbits, and in normal rabbits following injections of angiotonin. Richard G. Abell and Irvine H. Page.

It is well known that in hypertensive patients, and in animals made hypertensive experimentally, there is an increase in resistance to blood flow. Although it has been observed that the retinal arterioles of hypertensive individuals are narrower than normal, there are no reports of measurements of their diameters before, as well as after the development of hypertension.

In the present experiments, living arterioles in transparent moat chambers in ears of normal rabbits were observed with the microscope, and their diameters measured. The animals were then made hypertensive either by the method of Goldblatt (1934) or the method of Page (1939, 1940), and the diameters of the same arterioles measured again.

Of the 7 operated rabbits, 4 became hypertensive. The blood pressure rose to from 1.4 to 2.1 times the normal level. The arterioles constricted from approximately 0.4 to 0.7 their control diameters when the rabbits became hypertensive.

No constriction occurred in those rabbits which did not become hypertensive.

The capillaries and venules did not constrict in any of the experiments.

In order to see whether the arteriolar constriction that occurred in the hypertensive rabbits might have been due to angiotonin, 0.2 cc. of angiotonin was injected intravenously into a normal rabbit, and the resulting arteriolar constriction measured. After the arterioles had returned to their control diameters, the rabbit was made hypertensive, and the resulting constriction of the same arterioles measured.

In both instances the arterioles constricted to approximately 0.5 their original diameters, and the blood pressure rose to about 1.4 times its control level.

This suggests that the arteriolar constriction that occurred in these hypertensive rabbits might have been due to angiotonin.

It should be emphasized that these studies have been made on vessels in the ears of rabbits, which are notoriously active in dilatation and constriction; consequently the *extent* of constriction found here should not be applied to other peripheral arterioles without further study.

August 27

Catalysis of ionic exchanges by bicarbonates. M. H. Jacobs and Dorothy R. Stewart.

The acceleration of hemolysis in solutions of NH_4Cl by low concentrations of bicarbonates, first observed by Ørskov, was explained by Jacobs and Parpart as essentially a catalysis of diffusion involving entrance of CO_2 and NH_3 into the cell, conversion of CO_2 into HCO_3^-, exchange of HCO_3^- for Cl^-, reconversion of HCO_3^- into CO_2, and so on. This principle can be extended to other ionic exchanges in the erythrocyte in which ammonium salts are not concerned. In general, as long as the necessary pH differences exist between the anion-permeable cell and its surroundings, and anions such as Cl^- are available for exchange, a reaction which for brevity may be represented as $HCO_3^- \rightleftharpoons CO_2 + OH^-$ may take place in opposite directions on the two sides of the membrane, the resulting cycle leading to the final equilibrium distribution of ions far more rapidly than is possible in the absence of bicarbonates. This catalysis-like effect is illustrated by the volume changes of erythrocytes that occur on changing the reaction of the surrounding medium or on suspending the cells in a solution of a salt with a bivalent anion such as SO_4^{--}. With certain limitations and qualifications, changes in the amounts of bicarbonates in the solution affect only the rate of the process and not the position of the final equilibrium.

The rôle of carbonic anhydrase in the catalysis of ionic exchanges by bicarbonates. Dorothy R. Stewart and M. H. Jacobs.

The theory suggested in the preceding abstract for the catalytic effect of bicarbonates on the attainment of certain ionic equilibria involves the reversible reactions

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO^-.$$

The first of these reactions is known to be strongly accelerated in both directions by the enzyme carbonic anhydrase. Considerable support is therefore given to the theory by the observation that the catalysis-like effect of bicarbonates on ionic exchanges in the erythrocyte is in turn strikingly influenced by this enzyme. The importance of carbonic anhydrase in such processes can be shown either by adding it to the medium in which the cells are suspended or by inhibiting its action within the cells by means of sulfanilamide or cyanides. In general, under the conditions of these experiments, the enzyme is more effective inside than outside the cells, but under certain circumstances its external effect may also be very striking. Inhibition of the enzyme within the cell by cyanides occurs almost instantly, that by sulfanilamide reaches its maximum only after several minutes. Both effects may readily be reversed by washing the cells.

Some effects of desoxycortico-sterone and related compounds on the mammalian red cell. Martin G. Netsky and M. H. Jacobs.

The sodium salt of the phosphate ester of desoxycortico-sterone (Na DOC PO_4) was found to produce sphering of human erythrocytes in concentrations as low as 1 mg. per cent or 2×10^{-5} mols per liter. The sodium salt of the phosphate ester of di-desoxycortico-sterone (Na di DOC PO_4) produced sphering at 0.4 mg. per cent or 5×10^{-7} mols per liter; 21-sodium hydrogen phosphate of 3-acetoxy-Δ^5-pregnene-21-ol-20-on (Na AcO pregnene PO_4) also produced sphering, but sodium glucuronidate pregnanediol did not. Neither compound E, nor free desoxycortico-sterone, nor Kendall's amorphous fraction gave sphering. Sphering ability seems to be associated with molecules of polar : non-polar structure, although a special form of non-polarity is required. Sphering can be reversed or inhibited by the addition of serum protein, the amount of protein necessary being the same in either case. The reaction is quantitative, 1 mg. of either Na DOC PO_4 or Na di DOC PO_4 being equivalent to approximately 10 mg. of serum protein. In higher concentrations, those substances which produce sphering are directly hemolytic. Direct hemolysis is also prevented by the addition of serum protein, and apparently the same type of chemical structure is required for it as for sphering. The effect of some of these substances on permeability to ammonium chloride and to glycerol was studied by the hemolysis method. In the case of ammonium chloride, the hydroxyl-chloride ion exchange was inhibited and hemolysis slowed markedly, both in human and beef cells. The effect on glycerol hemolysis of beef cells was a decrease in the time of hemolysis at all pH levels. For human cells this was also true at pH levels more acid than about 6.8, but at higher pH values, the effect was a more complicated one, low concentrations increasing the time, higher ones decreasing it below the level of the control. For the three Na PO_4 salts, the order of activity was: Na di DOC PO_4 > Na DOC PO_4 > Na AcO pregnene PO_4. Similar effects on glycerol permeability, obtained with compound E and Kendall's amorphous fraction, indicate that an extremely polar : non-polar structure is not required for permeability changes.

Permeability of the Arbacia egg to potassium.[1] Herbert Shapiro and Hugh Davson.

The permeability of the *Arbacia punctulata* egg to ions, and the problem of the maintenance of concentration gradients in this cell, have hitherto not been investigated. Chemical analyses were made of the potassium content of the egg and of sea water. The eggs were found to have approximately twenty times as much potassium as the sea water. Fertilized eggs contained very nearly the same amount of potassium as unfertilized. Suspensions of eggs were placed in a shallow layer on the bottom of Erlenmeyer flasks, and oxygenated by passing moistened oxygen over the suspensions. The flasks were immersed in a thermostat maintained at 25.6° C. Samples of suspension were taken at regular intervals, centrifuged, and the supernatant fluid analyzed chemically for potassium content. Runs were made over periods varying from two to seven hours. Total cell volume was determined by measurement of cell diameters, and of cell concentration by a dilution method. Both unfertilized and fertilized eggs lost potassium on coming in contact with sea water, though at a slow rate; from about 1.5 to 8 per cent of the cellular potassium diffused out in a two-hour period. Eggs placed in nitrogen also lost potassium, though at a rate not markedly different from that of eggs in oxygen. Eggs in artificial sea water with five times the normal potassium content accumulated potassium, and did this against a gradient. When placed in artificial sea water

[1] This investigation has been aided by a grant from the Penrose Fund of The American Philosophical Society.

containing four times the normal amount of calcium, fertilized eggs lost potassium more rapidly than those in normal sea water. When the excess calcium sea water was replaced by normal sea water, potassium began to reenter the cell, once more against a gradient.

Lipo-protein complexes in the egg of Arbacia. A. K. Parpart.

Determinations of the total lipid were made on "lyophylled" eggs of *Arbacia*. Values of total lipid were 5.4 per cent of the whole egg or 26.9 per cent of the solids of the egg. By methods found applicable to erythrocytes it was found that 77 per cent of the total lipid behaves as though it were bound to protein.

Eggs exposed to a 30 parts NH_4Cl, 70 parts sea water mixture for four to ten hours showed no decrease in the amount of lipid bound to protein. Eggs undergoing development for five hours did not change in total lipid or in lipid bound to protein.

These data suggest that the major portion of the lipid acts as a structural component of the egg cell more than as a metabolic component.

The relation between protoplasmic streaming and the action potential in Nitella and Chara. Samuel E. Hill.

Much past work has shown that protoplasmic streaming is profoundly affected by passage of electrical currents through the cell, or by passage of action currents along the cell. Does presence or absence of streaming bear any relation to electrical irritability?

Under certain conditions, for example, soaking for three days in distilled water, *Nitella* cells lose their ability to give an electrical response to stimulation, yet the protoplasmic streaming continues. Streaming is thus not a sign of irritability.

The streaming may be stopped by very weak electrical stimulation. If the applied voltages are small enough, the streaming slows down and gradually comes to a stop, no action current appearing. If, however, a larger stimulating voltage is applied, an action current appears and the streaming stops abruptly. After 30 to 60 seconds the streaming begins anew, starting and stopping abruptly several times as if pulling against high viscosity. After a few seconds of this the streaming again becomes regular. These abrupt starts and stops are accompanied by no electrical changes. During the time while the protoplasm is at a standstill, it is possible to provoke an action current by electrical stimulation. This can be repeated at intervals (about 25 seconds) for 30 minutes or more, every stimulation being followed by an action current and the protoplasm at a standstill all the while. After a rest of a few minutes the streaming begins again.

The streaming appears to have no antecedent relation to the action current, since presence of streaming does not indicate electrical irritability, nor absence of streaming indicate failure of electrical response.

Observations on luminescence in Mnemiopsis. Aurin M. Chase.

Harvey and Korr (*J. Cell. Comp. Physiol.*, 1938) found that extracts or fragments of the photogenic cells of *Mnemiopsis leidyi* can luminesce even in the complete absence of oxygen. Under such conditions continuous luminescence occurs rather than the brief flashes characteristic of the living organism; an indication that nervous control of the process has disappeared.

The present experiments concern luminescence of the intact, living animal. Upon electrical stimulation through the sea water flashing occurs along the rows of swimming plates. After complete de-aeration (thirty to forty minutes flushing with purified hydrogen) no flashing can be elicited by electrical or mechanical stimulation. Three to five minutes after re-admitting air the animal again responds.

The cycle of de-aeration and re-aeration can be repeated as many as three times before the animal dies. Movement of the swimming plates stops at about the same time that luminescence on electrical stimulation ceases. As the animal begins to disintegrate, either in an atmosphere of air *or of hydrogen*, a dim, continuous luminescence gradually appears along the rows of swimming plates. This lasts for about an hour, and undoubtedly represents the basic luminescent reaction as studied by Harvey and Korr, freed from its normal nervous control.

Mnemiopsis in sea water (air present) loses its ability to luminesce on electrical stimulation within 15 seconds after addition of 0.0001 *M* KCN, although the swimming plates continue to move for ten to twenty minutes. Eserine (1 : 2,000) increases the sensitivity to luminesce on mechanical stimulation and also increases the duration of the luminescent flashes. Addition of acetylcholine (1 : 3,000) enhances this effect. Returning the animals to plain sea water gradually restores the normal response. Adrenaline (1 : 100,000) apparently decreases the sensitivity but the effect is less clear-cut than the increased sensitivity caused by eserin and acetylcholine.

Papers Read by Title

Photodynamic studies on Arbacia eggs. Fred W. Alsup.

Rose bengal in concentrations of 1 part dye to 20,000 or more parts sea water and eosin in a concentration of 1 : 2,000 have no effect, in the dark, on the relative viscosity of the inner protoplasm of the unfertilized eggs of *Arbacia punctulata*. However, when the eggs are exposed in either of these dye solutions to light from a 1000-watt electric bulb, i.e., exposed to photodynamic action, the viscosity is markedly increased. On the average, the viscosity of eggs exposed to photodynamic action is about 40 per cent higher than that of unexposed eggs. Increases in viscosity can be detected within 5 seconds after the exposures and reach a maximum in about 1–5 minutes after the exposures.

The unfertilized eggs of *Arbacia* become activated when exposed to photodynamic action. Most of them show marked amoeboid movement and do not cleave when left in the dye solutions or removed to sea water. A very low percentage of these eggs divide in an irregular fashion. If eggs are removed to mixtures of sea water and isotonic calcium chloride soon after they are exposed to photodynamic action, a much higher percentage cleave irregularly. Apparently calcium strengthens the cortex of the eggs, which has been liquefied by photodynamic action.

Disruption of mitosis in Colchicum *by means of colchicine.* Ivor Cornman.

Colchicum, the commercial source of colchicine, contains a concentration of the alkaloid sufficient to block mitosis in other plants. By growing excised roots of *Colchicum* corms as temporary cultures in small vials, it has been possible to test a wide range of conditions upon uniform material. During the 8½ hours of the experiments, mitosis continued normal in tap water, in 1 per cent colchicine, and in sucrose isomolar with the effective colchicine concentrations. Mitosis was blocked in *C. byzantinum* by 5 per cent colchicine and in *C. autumnale* by 10 per cent. The cytological picture in *C. byzantinum* is typical of colchicine effects: disappearance of the spindle structure and then on the spindle material; accumulation of blocked metaphases; inhibition of the cell plate; appearance of tetraploid and binucleate cells. Upon removal from the colchicine, the cells recovered and continued dividing. They appeared normal except for the abnormal orientation of the spindles. Onion roots cultured in the same way showed typical colchicine effects in 0.01 per cent colchicine. It is concluded that the spindle mechanism in

Colchicum is the same as in other angiosperms with regard to the colchicine effect, and that the immunity of *Colchicum* to its own alkaloid resides in some extra-mitotic protective mechanism.

The effect of roentgen radiation on the jelly of the Nereis zygote. T. C. Evans.

Several investigators have reported that irradiation of *Nereis* ova results in an extreme swelling of the fertilization membrane following insemination. The swelling of the fertilization membrane is related to the amount of jelly retained within the membrane. Within a few minutes after insemination, unirradiated eggs will exude the jelly rapidly and it will pass through the membrane to surround the zygote with a thick viscous layer.

In this investigation zygotes have been irradiated after the jelly has been exuded, and it has been found that the radiation disperses the jelly immediately. The dosage required is about 44,000 roentgens.

The action of the roentgen radiation on the jelly, after it has been produced externally, is similar to that previously reported for the jelly of *Arbacia* and *Asterias* eggs.

Tests of nucleoli and cytoplasmic granules in marine eggs. R. Ruggles Gates.

In testing the nucleoli of the eggs of *Asterias, Arbacia, Mactra* and *Chaetopterus* for phospholipids the absence of lipoids and phospholipids from these bodies was shown by negative tests with Sudan III, osmic acid, Scharlach R and Feulgen without hydrolysis. In *Mactra* eggs treated with chloroform the nucleolus was unchanged. Observation of fresh eggs of these genera in sea water under an immersion lens showed that the nucleolus consists of two parts, like immiscible fluids, one enclosed within the other. The outer part is more quickly soluble in tap water than the inner portion.

While the nucleus is unaffected when Feulgen is added without previous hydrolysis, the cytoplasm in all these eggs unexpectedly showed a gradually deepening magenta color, indicating the presence of substances which have a free aldehyde group. The same reaction was obtained with the muscles of *Chaetopterus*. The cytoplasmic substances are relatively insoluble in water but more soluble in alcohol. They appear to belong to the acetalphosphatids of Feulgen and Voit. *Fucus* eggs and oögonia showed no color change at ordinary temperatures, so these substances may be characteristic of animal cells; but on exposure to air or rise of temperature a pink color develops in certain tissues of the *Fucus* thallus in Feulgen.

When the cytoplasm of *Chaetopterus* eggs is examined under an immersion lens after the Feulgen reaction, many of the granules, both large and small, are magenta in color. In crushed eggs, some granules are seen to be deep magenta, some pale, some uncolored, and there is a diffuse pink in the cytoplasm. The smallest granules are most intensely colored, some granules of all sizes remaining uncolored.

Sex-linkage of stubby (sb) in Habrobracon. Russell P. Hager.

Linkage of fused (fu) with the sex alleles has been demonstrated to occur with crossing-over in 8.6 per cent (Speicher) to 17.6 per cent (Bostian) of the cases (*cf.* Whiting, P. W., *J. Morph.*, **66**: 323–355). Since stubby (sb) was known to be linked with fused, tests were made to determine the percentages of crossing-over between stubby and fused, and stubby and the sex alleles so that the order of the factors could be mapped. Females heterozygous for stubby and fused (sb/fu) yielded 479 sb, 507 fu, 136 sb fu, and 179 + haploid males. Crossing-over between

stubby and fused is therefore about 24.2 per cent as data collected previously by others have indicated.

Orange-eyed females heterozygous for stubby were mated to stubby males:—o sb xa/o xb × sb xa or sb xb. The crosses with sb xa yielded 370 (linked) heterozygous and 228 (cross-over) stubby females; 10 (cross-over) heterozygous and 32 (linked) stubby diploid males. The crosses with sb xb yielded 642 (cross-over) heterozygous and 1132 (linked) stubby females; 70 (linked) heterozygous and 57 (cross-over) stubby diploid males. The cross-over class among the biparental males is of the type opposite to the cross-over class among the females. The cross-over percentage was calculated by extracting the square root of the products of the comparable classes. $\sqrt{642 \times 228}/(\sqrt{642 \times 228} + \sqrt{1132 \times 370})$. Crossing-over between stubby and the sex factor was accordingly 37.5 per cent among the females and 33.6 per cent among the diploid males.

The order of factors is therefore:

sex—(8.6 per cent to 17.6 per cent)—fused—(22 per cent to 24.2 per cent) stubby: sex.....(33.6 per cent to 37.5 per cent).....stubby.

The elasmobranch interrenal; a preliminary note. The interrenal body of Alopias vulpinus (Bonnaterre). E. R. Hayes.

This description is based upon the examination of a thirteen-foot "thresher" shark, *A. vulpinus*. The interrenal is an unpaired, elongate, yellow body lying between the caudal portions of the two kidneys and immediately dorsal to the posterior cardinal sinus. Slightly asymmetric in position, the gland is more closely applied to the left kidney. Beginning 5 cm. from the posterior limit of the kidney, it extends craniad 30 cm. and is discontinuous at one point in its anterior half. In cross-section, the gland is roughly oval, the greatest diameter ranging from 8–10 mm. posteriorly and tapering to 2–3 mm. anteriorly.

Microscopically, a rather thin capsule is seen covering a strikingly uniform parenchyma which shows no lobulation. It consists of cords of lipid-laden cells interlacing with blood sinuses and clothed by the endothelium lining the sinuses. It is possible to distinguish only one type of cell in the parenchyma. This type closely resembles the "spongiocytes" of the mammalian adrenal cortex. The cells are heavily packed with droplets of Sudanophil lipid which also blacken with OsO_4 (labile in xylol) and with the Schultz test give a strongly positive reaction for cholesterol. Paralleling the uniformity of these reactions is the even distribution throughout the gland of a considerable amount of birefringent material. Certain cells show evidence of nuclear pycnosis although in other respects they are indistinguishable from neighboring cells. The life history of the cells of this gland remains to be worked out.

The cytology of Amoeba verrucosa. Dwight L. Hopkins.

Amoeba verrucosa is a fan-shaped form typified by longitudinal folds and grooves which are formed continuously on its superior surface. It feeds on a variety of plant and animal organisms including bacteria and rotifers. In feeding it flows over the prey, trapping it in a groove, or depression in the under-surface. Once the prey is trapped, the sides of the groove are extended downward and under until they meet and the victim is enclosed in an irregular tube. The folds or sides of the groove, when they meet, form closely approximating, irregular lines, which remain visible for some time. The tube is not closed completely. When the prey is rejected as food it is squeezed out from the posterior end of the tube; when accepted, only the water is squeezed out and the food is drawn into the interior. The food vacuole thus formed contains little or no fluid.

Once the food vacuole is inside, small neutral red staining vacuoles which are very numerous in the cytoplasm are attracted to its walls and enter by coalescence. This process renders the recently formed food vacuole stainable with neutral red. As digestion proceeds the color is first red, then yellow, and finally colorless, just before the vacuole is expelled. Due to the addition of neutral red vacuoles, food vacuoles during digestion may contain considerable fluid material, but most of this is absorbed by the cytoplasm before the residues are expelled.

Contractile vacuoles arise by coalescence and swelling of smaller vacuoles. The cytoplasm contains numerous small rod-shaped and spherical bodies which come to the region where contractile vacuoles form through the plasmagel and when the gel transforms into sol, they cluster around the old contractile vacuole. These small bodies, as well as the membrane of the contractile vacuole, take Janus Green B faintly. The small bodies which stain finally with Janus Green B form small contractile vacuoles by coalescence and swelling.

Observations on the melanophore control of the cunner Tautogolabrus adspersus (Walbaum). George W. Hunter, III, and Edward Wasserman.

Background responses under a constant source of illumination were studied in the cunner, *Tautogolabrus adspersus*. Black-adapted fish responded to a white background in 15 seconds, reaching a maximum in 50 minutes, while white-adapted fish placed in a black background required an average of about ten seconds for the first phase of the reaction and 50 minutes to complete the response. Responses of white- and black-adapted fish to yellow and blue backgrounds were intermediate to the controls on white and black backgrounds. Placing cunners in complete darkness produced paler fish but darker than the white-adapted ones, while enucleated cunners gave no response to background changes. Both experiments indicate the importance of the eye in normal responses to background changes.

Cutting of caudal fin rays and the accompanying nerves of white-adapted fish produced dark bands due to expanded melanophores in 40–45 seconds and a maximum response in 45–60 minutes. Windows gave similar results. Cuts proximal to the band produced darkening of the freshly cut area distally as far as the original cut. Electrical stimulation of the medulla and roof of the mouth at the lowest frequency possible on a Harvard inductorium using 6 volts, produced a temporary darkening of white-adapted fish lasting about a minute, while the highest frequency gave a partial but distinct blanching of black-adapted fish.

Observations on recutting of fading bands and the fading time of single and multiple fin ray cuts are still being carried on. Experiments on hypophysectomized fish as well as the effects of drugs and salts on melanophores are in progress and will be reported elsewhere.

The evidence accumulated thus far suggests that this northern representative of the wrasses, the cunner, has a melanophore system controlled by adrenergic and cholinergic sets of nerve fibers. While the pituitary has given a positive test for intermedin when injected into a blanched frog, its rôle in the normal control of melanophores has not yet been determined.

The control of melanophores in the cunner is being studied as one phase of the problem dealing with pigment production and its control about the cysts of the trematode metacercaria, *Cryptocotyle lingua,* which occur on the scales of the cunner.

The influence of temperature on reconstitution in Tubularia. Florence Moog.

The fact that the body size of Metazoa is generally greater at lower temperatures has been noted frequently. In *Tubularia* the size of the reconstituted

hydranths is similarly affected by low temperature. The table shows the length of the reconstituents and the time for their formation in one experiment consisting of batches of 25 sections of stems, each 6 mm. long, kept in 200 cc. of sea water.

Temperature	Time to constriction of primordium	Length of primordium
° C.	*hours*	*micra*
22.2	44.7	854
19.0	55.1	918
14.0	59.5	1050

In three experiments the reconstituents at 14° averaged 15.2 per cent longer than at 22°, and their time of formation was 39.3 per cent longer.

Some authors have pointed out, on the basis of studies on embryonic and adult vertebrates, that the larger body size might be accounted for by the fact that less food is needed to maintain tissues at low temperatures, so that more can be used in building new protoplasm. But since an external food supply is not a factor in reconstitution of *Tubularia*, it seems likely that the increased size is due at least in part to modification of chemical equilibria. Low temperature would most likely slow the chemical processes involved in the conversion of tissue, without markedly affecting diffusion, so that the change in size might result from the deeper penetration of oxygen.

It is interesting to note that low temperature increases the size of the reconstituted hydranth while decreasing its rate of formation. Other agents, such as low oxygen concentration, low pH, cyanide, azide, and urethanes, which decrease the rate, decrease the size also. Evidently the processes underlying reconstitution velocity and determination of size of the primordium are independent to a considerable degree.

Factors influencing the pigmentation of regenerating scales on the ventral surface of the summer flounder. Clinton M. Osborn.

From summer flounders which had been black- or white-adapted, scales were plucked in a definite pattern from the naturally white lower surface and the fishes returned to their original tanks. It was apparent within two weeks that the regenerating scales on the white-adapted flounders were white, while those on black-adapted fishes developed melanophores. When black-adapted flounders were blinded by enucleation, the regenerated scales were pigmented regardless of the shade of the background, indicating that intact eyes were not essential to pigment production.

To test the direct effect of light, flounders prepared in three different ways were illuminated strongly underneath through special glass-bottomed aquaria. In one experiment white-adapted fishes were illuminated ventrally. The regenerated scales were *white*. In the next experiment the fishes were black-adapted (black walls and ceiling) while ventrally illuminated and grew melaninated scales. Finally, blinded fishes (in the dark phase, but not maximally black) regenerated pigmented scales when ventrally illuminated. The results from these three experiments were qualitatively similar to the original observations, indicating that bright illumination had little influence on the color of the regenerating scales. This was further substantiated in experiments where the regenerated scales were melaninated on black-adapted fishes in tanks dimly lighted during the day and totally dark at night.

It is concluded that the color of scales regenerating on the naturally white lower surface of the summer flounder is influenced primarily by the physiological factors (nervous and endocrine) which cause the upper surface of the fish to assume the pale or the dark phase. Factors which produce the dark phase favor

the development of melanophores in regenerating ventral scales while physiological agents causing the pale phase allow white scales to regenerate. Light appears to have little direct effect on the color of regenerating scales although melanination is somewhat accelerated by light in physiological conditions which favor pigmentation.

Hypersensitization of catfish melanophores to adrenaline by denervation. G. H. Parker.

The melanophores in a denervated caudal band of a catfish of intermediate tint will concentrate their pigment after the fish has received 0.008 or even 0.004 of a milligram of adrenaline per 100 grams of fish. These dosages do not induce noticeable pigment changes in the innervated melanophores of the rest of the fish. Weaker doses of adrenaline have no obvious effect on either denervated or innervated melanophores. Stronger doses induce pigment concentration in both denervated and innervated melanophores. The greater sensitivity for adrenaline thus shown by the denervated melanophores as compared with the innervated ones may be due to the fact that, after the color nerves have been cut, not only the adrenergic but the cholinergic fibers degenerate. Consequently the injected adrenaline does not find in the denervated bands the local dispersing agent acetyl choline for an opponent as it does in the innervated regions. Hence in denervated bands adrenaline is able to act efficiently at lower concentrations than in innervated areas. There is no reason to suppose that denervation alters the melanophores themselves. Denervation in the catfish appears merely to remove an adrenaline opponent and thus to give this agent more effective sway. This explanation of melanophore hypersensitization to adrenaline may not apply to other instances of the special sensitization of effectors, but it appears to meet the requirements in the melanophores of the catfish.

Implants consisting of young buds, formed in anterior regeneration in Clymenella, plus the nerve cord of the adjacent old part. Leonard P. Sayles.

Worms were cut to regenerate anteriorly. After 2 to 9 days, any newly-formed material plus the adjacent anterior nerve cord was inserted into the thirteenth segments of hosts. In some cases the original buds dropped off. Then either (1) no material formed at the implant or (2) small new buds, each terminating in the dorsal half of an anal segment, developed. All of the latter terminated ventrally in truncate regions. These results were similar to those obtained when pieces of anterior nerve cord from non-regenerates were inserted at posterior levels of hosts.

When the original bud was retained, the results were modified. When only a small blastema had regenerated, the implant gave rise, in many cases, to a bud terminating in a partial anal segment dorsally and either a large, conical mass or a weakly developed peristomium ventrally. When a small, 3-day type head was present, this bud might continue to develop. Frequently, however, a partial anal segment appeared on the dorsal side of the bud. Then the portion of the original bud beyond this anal segment regressed until it was only a small ball of pigmented material. This ball then dropped off, leaving a growing, incomplete tail bud in place of the original head. When the implant included a well-developed head of the type produced after 5 to 9 days, no anal segment elements appeared. In some cases the bud continued to grow and organize, in others it regressed somewhat.

A young head bud, therefore, was not able to maintain itself against the more powerful tail-forming influence of the host's posterior segments, although this bud did modify the induced bud to some extent. Older buds, however, were retained without the host producing any additional structures.

Chaos nobilis Penard in permanent culture. A. A. Schaeffer.

From all published accounts of amoebas probably belonging to the genus *Chaos* Linnaeus, six well-attested species emerge. Three of these: *diffluens, nitida, ncos, leschcri,* are uninucleate and two: *chaos* and *nobilis* are multinucleate. Excepting *nobilis,* all these species are readily cultured. *Nobilis* has been reported in the past (Vonwillier, Penard) as dying out in cultures. A few *nobilis* found in a ditch near Willow Grove, Pennsylvania, on April 20, 1941, maintained themselves in laboratory culture until now (August 20) and are slowly increasing in number. The rate of multiplication is much slower than in the other five species, for from 4 to 10 days elapse between divisions. This slow rate may be due to improper culturing methods, although from their appearance, one would judge these amoebas to be normal in every respect.

Von Stein (1867) was probably the first to see a *nobilis.* Next Butschli (1876) studied it and counted and measured nuclei in a wild culture. Penard studied it in 1902 and regarded it as a distinct species. Vonwillier later inclined to the view that the multinucleate amoebas which he found were like those of Calkins and Penard, but not like those of Butschli, Schubotz and Gruber. Lucy Carter described a multinucleate amoeba similar to Butschli's. The Willow Grove amoeba is similar to Penard's amoeba, but does not show the variability in number and nuclear size of Butschli's amoeba.

The two multinucleate species, *chaos* and *nobilis,* are distinctly different in size range, in nuclear structure, size, and number, and in rate of reproduction. Thus, the largest *nobilis* are about 250 μ in diameter and have from 80 to 90 ovoidal nuclei measuring 16 $\mu \times 12\,\mu$; the smallest are from 100 μ to 115 μ in diameter with 10 to 15 nuclei of about the same size and shape as those of the larger amoebas. The Willow Grove amoeba therefore corresponds very closely with Penard's *nobilis,* with Lucy Carter's, and with Butschli's multinucleate amoeba, but differs in nuclear number from Vonwillier's.

Further studies on Mactra egg cells. Victor Schechter.

The study of the problem of longevity in unfertilized *Mactra* egg cells was continued this summer along two directions. It was found that a non-dialyzable factor detrimental to the life of the eggs gradually develops in sea water considerably before the eggs show structural or functional deterioration. With regard to beneficial factors, dextrose down to the surprisingly low concentration of 0.001 per cent was found to be effective.

*The effect of centrifugation upon the oxygen consumption of Arbacia eggs.** Sidney F. Velick.

When unfertilized eggs of *Arbacia punctulata* are centrifuged in the cushioned medium of E. B. Harvey at a speed sufficient to stratify the cellular elements and stretch the cells to an axial ratio of about 1.5 to 1, the oxygen consumption is increased 60 to 120 per cent over that of the unstratified controls from the same egg suspension. The increment usually persists for several hours in the respirometer and does not result in membrane formation or cleavage. Upon fertilization, the oxygen consumption of the stratified eggs increases by the same order of magnitude as do the unstratified eggs. Return of the unfertilized stratified eggs to the spherical form is not accompanied by a decline in respiration, but a decline to the original level does occur after the spontaneous redispersion of the stratified granules has proceeded to a sufficient extent in a stationary vessel. The fat globules remain aggregated in a cluster long after the decline has occurred. As in the

* This work was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

case of the unstratified egg, the oxygen consumption increases progressively upon too rapid shaking, an effect which has been observed by others on unstratified eggs and attributed to damage of the membrane. These experiments, undertaken at the suggestion of Dr. Kurt G. Stern, are being continued.

Ectodermization of the larva of Arbacia. Allyn Waterman.

A reinvestigation of previous work on *Arbacia* development has been attempted by exposing cleavage stages, swimming blastulae and early gastrulae to 2,4-dinitrophenol, 3,5-dinitro-o-cresol, pyocyanin, methylene blue, neutralized iodoacetic acid and to modifications of salt proportions in an artificial sea water formula which supported typical development through the pluteus stage. The reactions of these various stages differ little and then only in degree which is apparently correlated with the stage of development. Strong concentrations of respiratory affectors retarded or inhibited differentiation and gastrulation. Among the different abnormal types were found ectodermized embryos and undifferentiated exogastrulae. Weak concentrations accelerated development by several hours, without any differential effect upon the germ layer derivatives, and these larvae died earlier than the controls or abnormal types. Iodoacetic acid provoked ectodermization, indicating a shift of the control of development to the material at the animal pole. Omission of SO_4, $MgCl_2$, $CaCl_2$, or an excess of $MgSO_4$ in the artificial medium caused ectodermization. In all a great variety of abnormalities occurred which differed little from those provoked by many other methods. Undifferentiated exogastrulae cannot be considered a type of endodermization. During early development, at least, *Arbacia* embryos show a wide tolerance to most ionic variations in their environment.

The degree of ectodermization was variable within the same culture, showing a differential susceptibility between individuals. Completely ectodermized individuals often attained giant proportions, developed cilia and an exaggerated apical tuft, and survived as long as other types. Less completely ectodermized larvae often possessed some skeletal material and an undifferentiated gut.

While these results may be explained by the ectodermal-endodermal gradient hypothesis of Runnström, they appear to furnish no support to the suggestion of Lindahl that carbohydrate metabolism predominates at the animal pole (Needham and Needham).

Studies on Zoöchlorella-free Paramecium bursaria. Ralph Wichterman.

Isolation cultures were made of 40 specimens of *P. bursaria.* One strain disclosed *P. bursaria* to be completely free of the alga *Zoöchlorella* but to have instead great numbers of optically active crystals. These crystals were especially abundant in the posterior region of the ciliates. Unlike green *P. bursaria,* which generally settle to the bottom of the culture dish and congregate toward the strongest source of light, the "white" ones swim actively throughout the culture medium which consisted of desiccated lettuce infusion. White ones showed the mating reaction with green individuals of certain other clones. The mean daily fission rate of green specimens which mated with the white ones was 1.1 divisions while white *P. bursaria* showed a faster division rate, namely 1.5.

Since green *P. bursaria* contained no crystals and zoöchlorella-free individuals contained many, green individuals were kept in complete darkness for varying periods of time up to 25 days in order to find out whether the zoöchlorellae disappeared and crystals appeared. It was found that green individuals kept in darkness even for 25 days lost some but not all of their zoöchlorellae. On the other hand, optically active crystals appeared in such specimens and darkness did not prevent the mating reaction from taking place. However, large clumps of individuals, so characteristic in early stages of the mating reaction in the same

strains when subjected to light, did not form when they were kept in darkness for a considerable length of time.

Zoöchlorella suspensions from green individuals of the mating type opposite from the white *P. bursaria* were made. When a white specimen was placed in such a suspension of zoöchlorellae, the ciliate ingested the algae rapidly. Food vacuoles contained from one to five zoöchlorellae. However, in less than a day, the white *P. bursaria* became darkly granular, sluggish, then died. Yet when zoöchlorellae from another strain which also mated with the white ones were placed with them, no such lethal effect took place. Both of the above-mentioned clones of green *P. bursaria* showed the mating reaction with each other.

An experimental study of intracellular pH in the Arbacia egg. Floyd J. Wiercinski.

The problem of determining the exact pH of the living cell by the microinjection of indicators presents numerous difficulties. In addition to the salt and protein errors of the indicators these difficulties include membrane formation, granular breakdown, and most important of all, the uptake of indicators by the granules. In order to avoid factors due to the presence of granules, indicators were injected into the hyaline region of the centrifuged *Arbacia* egg.

Ten sulfonephthalein indicators and certain mixed indicators were used. A mixed indicator has the advantage of a sharp color transformation point at a given pH. Varied results were obtained with the indicators under different conditions of experimentation.

Tests with phenol red show a pH < 7.0, brom thymol blue < 6.8 and > 6.0, brom cresol purple + brom thymol blue < 6.6 and > 6.0, brom cresol purple < 6.8 and > 6.0, brom cresol green + chlor phenol red > 6.2 and < 5.8, chlor phenol red < 6.6 and > 6.0, chlor phenol red + aniline blue > 5.8, brom cresol green + Na Alizarine S > 5.8, methyl red > 5.8, and brom cresol green > 5.6. When eggs were immersed in 0.29 M $CaCl_2$ at pH 6.1 somewhat lower values were obtained. This may be due to an augmentation of the injury reaction.

The results indicate a pH somewhere in the neighborhood of 6.2 for the hyaline protoplasm of the *Arbacia* egg.

Heat produced by respiring whole blood of Tautoga onitis and Mustelus canis. E. Alfred Wolf, Maryon Dytche, John D. O'Neal and Milton Schaffel.

The colorimeter vessel was a triple-walled, silver-mirrored Dewar flask of about 100-cc. capacity. Temperature rise was measured with a Beckmann thermometer. Oxygen was supplied by bubbling. A method was found to prevent foaming which did not injure the cells. The fish used for the investigation were selected for their availability in Woods Hole waters. The constant temperature water bath was kept at 15.3° C.

The gas used for oxygen supply for *Tautoga* was oxygen and oxygen plus 5 per cent carbon dioxide; for *Mustelus,* because of lack of time, oxygen only was used. This high percentage of carbon dioxide was selected in order to have a basis of comparison for further work with higher vertebrates. The fish used in this investigation are normally not exposed to such high pressures of carbon dioxide and could not survive such conditions. At such pressures the blood of these fish could not be saturated with oxygen and the fish would suffocate in spite of the rich supply of oxygen (R. W. Root, E. A. Wolf and others). Our present work strikingly verifies these findings: in pure oxygen the rate of heat production remained constant for hours; in oxygen plus 5 per cent carbon dioxide this rate decreased after about one hour of exposure; all curves changed from straight lines

to curves of decreasing slope. We interpret this change as signs of approaching suffocation in an abundance of oxygen.

The rate of heat production of blood is of significant magnitude in the bioeconomics of fish. This rate in oxygen was 82 calories per kg. of blood per hour for *Tautoga* and 51 cal. for *Mustelus*. In oxygen plus 5 per cent carbon dioxide the rate decreased to 40 cal. for *Tautoga*. A fish of comparable size at 15° C. would produce about 360 cal. per kg. of body weight per hour.

Effect of differences between stages of donor and host upon induction of auditory vesicle from foreign ectoderm in the salamander embryo. C. L. Yntema.

Operations were performed on the embryo of *Amblystoma punctatum*. Foreign ectoderm from embryos at stages from 9 (early gastrula) to 28 (late head-process) was placed in the ear region of host embryos at stages from 12 (late gastrula) to 35 (onset of circulation). Prospective body ectoderm was used in experiments in which the donor was an early or middle gastrula. At older stages prospective gill ectoderm was transplanted. The animals were preserved at stage 46 (beginning of feeding). The contribution of the grafts was determined by retaining the Nile-blue sulfate stain of the grafts in sections.

The ability to induce a small vesicle was retained by a host as advanced as stage 35. The ability to induce was greatest, as measured by older grafts, during the neural groove stage (13) and during completion of neuralation (stages 19 and 20).

The response of the prospective body ectoderm from early and middle gastrulae was masked by induction of neural tissue from the grafts. Prospective gill ectoderm from stages 12 and 13 formed auditory vesicles if the hosts were neurulae. When older hosts were used, this potency was not realized. The most normal vesicles induced by hosts at stage 35 were from ectoderm taken from donors at stage 22 (early head process). Ectoderm from stage 28 formed small vesicles when placed in the ear region of stage 20.

The following are some general conclusions. If an embryo can no longer regenerate a labyrinth, its gill ectoderm possesses under certain conditions the capacity to form an auditory vesicle, and the ear region may still induce a vesicle. Ectoderm which is competent to form an auditory vesicle in the ear region of some stages is not competent to do so at certain other stages. Both the stage of the foreign ectoderm and the stage of the host are factors which determine the response to form a vesicle.

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THE EQUILIBRIUM BETWEEN HEMOGLOBIN AND OXYGEN IN WHOLE AND HEMOLYZED BLOOD OF THE TAUTOG, WITH A THEORY OF THE HALDANE EFFECT

R. W. ROOT AND LAURENCE IRVING[1]

(From the College of the City of New York; Swarthmore College; and the Marine Biological Laboratory, Woods Hole)

The combination of oxygen with the blood of certain fishes occurs far less readily when the CO_2 tension is raised, but in a number of these same fishes hemolysis largely eliminates the sensitivity of the hemoglobin toward CO_2 (Black and Irving, 1938; Root, Irving, and Black, 1939). The peculiar effect of hemolysis indicates that the properties of the whole blood, as regards O_2-combination in the presence of CO_2, do not parallel the properties of the hemoglobin when released from the cell. One cannot, therefore, infer the properties of fish hemoglobin in their entirety from a study of whole blood alone. It has been further shown that the whole and hemolyzed blood of the tautog not only differed with respect to O_2-combination in the presence of CO_2, but the reciprocal effect of oxygenation on CO_2-combination showed significant differences (Root and Irving, 1940). In hemolyzed blood the ratio $\frac{-\Delta BHCO_3}{\Delta O_2}$ is apparently constant for any given CO_2 tension, whereas this is not the case in whole blood. The behavior of the whole blood is apparently exceptional, since it is commonly considered that for any given hemoglobin $\frac{-\Delta BHCO_3}{\Delta O_2}$ is constant (Henderson, 1928; Redfield, 1933*a*).

The material to be presented in this paper is in part an amplification of the work done by us on the blood of the tautog, *Tautoga onitis* (Linn.). The equilibrium between hemoglobin and oxygen in both whole and hemolyzed blood has been examined in detail over a wide range of CO_2 tensions. From the study a clearer picture has developed

[1] The authors are indebted to the U. S. Bureau of Fisheries at Woods Hole for the provision of laboratory space and facilities during the course of this investigation.

of the contrast in behavior of whole and hemolyzed fish blood. It has enabled us to describe theoretically not only the equilibrium that exists between hemoglobin and oxygen in the two conditions of the blood, but also to give an interpretation of the effect of oxygenation on CO_2-combination (Haldane effect) as observed in the blood of this fish.

Throughout this investigation the methods of handling the blood, equilibrating it, and analyzing the gas phases were the same as those described in the paper by Root and Irving (1940).

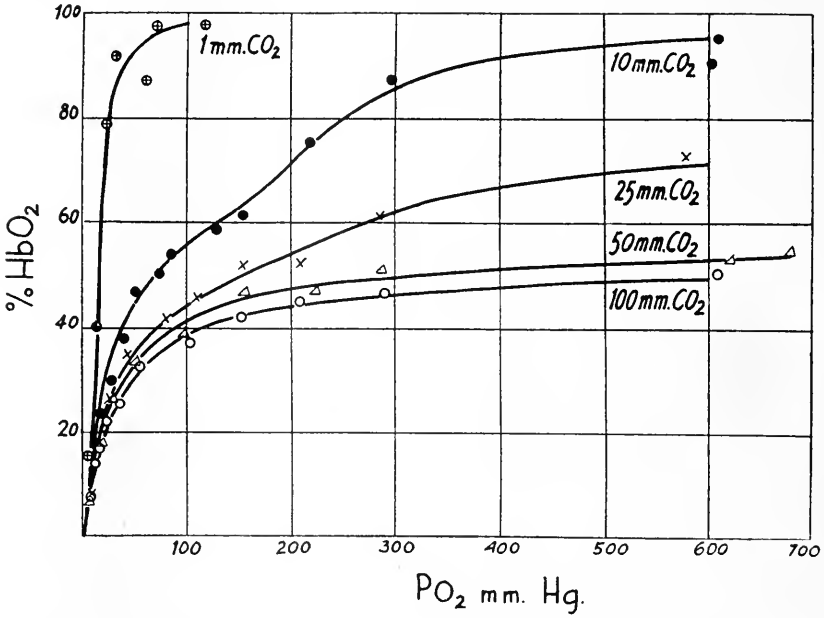

FIG. 1. Oxygen dissociation curves of whole tautog blood at 15° C. and constant CO_2-tensions. The curves have been drawn according to the equation indicated in the text, using the following constants:

PCO_2 mm. Hg.	α_1	α_2	α_4	α_0	K_1	$K_2 \times 10^2$	$K_4 \times 10^9$
0–1	0	1.0	0	0		.51	
10	.75	0	.25	0	.0286		.25
25	.60	0	.15	.25	.0286		.16
50	.56	0	0	.44	.0286		
100	.52	0	0	.48	.0286		

COMPARISON OF THE O_2-DISSOCIATION CURVES OF WHOLE AND HEMOLYZED BLOOD

The O_2-dissociation curves for both whole and hemolyzed blood of the tautog have been established at 10, 25, 50, and 100 mm. CO_2

tension. The former are shown in Fig. 1 along with a curve established in the virtual absence of CO_2 (data of Root, Irving and Black, 1939). Those for hemolyzed blood are presented in Fig. 2. Each curve represents the data from a single large sample of blood, with the exception of the one at 100 mm. CO_2 where two lots of blood were used. The curves at each CO_2 tension have been drawn from an equation which seemed best to fit the data, the constants used being shown in the table

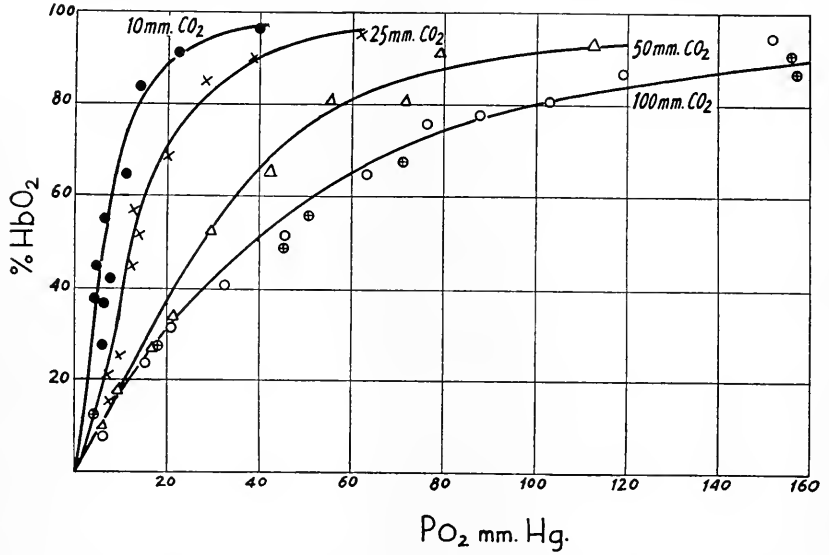

FIG. 2. Oxygen dissociation curves of hemolyzed tautog blood at 15° C. and constant CO_2-tensions. The curves have been drawn according to the equation given in the text, using the following constants:

PCO_2 mm. Hg.	α_1	α_2	α_0	K_1	$K_2 \times 10^2$
10	0	1.0	0		2.0
25	0	1.0	0		.59
50	.2	.8	0	.10	.11
100	.5	.5	0	.05	.033

beneath the figures. Reference to this equation and its implications will be made later.

If one compares in a general way the family of curves obtained for each type of blood the following major differences become evident: (1) the hemolyzed blood has a greater affinity for oxygen at comparable CO_2 tensions than has the whole blood; (2) the dissociation curves for hemolyzed blood at comparable CO_2 tensions are different in shape from those of whole blood; (3) the hemolyzed blood, up to 100 mm. CO_2

tension, shows no evidence of hemoglobin inactivation, whereas whole blood does; and (4) the reduction in affinity for oxygen with rise in CO_2 tension is different in magnitude in the two types of blood.

In order to further the comparison of the behavior of the two types of blood it is desirable to study as closely as possible the shape of the O_2-dissociation curves in each case. For our purpose this has consisted in an attempt to fit certain existing equations describing the equilibrium between hemoglobin and oxygen to the data for the curves. Once a suitable fit is obtained the theoretical implications of the particular equation employed offer some insight into the behavior of the hemoglobin at any particular CO_2 tension. Any attempt to fit existing equations to dissociation curves established at constant CO_2 tension, instead of constant pH, is perhaps open to criticism, but we have reason to believe that the results are not so totally different that the general conclusions drawn from such an analysis would be invalidated when the pH is kept constant.

It is obvious from examination of the data that the classical equation of Hill (1910) is too simple an expression adequately to describe all of the dissociation curves. We have therefore resorted to the equation suggested by Redfield (1933*b*) and used by Green and Root (1933) in describing the equilibrium between hemoglobin and oxygen in certain fish bloods. This equation is based on the theory that there are different components of the respiratory protein which act independently of each other in compliance with Hill's equation but with different values of n. If the O_2-dissociation constants of the components having values of n of 1.0, 2.0, 3.0, 4.0 etc., are designated by K_1, K_2, K_3, K_4, etc. and the fraction of the total O_2 bound by each of these components as α_1, α_2, α_3, α_4, etc., the fraction of the total respiratory protein present in the oxygenated form, Y, at any particular O_2-tension, χ, is given by the equation:

$$Y = \frac{\alpha_1 K_1 \chi^1}{1 + K_1 \chi^1} + \frac{\alpha_2 K_2 \chi^2}{1 + K_2 \chi^2} + \frac{\alpha_3 K_3 \chi^3}{1 + K_3 \chi^3} + \frac{\alpha_4 K_4 \chi^4}{1 + K_4 \chi^4}.$$

It is necessary in tautog whole blood to introduce the term α_0 to take into account the fraction of hemoglobin inactivated at high CO_2 tensions. The sum of α_0 plus the other fractions will equal 1. Fortunately it has not been necessary for us to use more than two terms of the general equation in describing the more complicated dissociation curves obtained with the blood of this fish. The simple curves require but one term and could as well be described with Hill's equation, providing we take into account the fraction of hemoglobin inactivated in whole blood at high CO_2 tensions.

The general results of our analysis of the O_2-dissociation curves, using the above equation, can be obtained by reference to the table of constants beneath Figs. 1 and 2, and to the curves drawn according to the equation, using these constants. It is clear that in whole tautog blood the O_2-dissociation curve in the virtual absences of CO_2 is sigmoid and is characterized by a value of $n = 2$, i.e. there is a single component of the hemoglobin uniting with two molecules of oxygen at a time. As CO_2 is added the dissociation curve not only moves to the right but becomes more complicated. Components with different values of n and dissociation constants sufficiently different to produce definite undulations in the curve come into view. Further addition of CO_2 brings about inactivation of some of the hemoglobin and finally simplifies the O_2-dissociation curve to the form of a rectangular hyperbola, i.e. there is now a single component of the hemoglobin combining with one molecule of oxygen at a time. We see, then, that CO_2, in addition to inactivating a portion of the hemoglobin, completely changes the O_2-dissociation curve of whole blood from a second to a first power curve, and that the intermediate stages in the conversion apparently produce different components that unite with different amounts of oxygen at a time, thus complicating the dissociation curves in this region.

The picture presented is essentially that obtained earlier by Green and Root (1933) on the same blood at 25° C. It differs in that their intermediate curves did not show the marked inflections that ours show. However, a too rigorous comparison of the intermediate curves is not justified since ours were established at a constant CO_2 tension instead of constant pH, as theirs were, and at 15° C. instead of 25° C.

In hemolyzed blood, as the table beneath Fig. 2 will indicate, not only are the dissociation constants for the curves much larger than those for whole blood at comparable CO_2 tensions, but those components characterized by a value of n greater than 1 persist at CO_2 pressures at which they have definitely disappeared in whole blood. Furthermore, it can be seen that there is no necessity for assuming that any of the hemoglobin has become inactive, as was the case in whole blood. There is this similarity, however, between whole and hemolyzed blood: added CO_2 decreases the magnitude of the dissociation constants (Bohr effect) and changes the behavior of the hemoglobin in the direction of components which react with only a single molecule of oxygen at a time ($n = 1$). The latter process requires a much higher CO_2 tension in the hemolyzed blood, not being completed even at 100 mm. CO_2 tension.

By way of summary, the study of the O_2-dissociation curves of

whole and hemolyzed blood, both comparatively and individually, has yielded sufficient information to enable us to visualize, at least partially, what the addition of CO_2 does to the hemoglobin of this fish. In whole blood it changes the dissociation constants, modifies the behavior of the components combining with oxygen, and effects considerable inactivation of the hemoglobin. In hemolyzed blood, at least up to 100 mm. CO_2, we obtain the first two effects, but not the latter. However, the dissociation constants are all much larger in magnitude, and the change in the components of the hemoglobin requires a much higher tension of CO_2. We are confronted then with the apparent fact that liberation of the hemoglobin from the cell, in the presence of CO_2, in some way decreases the dissociation of oxygen from the hemoglobin, prolongs the existence of those components of the hemoglobin which act as if they were combining with more than one molecule of oxygen at a time, and abolishes, or greatly postpones, any inactivation of the O_2-combining groups. The reason for this is yet to be elucidated.

Theoretical Interpretation of the Effect of Oxygenation on the CO_2 Bound by the Blood (Haldane Effect)

In a previous paper (Root and Irving, 1940) evidence was presented to show that the effect of oxygenation on CO_2 transport in tautog blood was different from that in the blood of mammals. It was tentatively suggested that a part of the difference might be explained on the basis of the theory that there were several O_2-combining components of the hemoglobin behaving differently with respect to O_2-combination and CO_2 sensitivity. At the time we could not see how such an interpretation could apply to hemolyzed blood, since the Haldane effect here was quite typical, and suggested that perhaps there were fundamental changes in the properties of hemoglobin upon hemolysis. With the combined picture we now have of the effect of CO_2 on the O_2-combining power and the reciprocal effect of oxygenation on the CO_2-combining power, we are in a position to give a more adequate interpretation of the Haldane effect as observed in this blood.

The primary fact to be explained is the inconstant $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratio found at 10 and 25 mm. CO_2 pressure for whole blood. To those familiar with mammalian blood it is well known that these ratios are considered to be constant for any single hemoglobin, and it is usually believed that they are constant, though of different magnitude, for the hemoglobin of any species (Redfield, 1933*a*). An inconstant ratio, then, would be considered atypical as compared with the usual constant ratios.

Our interpretation rests on the fundamental postulate that the hemoglobin consists of O_2-combining components which can combine either with a single molecule of oxygen at a time, or more, depending upon how many prosthetic groups the components contain. On this basis let the following assumptions be made:

1. For any single O_2-combining component the $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratio is constant. Let this constant be called R.

2. Different O_2-combining components have different values for R. This is not a groundless assumption for R varies among hemoglobins of different species (Redfield, 1933*a*).

3. Hence, if the proportions of the several O_2-combining components change, the ratio $\frac{-\Delta BHCO_3}{\Delta O_2}$ for the combined components of the whole hemoglobin *may be* inconstant.

We are now in a position to apply these assumptions. Let the components of the hemoglobin be designated as α_1, α_2, α_3, α_4 etc. in accordance with the previous treatment (see page 310), and the corresponding $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratios be written as follows:

$$\frac{-\Delta BHCO_3{}^{\alpha_1}}{\Delta O_2{}^{\alpha_1}} = R_{\alpha_1}; \qquad \frac{-\Delta BHCO_3{}^{\alpha_2}}{\Delta O_2{}^{\alpha_2}} = R_{\alpha_2};$$

$$\frac{-\Delta BHCO_3{}^{\alpha_3}}{\Delta O_2{}^{\alpha_3}} = R_{\alpha_3}; \qquad \frac{-\Delta BHCO_3{}^{\alpha_4}}{\Delta O_2{}^{\alpha_4}} = R_{\alpha_4}. \quad (1)$$

Any given increment of oxygenation of the whole hemoglobin, $\Delta O_2{}^{\omega}$, will be equal to the sum of the increments for each of the components, i.e.

$$\Delta O_2{}^{\omega} = \Delta O_2{}^{\alpha_1} + \Delta O_2{}^{\alpha_2} + \Delta O_2{}^{\alpha_3} + \Delta O_2{}^{\alpha_4}. \quad (2)$$

The base correspondingly released by the whole hemoglobin, $-\Delta BHCO_3{}^{\omega}$, will be equal to the sum of that released by each of the components, i.e.

$$-\Delta BHCO_3{}^{\omega} = -\Delta BHCO_3{}^{\alpha_1} + -\Delta BHCO_3{}^{\alpha_2} + -\Delta BHCO_3{}^{\alpha_3} + -\Delta BHCO_3{}^{\alpha_4}. \quad (3)$$

Combining equations (2) and (3) we have

$$\frac{-\Delta BHCO_3{}^{\omega}}{\Delta O_2{}^{\omega}} = \frac{-\Delta BHCO_3{}^{\alpha_1} + -\Delta HBCO_3{}^{\alpha_2} + -\Delta BHCO_3{}^{\alpha_3} + -\Delta BHCO_3{}^{\alpha_4}}{\Delta O_2{}^{\alpha_1} + \Delta O_2{}^{\alpha_2} + \Delta O_2{}^{\alpha_3} + \Delta O_2{}^{\alpha_4}}. \quad (4)$$

It is evident from (1) that equation (4) can be rewritten in the following form:

$$\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}} = \frac{R_{\alpha_1}\cdot\Delta O_2^{\alpha_1} + R_{\alpha_2}\cdot\Delta O_2^{\alpha_2} + R_{\alpha_3}\cdot\Delta O_2^{\alpha_3} + R_{\alpha_4}\cdot\Delta O_2^{\alpha_4}}{\Delta O_2^{\alpha_1} + \Delta O_2^{\alpha_2} + \Delta O_2^{\alpha_3} + \Delta O_2^{\alpha_4}}. \quad (5)$$

By the use of this fundamental equation curves can be drawn which relate the total CO_2 to the degree of oxygenation of the hemoglobin. Since the effect of oxygenation upon CO_2-combination is called the Haldane effect, the curves which describe the effect of change in combined oxygen (ΔO_2) upon the combined CO_2($\Delta BHCO_3$) will be called Haldane curves. These curves have been constructed from data calcu-

TABLE I

Data for construction of Haldane curve for tautog whole blood at 10 mm. CO_2. $R_{\alpha_1} = .05$; $R_{\alpha_4} = .135$; $\Delta O_2^{\omega} = 10$ per cent HbO_2.

HbO_2	$\Delta O_2^{\alpha_1}$	$\Delta O_2^{\alpha_4}$	$-\Delta BHCO_3^{\alpha_1}$	$-\Delta BHCO_3^{\alpha_4}$	$-\Delta BHCO_3^{\omega}$	Total $[CO_2]$
per cent	*per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
0	0	0	0	0	0	20.50
10	10	0	.50	0	.50	20.00
20	10	0	.50	0	.50	19.50
30	10	0	.50	0	.50	19.00
40	10	0	.50	0	.50	18.50
50	10	0	.50	0	.50	18.00
60	8.5	1.5	.43	.20	.63	17.37
70	5.0	5.0	.25	.68	.93	16.44
80	2.5	7.5	.13	1.01	1.14	15.30
90	2.0	8.0	.10	1.08	1.18	14.12

lated according to the principle of equation (5) and presented in Tables I and II. The change in oxygenation is expressed as an increment of the percentage saturation of the whole hemoglobin, since the original O_2-dissociation curves are drawn in that manner; furthermore we have given ΔO_2^{ω} the arbitrary value of 10% HbO_2. This is a sufficiently small increment to provide an adequate number of points on a theoretical Haldane curve. It is to be understood that in assigning a value of 10% HbO_2 to ΔO_2^{ω} it means that the fully reduced hemoglobin is oxygenated in steps of 10 per cent and for each step the $BHCO_3^{\omega}$ released is calculated according to equation (5). The value obtained when subtracted from the total CO_2 remaining in the preceding step of oxygenation will provide a point on the Haldane curve.

By breaking down the O_2-dissociation curve at any given CO_2 pressure into its components (see Figs. 3 and 5) the values for the

ΔO_2 of the components can readily be determined for any value of ΔO_2^{ω}. In addition to knowing these values, the R values for each of the components must be known in order to calculate the $BHCO_3^{\omega}$ released on oxygenation of the hemoglobin. These can be determined from the slope of the experimentally established Haldane curve providing there is any part of it where the slope is due to only one component acting. The latter can be determined by consulting the corresponding O_2-dissociation curves for the components. Fortunately we have had to deal with only two components at any one time and this has simplified the work of calculating the R values. Once the value for one component is known, the other can be readily determined.

As an example of the determination of the R values, we refer to the whole blood of the tautog at 10 mm. CO_2 pressure where there are two

TABLE II

Data for construction of Haldane curve for tautog hemolyzed blood at 100 mm. CO_2. $R_{\alpha_1} = .04$; $R_{\alpha_2} = .06$; $\Delta O_2^{\omega} = 10$ per cent HbO_2.

HbO_2	$\Delta O_2^{\alpha_1}$	$\Delta O_2^{\alpha_2}$	$-\Delta BHCO_3^{\alpha_1}$	$-\Delta BHCO_3^{\alpha_2}$	$-\Delta BHCO_3^{\omega}$	Total $[CO_2]$
per cent	*per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
0	0	0	0	0	0	35.80
10	10	0	.40	0	.40	35.40
20	8.0	2.0	.32	.12	.44	34.96
30	5.0	5.0	.20	.30	.50	34.46
40	4.0	6.0	.16	.36	.52	33.94
50	4.0	6.0	.16	.36	.52	33.42
60	3.5	6.5	.14	.39	.53	32.89
70	3.0	7.0	.12	.42	.54	32.35
80	3.0	7.0	.12	.42	.54	31.81
90	3.0	7.0	.12	.42	.54	31.27

components, α_1 and α_4 acting. By examining the O_2-dissociation curves for these components, as shown in Fig. 3, it becomes evident that below 50 per cent O_2-saturation of the whole hemoglobin the α_4 component is contributing nothing to the O_2-saturation of the hemoglobin. Therefore the slope of the corresponding Haldane curve in this region is due solely to the α_1 component, i.e.:

$$\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}} = \frac{-\Delta BHCO_3^{\alpha_1}}{\Delta O_2^{\alpha_1}} = R_{\alpha_1}.$$

The value for R_{α_1} when $\Delta O_2^{\alpha_1}$ is put on a percentage O_2-saturation basis, turns out to be equal to 0.05 at this particular CO_2 tension. To calculate R_{α_4} one may go to a position of the Haldane curve where both components are clearly contributing to the slope of the curve. Between 60 per cent and 90 per cent O_2-saturation there is such a region.

Again, by consulting the O_2-dissociation curves for the components, one can find just how much of this 30 per cent increment of O_2-saturation is due to each of them. It happens that approximately one-third (10 per cent HbO_2) is contributed by the α_1 component, and the rest (20 per cent HbO_2) by the α_4 component. R_{α_4} may now be found as follows:

$$-\Delta BHCO_3^{\omega} = 3.2 \text{ vol. per cent (from experimental Haldane curve)}$$
$$-\Delta BHCO_3^{\alpha_1} = R_{\alpha_1} \cdot \Delta O_2^{\alpha_1} \quad \text{or}$$
$$-\Delta BHCO_3^{\alpha_1} = 0.05 \times 10 = 0.5 \text{ vol. per cent}$$
$$-\Delta BHCO_3^{\alpha_4} = -\Delta BHCO_3^{\omega} - -\Delta BHCO_3^{\alpha_1} \quad \text{or}$$
$$-\Delta BHCO_3^{\alpha_4} = 3.2 - 0.5 = 2.7 \text{ vol. per cent}$$
$$\frac{-\Delta BHCO_3^{\alpha_4}}{\Delta O_2^{\alpha_4}} = R_{\alpha_4} = \frac{2.7}{20} = .135.$$

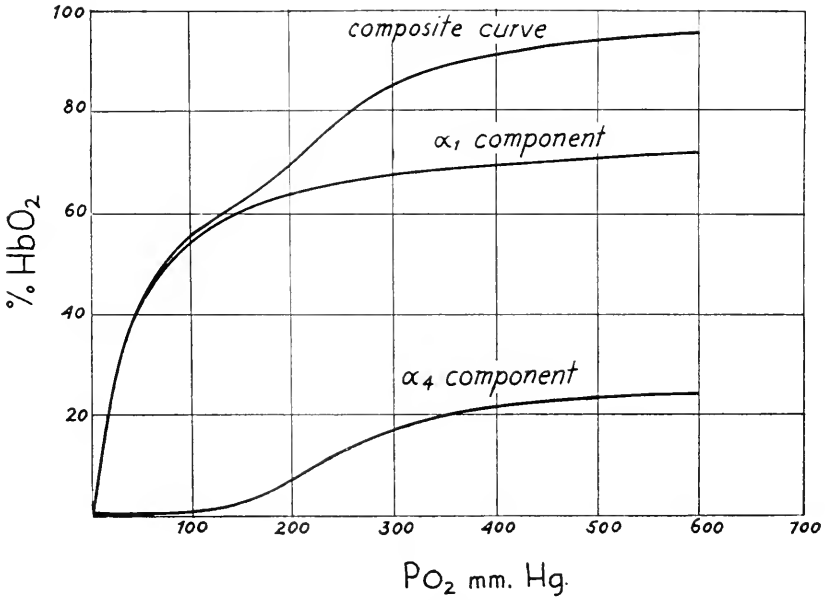

FIG. 3. Oxygen dissociation curves for the components of whole blood hemoglobin at 10 mm. CO_2-pressure. The upper curve represents the O_2-dissociation curve for the entire hemoglobin, obtained by adding the component curves together.

When there is no appreciable region where one component alone is contributing to the slope of the Haldane curve, an accurate determination of the R values is difficult or impossible and one must be satisfied with assumed values which will yield a theoretical curve closely fitting the experimental.

Having the R values, one is now in a position to construct a Haldane curve on the basis of the foregoing theory. With the hemoglobin fully

reduced, and the total CO_2 known under these conditions by extrapolating the experimental Haldane curve to 0 per cent O_2-saturation, one oxygenates the blood in steps of 10 per cent, determining for each step the amount of $BHCO_3$ released by each of the O_2-combining components. The total base released, $BHCO_3^{\omega}$, subtracted from the

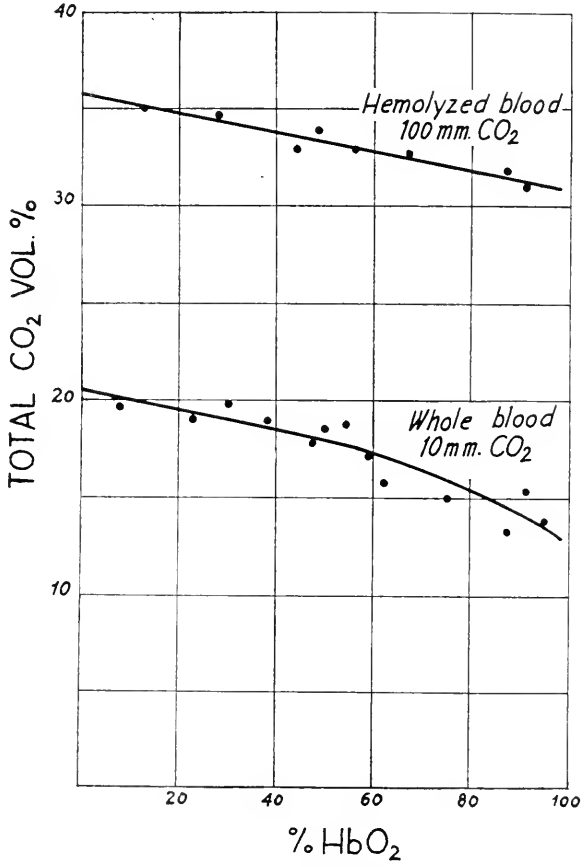

FIG. 4. Theoretical Haldane curves for whole blood at 10 mm. CO_2-pressure and hemolyzed blood at 100 mm. CO_2-pressure, drawn according to the theory discussed in the text. The points on the curves are those obtained by experiment.

total CO_2 present at the end of the preceding step in oxygenation will give a point on the Haldane curve for each increment of oxygenation. In Table I the data, derived in such a fashion, for the construction of a theoretical Haldane curve for whole tautog blood at 10 mm. CO_2 pressure are presented. The values in the first and last columns of this table have been used to plot the curve presented in Fig. 4, and the

points on the theoretical curve are those actually obtained by experiment. It is obvious that there is good agreement between theory and fact.

In the basic assumption for the interpretation of the anomalous Haldane curve for tautog blood it was pointed out that the $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratio for the entire hemoglobin *may be* inconstant. It has been demonstrated that such is the case for tautog whole blood at 10 mm. CO_2 pressure. However, it does not follow that the underlying theory can apply only to inconstant ratios, i.e. that the ratios *must* be inconstant at all times. A moment's consideration of equation (5) will make it clear that there could be such a set of R and ΔO_2 values for the components as to provide a practically constant release of base from the hemoglobin for each step in oxygenation. Should it so happen, for example, that the R values for the components are not too different, one might readily conclude experimentally that the $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratio for the whole hemoglobin is constant—at least one would be tempted to draw a straight line through the experimental points. Or, what is more important, if it should so happen that the ΔO_2 values for each of the components remains practically constant over an extended range when the hemoglobin is oxygenated by equal steps, then it would follow, no matter what the R values, that the $\frac{-\Delta BHCO_3{}^{\omega}}{\Delta O_2{}^{\omega}}$ ratio would be practically constant in this same range. Whether such a circumstance would occur or not would be determined both by the values of the O_2-dissociation constants for the components and the shape of the O_2-dissociation curves they yield.

To illustrate the possibilities outlined above, we will consider the Haldane curve for hemolyzed tautog blood at 100 mm. CO_2 pressure. At this CO_2 tension experiment shows that the $\frac{-\Delta BHCO_3{}^{\omega}}{\Delta O_2{}^{\omega}}$ ratio is best represented as constant, yet analysis of the O_2-dissociation curve indicates that one is dealing with two components, α_1 and α_2. It is difficult to determine the exact values for R_{α_1} and R_{α_2} from the experimental Haldane curve since there happens to be no appreciable part of it where one component alone is acting. Such will be made clear by consulting the O_2-dissociation curves for the components presented in Fig. 5. It is evident that one must reduce the hemoglobin below 10 per cent O_2-saturation before there is any significant separation of the components. Since there is no apparent inflection in the Haldane curve even in this region, one must conclude that the R values are not

too different. The values we have finally taken are indicated in Table II. It is clear, furthermore, from Table II that the ΔO_2 values for the components remain about the same from 30 per cent O_2-saturation to 90 per cent for each 10 per cent step in oxygenation of the hemoglobin. Such a combination of factors can only mean that the theoretical Haldane curve will be practically a straight line, i.e. the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio is apparently constant. Figure 4 shows the theoretical curve for hemolyzed blood at 100 mm. CO_2-pressure drawn from the data of Table II. The points on the curve are those actually obtained

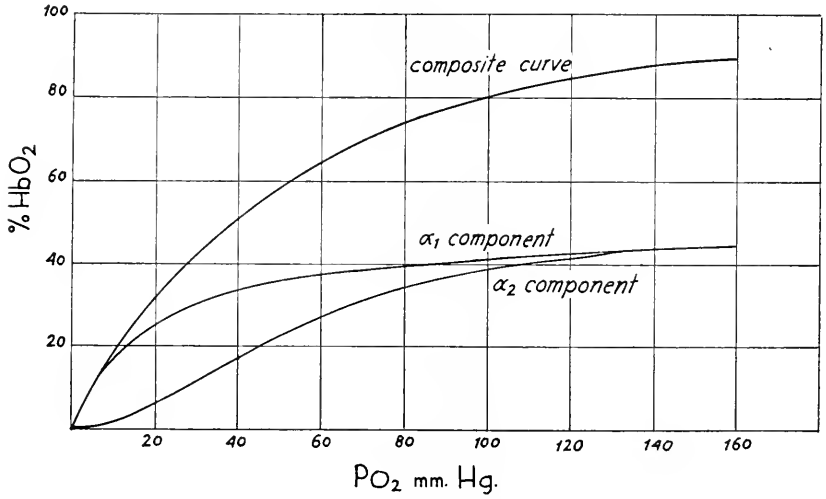

FIG. 5. Oxygen-dissociation curves for the components of hemolyzed blood hemoglobin at 100 mm. CO_2-pressure. The upper curve represents the O_2-dissociation curve for the entire hemoglobin, obtained by adding the component curves together.

by experiment. It must be concluded that under the right circumstances it is possible to have what appear to be constant $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratios even though more than one O_2-combining component is contributing to the oxygenation of the hemoglobin and the release of base. Such a state of affairs does not necessarily constitute an exception to the theory we have presented, but merely a special case.

Considering both the Haldane effect in whole blood at 10 mm. CO_2 pressure, where there is obviously an inconstant $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio, and the same effect in hemolyzed blood at 100 mm. CO_2 pressure, where

the ratio appears constant, it is clear that one must set forth certain qualifications concerning the type of ratio one might expect. If the values of R are quite different for each of the components and their equilibrium with oxygen is such as to yield widely varying ΔO_2 values for each step in the oxygenation of the hemoglobin (this would be dependent not only on the value of n but especially on the value of the O_2-dissociation constants for the components, which would have to be quite different in magnitude) then there should be no difficulty in demonstrating inconstant $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratios. If, on the contrary, the R values for the components are closely similar, or especially if the components have such an equilibrium with oxygen as to provide nearly

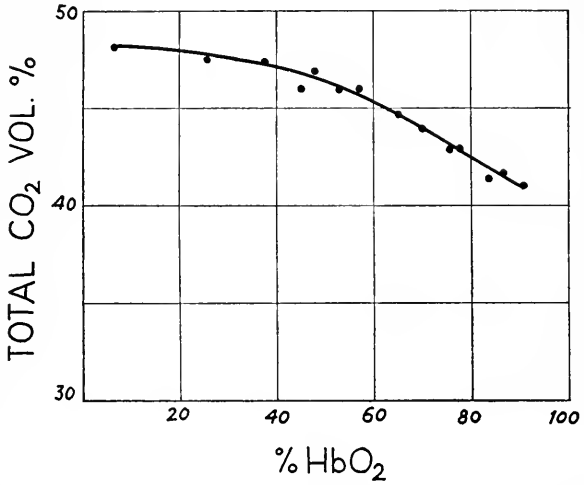

FIG. 6. Haldane curve for toadfish blood at pH 7.2. Data of Green and Root (1933)

constant ΔO_2 values for each step in the oxygenation of the hemoglobin (again this would be dependent on the value of n for the components, and their O_2-dissociation constants, which in this case would have to be more nearly alike) then the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratios would be practically constant, and experimentally would probably not show otherwise.

The inconstant $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio shown in whole tautog blood led us to re-examine some of the data of Green and Root (1933) on the blood of the toadfish. This blood is characterized by anomalous inflections in the O_2-dissociation curves adequately explained by the theory of components. At pH 7.2, for example, the O_2-dissociation

curve is satisfactorily described by assuming two O_2-combining components with widely different dissociation constants. Clearly, if the equilibrium with oxygen of these components at this pH is such as to provide anomalous inflections in the O_2-dissociation curve, then, if our theory is correct, the corresponding Haldane curve should present inflections, i.e. the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio should be inconstant. We have plotted the values of Green and Root for total CO_2 against the percentage of O_2-saturation and obtained the curve presented in Fig. 6. Although the slope of the curve is enhanced due to the fact that the CO_2 pressure was not kept constant (constant pH instead), it is evident from the inflection that the type of curve obtained is similar to that for whole tautog blood at 10 mm. CO_2 pressure, substantiating the theory we have presented.

There is a further matter of interest regarding the Haldane effect in the whole blood of the tautog. In our previous paper (Root and Irving, 1940), it was pointed out that when the CO_2-tension is raised sufficiently the Haldane effect tends to disappear, i.e. the ratio $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ approaches zero value. This happens when the hemoglobin has been partially inactivated and the remainder has been modified to a point where there is but a single O_2-combining component with a value of n equivalent to 1. The explanation probably is that if one decreased the pH sufficiently, the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio would diminish for a particular hemoglobin, even if the components themselves did not change their behavior, for one might eventually reach a point where the titration curves for the reduced and oxygenated forms of the hemoglobin are converging toward each other. In other words, it is not safe to assume in any situation where CO_2 modifies the behavior of hemoglobin that the change in slope of the Haldane curve is entirely due to this effect of CO_2. It will hold only as long as the pH of the blood remains in the region where the titration curves for reduced and oxygenated hemoglobin parallel each other. Outside these limits the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio will change in a manner quite independent of any modification of the hemoglobin or its O_2-combining components.

It can be seen from the major part of the foregoing discussion that the argument for the peculiarities of the Haldane effect in tautog blood rests primarily on the theory that the hemoglobin is made up of different O_2-combining components. One might turn the argument around and say that the peculiarities of the Haldane effect offer strong

support for the theory of components; for it is difficult to see how one could get inflected Haldane curves, such as we have found in whole blood, without having different O_2-combining components present, each of which independently affects the CO_2-combining power of blood on oxygenation. As the situation now stands it has been shown that a single scheme can be used to describe both the effect of CO_2 on the oxygenation of hemoglobin and the reciprocal effect of oxygenation on CO_2-combining power.

The authors wish to acknowledge their indebtedness to Virginia Safford and Henry Brown for technical assistance, and to Dr. Paul S. Galtsoff, Director, and Mr. Robert Goffin, Superintendent of the U. S. Bureau of Fisheries at Woods Hole for their coöperation during this investigation. They also wish to express to Professor A. C. Redfield their appreciation for his suggestions and criticisms in the preparation of the manuscript.

Summary

1. A detailed study has been made of the effect of CO_2 on the equilibrium between hemoglobin and oxygen in whole and hemolyzed blood of the tautog.

2. The study of the O_2-dissociation curves of whole blood has shown that the addition of CO_2 up to 100 mm. pressure changes the shape of the curves from sigmoid to rectangular hyperbolae with approximately 50 per cent of the hemoglobin inactivated. The intermediate stages in the transformation produce complex dissociation curves which can be described by assuming that fish hemoglobin is made up of different O_2-combining components acting independently of each other and combining with different amounts of oxygen at a time.

3. Hemolysis renders the hemoglobin less sensitive to CO_2 as evidenced by the fact that the O_2-dissociation curves move far to the left of those for whole blood; that the O_2-combining components which combine with more than one molecule of O_2 at a time show greater stability than they do in whole blood as the CO_2 tension is raised; and that there is no hemoglobin inactivation up to at least 100 mm. CO_2. There is still a prominent Bohr effect, however, and the O_2-combining components still gradually change their behavior as the CO_2 tension is raised.

4. Based primarily upon the characteristics of the equilibrium between hemoglobin and oxygen, a theory is offered to explain certain peculiarities of the effect of oxygenation on the CO_2-combining power of the blood (Haldane effect). The theory offered provides a common

explanation for the anomalies in the effect of CO_2 on oxygenation of the hemoglobin and in the reciprocal effect of oxygenation on the CO_2-combining power of the blood.

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OBSERVATIONS ON THE FOOD HABITS OF ENTAMOEBA MURIS AND ENTAMOEBA RANARUM

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I. Introduction

While studying nuclear structure and nuclear division in *Entamoeba muris* (Wenrich, 1940), casual observations indicated that there were food preferences among different individuals and among different populations of these amoebae. Further investigation showed that these amoebae often developed temporarily differentiated ingestion tubes which stain intensely with Heidenhain's haematoxylin. A preliminary report on these observations was made at the Marine Biological Laboratory and an abstract published (Wenrich, 1939). Subsequent studies extended the survey to other sets of slides showing *E. muris* from both rats and mice and similar conditions were then discovered for *E. ranarum* from frogs and toads. The present more extended and illustrated report covers the entire set of observations.

These studies have been made partly at the Marine Biological Laboratory and partly at the University of Pennsylvania. They have been made entirely on fixed and stained slides. A variety of fixing and staining agents have been employed in preparing the slides from caecal and rectal contents but the majority of the smears have been fixed in Schaudinn's sublimate-alcohol-acetic and stained with Heidenhain's haematoxylin.

The rats and mice from which slides were made were secured from a variety of sources but a good many rats were obtained from the Wistar Institute and to The Institute, and especially to Doctor Helen Dean King, grateful acknowledgment is made. Acknowledgment is also made to the staff of the Department of Zoölogy of the University of California at Berkeley, for aid in securing frogs and tadpoles and for facilities for their examination. Most of the observations on *Entamoeba ranarum* were made on material from *Rana pipiens* examined at the University of Pennsylvania.

Observations

Entamoeba muris

Entamoeba muris lives in the caecum of rats and mice. Of the more than 500 rats and more than 100 mice that have been examined during the past twenty-five years, relatively few have had amoebae in sufficient numbers to warrant detailed study.

The more striking results of this study of the food habits of *E. muris* are: (1) that there is a great diversity in the kinds of objects selected as food; (2) that some individuals may limit themselves, for a time at least, to a single type of food with which they may engorge themselves, while others may engulf a considerable variety of food objects; (3) that certain populations of amoebae, that is, those from a single host rat or mouse, may show strong tendencies to select one kind of food material, while in other populations, such tendencies are not manifested; and (4) that differentiated ingestion tubes are developed for the ingestion of some kinds of food.

The food objects fall into two natural divisions or groups; those of a plant nature and those of an animal nature. The former group is represented by a great variety of bacterial organisms, a few of which are illustrated by Figs. 1 to 4 and 28, yeasts (Fig. 5), *Blastocystis* (Fig. 12), plant filaments (Figs. 6, 24–27), all of which are apparently resident within the caecum; and starch grains (Figs. 7, 13, 15–18) from the host's diet. Animals are represented by the other Protozoa resident in the host's caecum and small intestine and by various types of cells derived from the host. Among the Protozoa are the trichomonads (Figs. 11, 14, 29–32), *Chilomastix* and *Hexamitus pulcher* from the caecum; and *Giardia* (Figs. 10, 13) and *Hexamitus muris* which come down from the small intestine. Host cells found ingested were erythrocytes (Fig. 8), leucocytes (Figs. 9, 19), and epithelial cells.

Diversity of food preferences among individuals of a population as well as instances of specialization by individuals are illustrated by Figs. 3, 6, 7, and 10, all from the caecum of one mouse. Figures 15, 17, 18, 20–27 are also from the same population. Preferences by individuals are illustrated on Plate I, where each amoeba has filled its cytoplasm with one kind of food. Figures 1 to 7 show ingested plant materials so that these individuals might be considered to have been "herbivorous," at least temporarily. Figures 8 to 11 illustrate individuals which were "carnivorous" at the time that they were killed, and the amoebae in Figs. 13 and 14 could be called "omnivorous" since they contain food objects of both plant and animal nature.

Population food preferences are occasionally noteworthy. The fusi-

form bacillus shown in Fig. 1 is probably the most common food object seen and many populations show a high percentage of their members containing this organism. The colonial species seen in Fig. 2 is sometimes given preference by a population. On the set of slides from which this figure was drawn, about 65 per cent of the amoebae had ingested one or more of these colonies, most of which, however, were much smaller than the one shown in Fig. 2 (cf. Fig. 14). Two quite different types of diplococcoid species are shown in Figs. 3 and 4. These are not uncommon food inclusions, but it is unusual to see so many of either kind in any single individual. Many other types of bacteria are found in the amoebae, but they have not been identified or drawn.

Yeasts are not uncommon food objects, but specialization on yeasts, as shown in Fig. 5, is uncommon. Several populations were found in which ingested filaments were more than occasionally seen, although the proportion of individuals enclosing filaments in any one population was never more than 2 or 3 per cent. Starch grains were not very commonly seen, although a number of populations included individuals which had ingested such grains.

Populations with ingested host cells were uncommon. Epithelial cells inside amoebae were seen only on a few occasions. Erythrocytes taken as food were noted in only two populations which were from mice. In one the number of individuals showing erythrocytes was greater than in the other, but in both there was a tendency for the same individual to ingest several red cells, as illustrated by Fig. 8. Ingestion of leucocytes was also uncommon and the one population in which a number of

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PLATE I

Figs. 1–11 showing examples of specialization by individual amoebae. Figs. 3, 6, 7, and 10 are from the same mouse. Figures 8 and 9 are also from mice. Figures 1, 2, 4, 5, and 11 are from rats.

Figs. 1–7. Examples of "herbivorous" food preferences.

Fig. 1. Amoeba filled with fusiform bacillus—the most common type of food.

Fig. 2. Amoeba containing a large colonial organism.

Fig. 3. Amoeba containing many small diplococcoid bacteria.

Fig. 4. Amoeba with large diplococcoid species.

Fig. 5. Amoeba with a dozen yeast cells.

Fig. 6. Amoeba with long coiled filament.

Fig. 7. Amoeba containing six starch grains. Note deeply-stained granules on side of two of them.

Figs. 8–11. Examples showing "carnivorous" habits.

Fig. 8. Amoeba showing five erythrocytes; one more was under the nucleus.

Fig. 9. Amoeba with four leucocytes.

Fig. 10. Amoeba with three specimens of *Giardia*.

Fig. 11. Amoeba with eight specimens of *Trichomonas muris*.

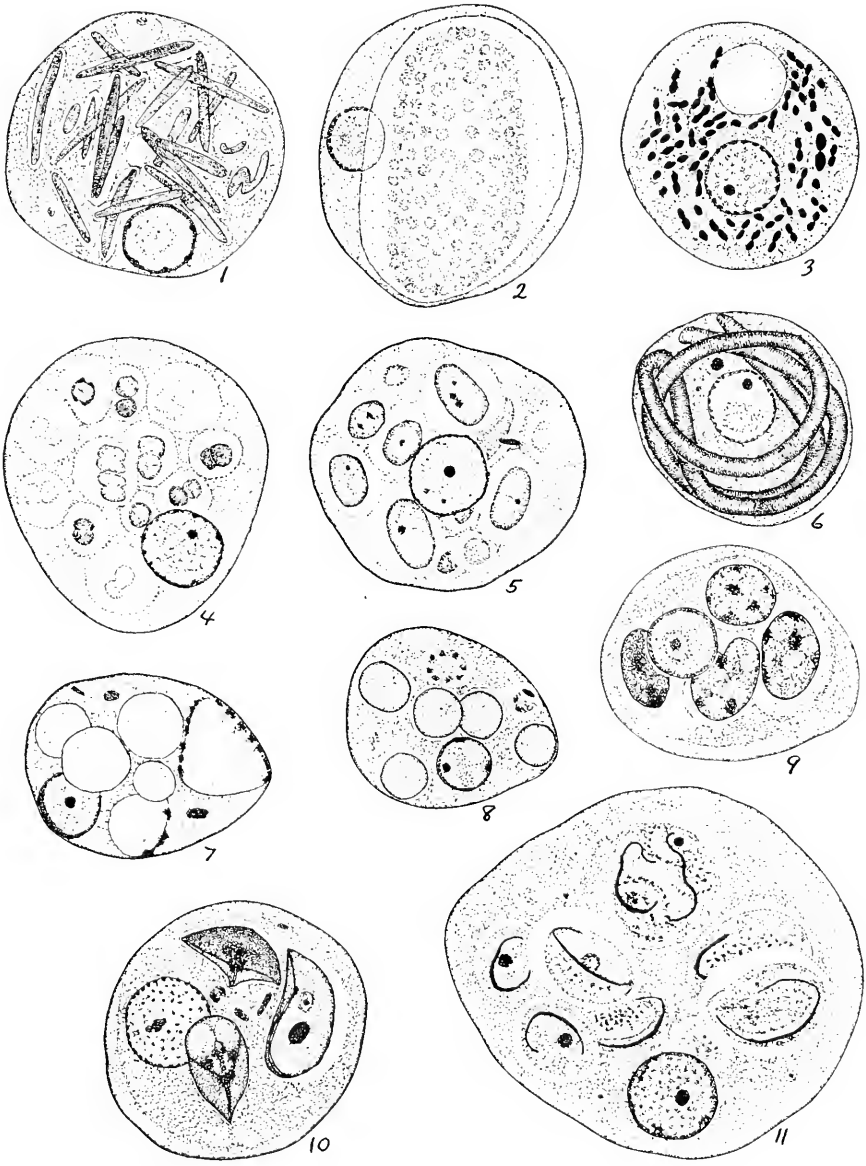

PLATE I

All figures are from fixed and stained slides. They have been drawn with the aid of a camera lucida at an initial magnification of × 3000 and reduced about one-third in printing. Figures 1–33 are of *Entamoeba muris* and Figs. 34–42 are of *E. ranarum*.

amoebae were found with ingested leucocytes was in a mouse. Individual preference is illustrated in Fig. 9.

Among the ingested Protozoa, *Trichomonas muris* was the most common. In many populations it was rarely seen as a food object, while in others it was the population preference. In one count from a slide from a rat about 80 per cent showed one or more trichomonads in various stages of digestion. Individual preferences for this flagellate to the exclusion of other food objects were common in such populations. *Chilomastix bettencourti, Hexamitus pulcher* and *Hexamitus muris* were seen within the amoebae on only a few occasions. *Giardia* was seen more frequently but was not commonly observed. In the population from a mouse, from which Fig. 10 was taken, between 3 and 4 per cent showed one to three individuals of this flagellate. In this population only the trophic stages of *Giardia* were ingested, although the cysts were available.

Ingestion Methods.—Entamoeba muris apparently adopts somewhat different methods for the intake of food, depending upon the nature of the material to be ingested. In the case of starch, it appears from Figs. 15 to 18 that food cups are formed which are just big enough to take in the granules with no vacuolar space between the food body and the cytoplasm. The absence of a vacuole around starch grains is also indicated in Figs. 7 and 13. In some cases (Figs. 7 and 13), deeply-stained bodies are seen in the cytoplasm which is in contact with the starch grain. These bodies are absent in other cases (Figs. 15–18) and in Fig. 7 only

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PLATE II

Figs. 12, 14, from rat. Figs. 13, 15, 17, 18, 20 and 21–33 from one mouse. Figs. 16 and 19 from another mouse.

FIG. 12. Amoeba with specimen of *Blastocystis.*

FIG. 13. Amoeba containing two starch grains, a specimen of *Giardia* and several bacteria.

FIG. 14. Amoeba containing *T. muris,* six colonial organisms and two bacilli.

FIG. 15. Amoeba with starch grain half ingested. Note that edge of food cup and cytoplasmic layer in contact with starch are deeply stained.

FIG. 16. Amoeba containing large starch grain.

FIGS. 17 AND 18. Show ingestion of starch grain almost completed. Note deeply-stained edges of closing-in pseudopodia.

FIG. 19. Amoeba with two partly ingested leucocytes. Note constriction of leucocytes.

FIG. 20. Amoeba with empty food cup. Wall of cup composed of denser cytoplasm but not deeply stained.

FIG. 21. Amoeba with empty food cup. Wall of cup deeply stained.

FIG. 22. Amoeba with food cup turned "wrong-side-out."

FIG. 23. Amoeba with food cup with partly ingested food object and deeply-stained walls.

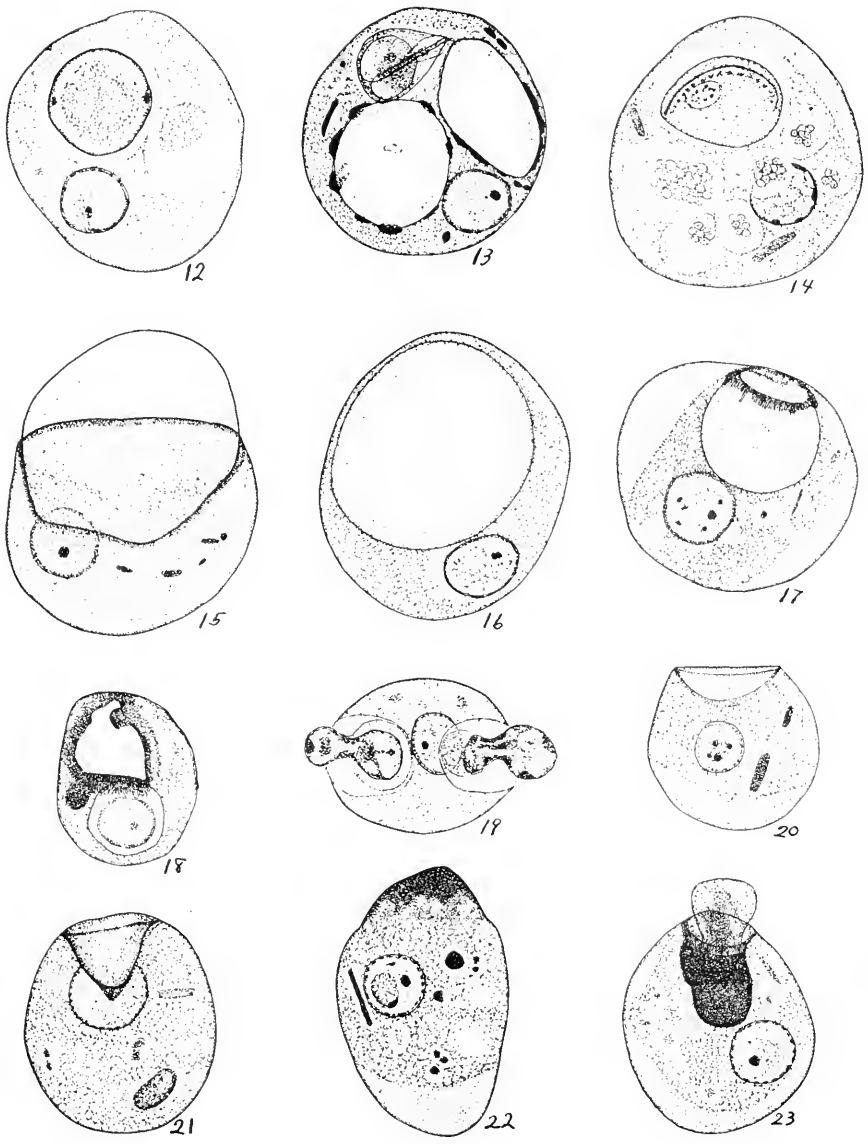

PLATE II

two of the six grains show them. Their nature is problematical, but it is assumed that they are related to digestion. In a number of cases the edges of the advancing pseudopodia which were closing in on a starch grain were deeply stained (Fig. 15) and more especially during the later stages of the enclosing process (Figs. 17 and 18).

In a number of instances, empty food cups were seen (Figs. 20 and 21) and the walls of these cups were obviously composed of denser cytoplasm which might (Fig. 21) or might not (Fig. 20) stain intensely. The condition shown in Fig. 22 is interpreted to be a food cup turned "wrong-side-out." In Fig. 23 a food cup is shown with a partially ingested object and with deeply-stained walls. The middle part of this tube-like cup is more deeply stained than the rest, suggesting greater thickness or greater density.

Figure 19 shows a small specimen of *E. muris* which was fixed while ingesting simultaneously two leucocytes, one at each side. Constriction of the leucocytes is indicated, but the edges of the two food cups are not deeply stained.

Ingestion of filaments was in some cases (Figs. 24 and 27), but not in others (Figs. 25 and 26), accompanied by the formation of deeply-stained ingestion tubes in connection with the ingestion cones. In Fig. 25 a filament has been surrounded at a region away from either end. Ingestion cones were formed and were advancing along the filament in both directions. In Fig. 27, an especially long, deeply-stained food tube is shown. From the left end of this tube and proceeding toward the right, there are three thickenings in the wall of the tube on alternate sides, suggesting a spiral band of more intensely staining material.

A definite "mouth" or ingestion cone and deeply staining "pharynx"

PLATE III

Figs. 24–27 from same mouse. Fig. 30 from another mouse. Figs. 28, 29, 31–33 from rats.

Fig. 24. Amoeba with long filament partly coiled inside. Note deeply-stained ingestion cone and "pharynx."

Figs. 25 and 26. Amoeba with partly ingested filaments; ingestion cones not deeply stained.

Fig. 27. Amoeba with partly ingested filament; long, deeply-stained "pharynx."

Fig. 28. Differentiated "mouth" and "pharynx" with partly ingested bacillus.

Fig. 29. Amoeba with ingestion of *T. muris* almost completed. Note deeply-stained "mouth" followed by undifferentiated food cavity with deeply-stained constriction farther in.

Fig. 30. Amoeba ingesting *T. muris* through differentiated "pharynx."

Figs. 31 and 32. Amoebae with apparently broken ingestion tubes, due to traumatism. In Fig. 32 the lower tube is all inside and is possibly a constriction tube.

Fig. 33. Amoeba with internal constriction tube.

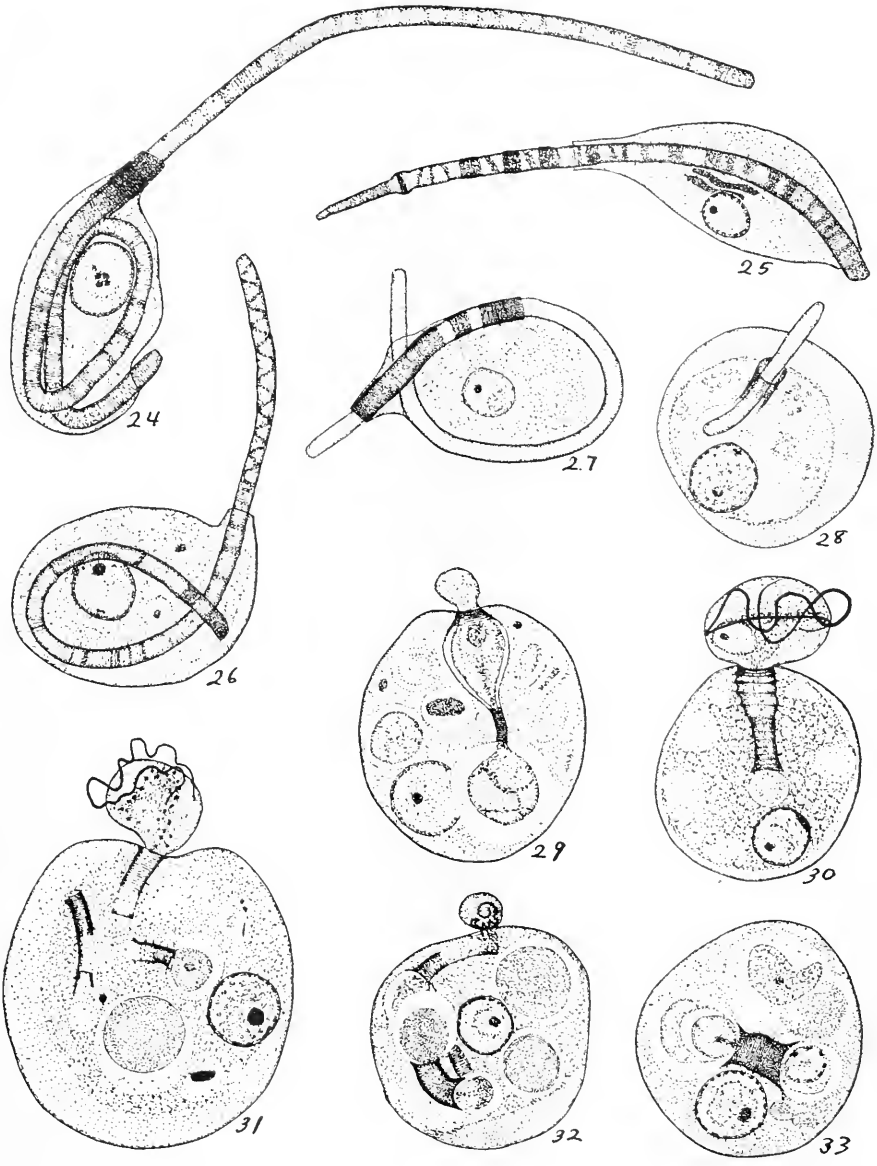

PLATE III

for the ingestion of rod-shaped bacteria are shown in Fig. 28. A number of narrow tubes of this type were seen containing partly ingested bacterial rods.

Trichomonas is apparently ingested by differentiated tubes. A typical case is illustrated in Fig. 30 and many variations of this picture have been seen. In one case the axostyle had been drawn into such a tube while the remainder of the victim remained outside. In another case the anterior flagella had been taken in and the prey had descended a short distance "head first." In still another case the posterior flagellum had been ingested ahead of the rest of the animal. Apparently the amoeba is able to "seize" the flagellate at any point on the latter's surface. In one instance two converging tubes were attached to one trichomonad. The ingestion tube varies in length, up to more than half the width of the amoeba. In some cases the diameter varies in different regions (Figs. 29 and 30). In Figs. 31 and 32 traumatic fragmentation of ingestion tubes is indicated. The amoeba illustrated by Fig. 31 showed definite signs of injury. In a few cases trichomonads were found partly incased in broad food cups, such as shown in Figs. 21 and 23. It is possible that early stages of ingestion may involve such food cups, to be followed by the gradual development of the differentiated tubes such as seen in Fig. 30.

There is evidence that similar tubes are employed to break up food masses, as illustrated in Fig. 33. It is possible that in Fig. 32 a combination of an ingestion tube and a constriction tube is indicated.

Entamoeba ranarum

The finding of the conditions just described for *Entamoeba muris* led to an examination of smears made from the rectum of frogs and

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PLATE IV

All figures of Entamoebae from frogs. Figs. 34, 35, 36, 38, 39, and 40 of *E. ranarum* from *Rana pipiens*. Figs. 37, 41, 42, of possibly different species, from California frog, *Rana draytonii*.

FIG. 34. Amoeba with a dozen specimens of *Hexamitus*.

FIG. 35. Amoeba with a specimen of *Trichomonas augusta*.

FIG. 36. Amoeba with four specimens of *Chilomastix*.

FIG. 37. Amoeba with five host cells.

FIG. 38. Amoeba with partly ingested filament; two deeply-stained "pharyngeal" regions.

FIG. 39. Amoeba with partly ingested short filaments; not deeply-stained "pharynx."

FIG. 40. Amoeba containing one host cell nucleus and a partly ingested second host cell nucleus.

FIG. 41. Amoeba with empty food cup. (cf. Figs. 20, 21.)

FIG. 42. Amoeba showing constriction of food inside cytoplasm.

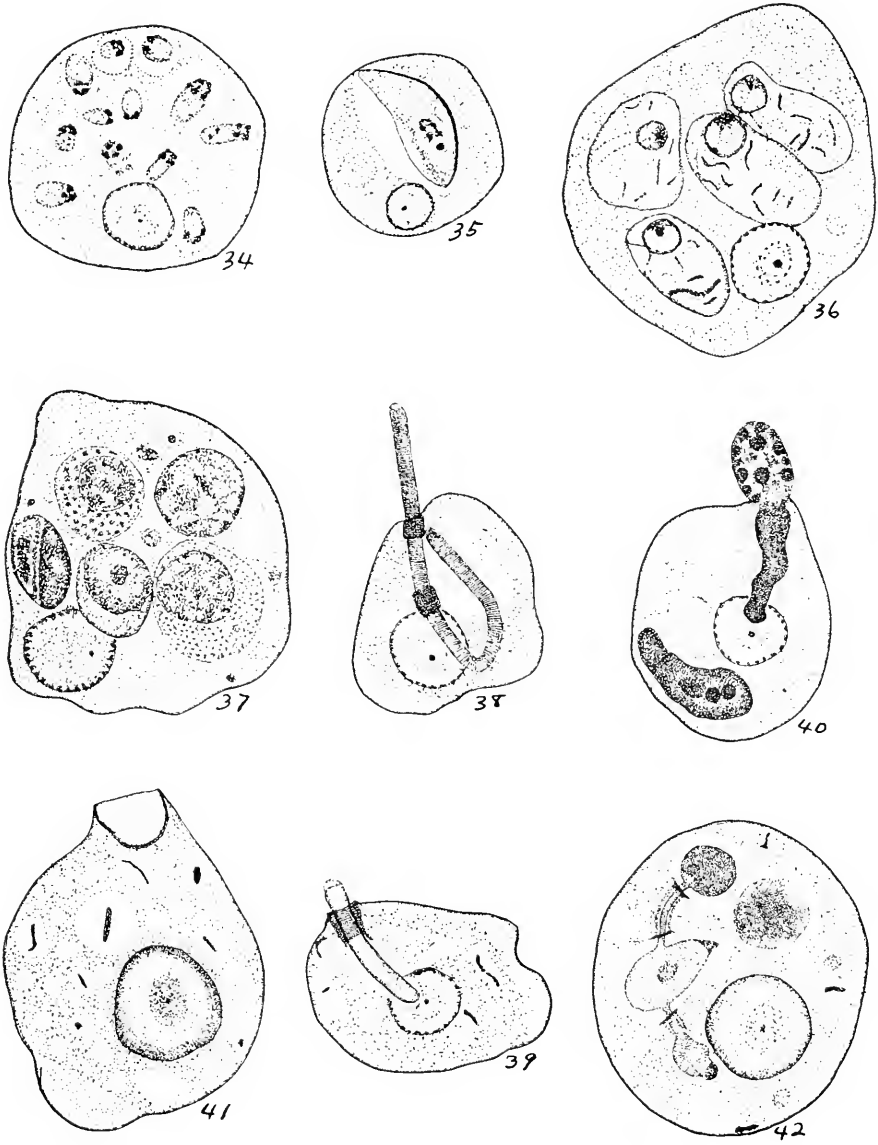

PLATE IV

toads. Here similar conditions were observed for *E. ranarum*. Some of these conditions are illustrated in Figs. 34–42. Individual specialization is indicated by Figs. 34–37. The amoeba in Fig. 34 contains twelve specimens of *Hexamitus*. The population in this case tended to favor *Hexamitus* as a diet, since 17 per cent had ingested one or more individuals. On the same slide 3 per cent of the amoebae contained trichomonads. In a count on a slide showing another population, 50 per cent of the amoebae contained *Hexamitus* as food. The population on this other slide showed diversity of choice, however, as indicated by the ingestion of filaments (Figs. 38 and 39) and host cell nuclei (Fig. 40). In another frog, *Chilomastix* was favored by a considerable number of the amoebae (Fig. 36).

On slides from the California frog, *Rana draytonii*, a large majority of the amoebae contained host cells, apparently leucocytes, although some may have been erythrocytes (Fig. 37). As many as twelve such cells were counted in a single amoeba. A more balanced diet was represented by an amoeba with four host cells and four individuals of the plant, *Blastocystis*. (The amoebae from this species of frog have a nuclear structure considerably different from that typical of *E. ranarum*, and may therefore be a different species.) In a similar amoeba from a California tadpole, a starch grain was seen.

The methods of ingestion employed by these amoebae from frogs and toads are apparently the same as those employed by *E. muris*. In the ingestion of filaments, the formation of ingestion tubes with deeply-stained annular thickenings is shown in Fig. 38. A short differentiated "pharynx" is shown in Fig. 39. Figure 41 shows an empty food cup similar to that seen in *E. muris* (cf. Figs. 20, 21). Constrictions for the breaking up of food masses are shown in Fig. 42, where there are two constrictions being applied simultaneously to a single food body. An internal constriction tube similar to that shown in Fig. 33 for *E. muris* was also seen on the same slide as that from which Fig. 42 was taken. Altogether, the food habits of *E. ranarum* are quite parallel to those of *E. muris*.

Discussion

Most of the extensive literature dealing with the feeding activities of amoebae is concerned with free-living species, there being relatively few reports on the food habits of those that are parasitic. Since the present study has been limited to fixed and stained specimens, the behavior aspects must be inferred, and an extensive discussion of amoeboid nutrition would be inappropriate. However, some interesting interpretations

can be made and their relation to existing literature can be noted. The following items seem worthy of attention here: (1) the diversity of food objects ingested; (2) preferences of individuals and populations for certain kinds of food; (3) methods of ingestion; (4) the breaking up of food bodies after their ingestion; and (5) the appearance of secretion bodies in contact with ingested starch grains.

Diversity of Food Materials Ingested.—Most of those who have studied *Entamoeba muris* have remarked upon the variety of food objects in the cytoplasm of the amoebae. This diversity has been compared to that frequently mentioned for *E. coli* from man. In amoebae from the caecum of mice, Wenyon (1907) noted bacteria of various kinds, *Trichomonas, Giardia, Hexamitus* and its cysts, yeast cells, and epithelial cells. Kessel (1924) noted the inclusion of *Chilomastix* and smaller amoebae besides different kinds of bacteria, and Wang and Nie (1934) state that ingested food consists mainly of starch grains, intestinal bacteria and plant debris. To these lists the present study adds *Blastocystis,* long plant filaments, and host erythrocytes and leucocytes.

A similar diversity of food inclusions in *E. ranarum* was noted in the present study. Dobell (1909) made few comments on the food of this species but remarked that when blood corpuscles and broken-up epithelial cells were available in the large intestine the amoebae readily ingested them. In the present study host cells, not clearly identifiable, but possibly including both erythrocytes and leucocytes, were conspicuous food objects in the amoebae from *Rana draytonii.*

Individual and Population Food Preferences.—The tendency for a single amoeba to ingest repeatedly the same kind of food object is well known for *E. histolytica,* individuals of which may contain as many as thirty to forty erythrocytes at one time; and, in cultures, these amoebae may engorge themselves with starch grains. Frye and Meleney (1936) noted that in cultures this species varied considerably in its tendency to ingest erythrocytes, depending upon the conditions in the medium with which the amoebae were surrounded. Pavlova (1938) has confirmed some of these results and states further that the capacity of *E. histolytica* to ingest red cells depends upon the pH of the medium, the capacity being greatest at pH values between 5.6 and 6.5. Semenoff (1938) reported that ingestion of erythrocytes did not take place unless the latter adhered to the surface of the amoebae. It is reasonable to suppose that *E. muris* and *E. ranarum* capture bacteria and active flagellates by an initial adhesion of the latter to the surface of the amoebae, and that this adhesion would, in turn, be controlled by various external and internal factors. One wonders if such factors would be sufficiently limited or specific in their effects to explain repeated ingestion of one kind of food

body by an individual amoeba. If this were so, then the preference of a large percentage of some populations for one kind of food might be similarly explained. However, the divergence of choice commonly exhibited within a population would indicate that individuals tend to vary among themselves as to their physiological state. It is doubtful if population preferences represent racial, that is, genetic, differences, although such a possibility cannot be ignored.

Methods of Ingestion.—Much has been written about the methods by which amoebae take food into their bodies, but Ivanić (1933) was apparently the first to call particular attention to the formation of a "cytostome" and accompanying tube sufficiently differentiated to stain deeply with iron hematoxylin. He first noted such structures in *Amoeba vespertilio, Amoeba iuvenalis* and an unnamed species of *Hartmanella,* but extended the observations to *Amoeba entzi* (1936), *Hartmanella maasi* (1936*a*) and *H. blattae* (1937). I have also seen a deeply-stained (iron hematoxylin) ingestion apparatus in two different small free-living amoebae of the *Hartmanella* type, where the "cytostome" was funnel-shaped, the funnel opening outwardly. On one slide showing these amoebae nearly every individual displayed from one to a dozen of these funnels at various points on the periphery.

In *E. muris,* ingestion methods seem to be much more diversified, apparently adapted to different kinds of food, but they include the formation of specialized tubes which stain intensely with iron hematoxylin.

In many of his illustrations Ivanić shows, proximal to the "cytostome," capacious vacuoles and speaks of food bodies as being drawn into them. Some of the conditions seen in the present study would lend support to this interpretation. In Fig. 19 the concentration of the more fluid cytoplasm at the inner ends of the partly ingested leucocytes together with the constriction at the "mouth" suggests suction. Suction is also suggested in the ingestion of *Trichomonas* by *E. muris;* here various portions, anterior flagella, posterior flagellum, etc., can be identified as having gone down the "pharynx" in advance of other portions, and often a rounded globule of trichomonas protoplasm occupies an internal vacuole while other portions of the flagellate remain outside and the two parts remain connected through the tube-like "pharynx" (Figs. 29, 30). The enlarged vacuoles into which the short filaments are entering in Figs. 28 and 39 suggest the same thing. Semenoff (1937, 1938) found that *E. histolytica* frequently extracted the nuclei from frog and chick erythrocytes although sometimes ingested fragments might include some cytoplasm. It is difficult to understand how suction

can be developed within an amoeboid cell, but the evidence at hand favors that interpretation.

The ingestion of starch appears to take place by simple extension of pseudopodia over the food object and in contact with it (Figs. 15, 17, 18). This method resembles that frequently reported for free-living amoebae during the act of ingesting starch or other solid bodies. Brug (1928) saw a living specimen of *E. histolytica* enter a group of starch grains and emerge two or three minutes later with four larger and two smaller grains in its cytoplasm, but he did not see the method of intake.

E. muris and *E. ranarum* apparently ingest filaments in a manner similar to that described for free-living species in such classical papers as those of Leidy (1879) and Rhumbler (1898); and more recently Comandon and Fonbrune (1936), have recorded their observations with motion pictures. Ivanić (1933) showed that ingestion of filaments by *A. vespertilio* is accompanied by deeply stainable thickenings along the ingestion tube and the present study reveals similar conditions for *E. muris* and *E. ranarum* (Figs. 24, 27, 38). That a differentiated tube, such as shown in Fig. 27, is fairly stable—for a time at least—is indicated by the finding of a similar tube attached to a bent filament but with the remainder of the amoeba missing—probably having been torn off during the smearing process. It is probable that Fig. 25 represents an early stage in the bending of the filament, a process which might well result in the condition seen in Fig. 27. It is interesting that Figs. 25 and 26 do not show the deeply-stained walls of the ingestion tube that are seen in Figs. 24 and 27. It is doubtful if these differences are the result of variations in the destaining process, since Figs. 24 and 26 were drawn from the same slide. Stainability seems to vary with density of protoplasm and the density is doubtless correlated with degree of contraction.

Peristaltic contractions may be indicated by the successive thickenings on alternate sides of the "pharynx" shown in Fig. 27. Comandon and Fonbrune (1936), employing motion pictures, record the observation of waves of contraction along the ingestion cone surrounding a filament in *A. verrucosa*. Peristaltic action during ingestion of trichomonads by *E. muris* may also be indicated by the differences in diameter of the "pharynx" shown in Figs. 29 and 30.

The Breaking Up of Food Bodies After Their Ingestion.—The ability of amoebae to break up food masses into smaller units has been noted by a number of observers, for example by Leidy (1879) and Penard (1912). More recently Entz (1925) has provided a good description of successive constrictions of food objects as seen in *Amoeba vespertilio;* and, in a later paper (1932) he reviewed the literature show-

ing instances of the breaking up of food masses in both amoebae and ciliates, and also in the flagellate, *Collodictyon*. Ivanić (1936) described the constriction of ingested food masses, sometimes several such constrictions taking place simultaneously; and Mast (1938) reported the breaking up of ingested *Colpidium* in the cytoplasm of *Amoeba proteus*.

The present record seems to be the first for the breaking up of food in a species of *Entamoeba* and none of the observers referred to above have reported the presence of deeply-stained constriction tubes, such as shown in Figs. 33 and 42. It is possible that the deeply-stained tube in Fig. 33 represents a "pharynx" which has persisted after the prey was ingested, although the vacuole at each end does not suggest that interpretation; and such an interpretation would not be applicable to the condition seen in Fig. 42.

Wenyon (1907) speaks of seeing several specimens of *Trichomonas* in a single vacuole in *E. muris* (see his Fig. 1). On the slides used in the present study, flagellates, or their fragments, were almost always in segregated vacuoles. However, large vacuoles, each containing many bacteria, were sometimes seen and one wonders if fusion of vacuoles may take place as well as their subdivision. Ivanić, however, believed that a succession of objects would be taken in through a single "cytostome."

Digestive Granules in Contact with Ingested Starch Grains.—Figure 13 shows a specimen of *Entamoeba muris* containing two starch grains, each of which has deeply-stained masses at its periphery. Figure 7 shows an amoeba with six starch grains and similar stained bodies are seen at the sides of two of them. That it takes some time for such bodies to appear is indicated by their absence in Figs. 15 to 18 where starch grains are being ingested, and also their absence from four of the six grains in Fig. 7. It seems reasonable to assume that these bodies in contact with food represent secreted material having a digestive function. Very similar bodies are shown by MacLennan (1936) for food bodies in *Ichthyophthirius* and he identifies them as elements of the vacuome since they react positively to neutral red and to the Kolatchev-Nassanov method for impregnation of Golgi material. Volkonsky (1934) shows similar neutral red staining bodies outside starch grains ingested by a large granulocyte of *Phascolosoma*, and also by a choanocyte of *Clathrina coriacea*. In his general review of cytoplasmic inclusions in Protozoa, MacLennan (1941) refers to such bodies as digestive granules.

Various observers have denied to free-living amoebae the capacity to digest starch. However, the avidity with which *E. histolytica* and other endamoebae ingest this form of carbohydrate is well established. It

should not be surprising therefore, if, as in the other cells referred to, digestive secretions should be elaborated by such amoebae for the digestion of starch.

Summary

On the basis of observations on fixed and stained slides showing *Entamoeba muris* and *E. ranarum,* the following observations and interpretations have been made.

In general, these species of *Entamoeba* show great diversity in the kinds of food ingested. *E. muris* more commonly feeds on a fusiform bacillus but its diet includes many other types of bacteria, *Blastocystis,* yeasts, plant filaments, starch grains, *Trichomonas, Chilomastix, Hexamitus,* and host erythrocytes, leucocytes and epithelial cells. *E. ranarum* shows a similar range of food objects.

Individuals often select for a time, at least,—a single kind of food, with which they may engorge themselves. Others are more omnivorous in their selection.

Populations from a single host may show decided preferences for one type of food; for example, about 80 per cent of one population of *E. muris* contained one or more specimens of *Trichomonas.*

A diversity of methods of ingestion is indicated. Starch grains are surrounded by enveloping pseudopodia without the formation of a fluid-containing vacuole around them. Trichomonads appear to be drawn through an ingestion tube with walls sufficiently differentiated to stain heavily with iron hematoxylin. Plant filaments are taken in through similar tubes some of which show the deeply-stained walls. There is evidence that differentiated tubes are employed to constrict food bodies into smaller units.

Bodies which stain with iron hematoxylin have been seen in contact with ingested starch grains in *E. muris.* These are interpreted as digestive granules in the sense that this term is used in the review by MacLennan (1941).

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STUDIES ON THE GROWTH OF INTEGUMENTARY PIGMENT IN THE LOWER VERTEBRATES

I. The Origin of Artificially Developed Melanophores on the Normally Unpigmented Ventral Surface of the Summer Flounder (Paralichthys dentatus) [1]

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Considerable evidence has accumulated to indicate that melanophores may be grown experimentally on certain fishes and amphibians in areas where these cells fail to develop naturally. Cunningham (1891, 1893, and 1895), working with several species of flatfishes; von Frisch (1911), using *Esox* and *Nemadulus;* and Osborn (1940*a*, *b*, and *c*), studying the summer flounder (*Paralichthys dentatus*) and the common bullhead (*Ameiurus melas*) have all reported success in growing melanophores on the normally unpigmented ventral [2] surfaces of these teleosts. Experimenting with the urodele, *Salamandra maculosa,* Herbst and Ascher (1927) were able to develop abnormal amounts of pigment ventrally. In spite of these observations the origin of the newly developed melanophores has remained an open question. Alternative possibilities are obvious: either they differentiate *in situ* or they migrate in from other areas.

This paper brings together the results of experiments referred to in an earlier report (Osborn, 1940*a*) designed to gain more information concerning the source of experimentally developed melanophores.

Materials and Methods

A freshly caught stock of adult flounders 15 to 18 inches long was maintained to avoid abnormal conditions in pigmentation which sometimes arise from prolonged sojourns in unnatural laboratory surroundings. The desired amount of pigmentation was developed ventrally in

[1] Contribution No. 296 of the Woods Hole Oceanographic Institution, whose research facilities and financial aid provided for this investigation are genuinely appreciated.

[2] The term "ventral" will be used to refer to the lower normally unpigmented surface of the animal. In the flatfishes the unpigmented side is more strictly the right or the left side, depending upon the species. In the summer flounder the right side is white.

an apparatus similar to that previously described (Osborn, 1940*a*) with minor improvements. When a fish was sacrificed, at least 40 scales plucked from widely separated areas on the ventral surface were fixed in 5 per cent neutral formalin. Of these, ten were dehydrated in alcohol, cleared in xylol and mounted in Clarite; ten were mounted in glycerine jelly directly following fixation; and 20 were treated according to Laidlaw's modification (1932*a*) of Bloch's (1917) "Dopa"[3] reaction. These preparations were finally mounted in balsam. All scales were studied by both transmitted and reflected light and photographic records made. Appropriate control preparations were reserved for each condition. The ventral surface was carefully examined for pigmentation with a dissecting microscope before each animal was sacrificed and daily observations were made during longer experiments.

Experimental

In connection with studies previously reported (Osborn, 1940*a*), it was observed that experimentally developed melanophores appeared in random positions and patterns on the ventral surface. Pigmented spots of macroscopic size and irregular in shape, differing in intensity from gray to black, appeared here and there over any part of the ventral surface. The only position where melanophores developed with considerable regularity was at the base of the tail. In this area normal control fishes also usually possess some pigment, probably because considerable light reaches this narrow region where the surface is somewhat rounded and unprotected by fins.

The "Dopa" Reaction

This reaction first described by Bloch (1917) and later modified by Laidlaw (1932*a*) has been used for the identification of melanoblasts. Although these cells contain no melanin pigment, Bloch observed the formation of a black substance which he called "dopa-melanin" when treated with "Dopa." He believed this was due to an oxidizing ferment, dopa-oxidase, in the cell which reacted with the "Dopa." Thus, many investigators have interpreted the "Dopa-positive" cell as a potential melanophore even though it had not yet differentiated.

In order to test ventral scales for the presence of melanoblasts by the "Dopa" technique, three groups of flounders (12 fishes in each group) were chosen. The first group was black-adapted, then totally blinded and finally illuminated ventrally to insure optimum conditions

[3] Throughout this paper the term "Dopa" will be used to refer to 3–4 dihydroxyphenylalanin (levorotatory).

for rapid growth of melanophores (Osborn, 1939, 1940*a*). This treatment was continued until considerable ventral pigment had developed (Fig. 6). When a random sampling of ventral scales from such a fish was studied it became apparent that all degrees of pigmentation (melanogenesis) were represented by the various scales (Figs. 8, 9, and 10). In some scales there were no melanophores, in others the small number of melanophores had only scattered melanin granules while still others were melaninated so heavily as to be indistinguishable from scales plucked from the dorsal surface. Such scales arranged in a progressive series show all stages in the acquisition of a full complement of melanin in melanophores, suggesting that the process of pigmentation occurs in the cells *in situ* as they differentiate on a particular scale. Furthermore, there appears to be no tendency for scales adjacent to naturally pigmented areas (the edges of the fins etc.) to become pigmented first with subsequent spreading from originally pigmented surfaces. Rather, melanophores suddenly appear containing a few pigment granules quite independently of neighboring cells. In an attempt to obtain more than circumstantial evidence on this point some scales possessing no melanin-containing cells (microscopic examination—Fig. 2) and others containing but few young melanophores (the exact number and their position on the scale recorded in each instance) were subjected to the "Dopa" treatment. An average of 14 out of 20 scales from each of the 12 fishes gave a positive "Dopa" test (Fig. 3).[4] In some ventral scales positive cells were as numerous as the melanophores on dorsal scales while in other instances only scattered cells responded positively. An entirely satisfactory explanation for the failure of some scales to react positively cannot be given. Two possibilities are suggested: either these scales possessed no melanoblasts, as is apparently the case in scales occasionally found on the dorsal surface, or the technique may not be entirely dependable even though precautions were taken that the solutions were fresh and the incubation temperature accurately controlled.

The flounders in group 2 were illuminated ventrally for a shorter period (4 to 10 days), only until the first appearance (macroscopic) of partially pigmented scales here and there over the surface. For the "Dopa" test scales were chosen which possessed no melanin-containing cells or but few melanophores (again carefully recorded). In this group an average of 16 out of 20 scales per animal responded positively. The range of variation was wide as in the first group.

[4] It is of interest to note that these "Dopa" positive cells appear similar to the round melanoblasts pictured by Laidlaw (1932*b*; Fig. 5, Plate 84; and Fig. 8, Plate 85) in human skin.

The third set of flounders was not subjected to ventral illumination or any other laboratory conditions. They were used immediately without allowing time for adaptation to any unnatural background. Adequate scales were plucked from the ventral surface for each of the three types of preparations previously listed and routine "Dopa" tests were run. None of the cells of the scales used possessed microscopically detectable melanin granules. An average of 13 out of 20 scales from each fish reacted positively to "Dopa." Again the range of variation in the number of positive cells from scale to scale was wide. It is of considerable interest, however, that flounders taken directly from nature should possess numerous potential melanophores on a surface so free from melanin.

Observations Using Transmitted Light

A brief summary follows for the microscopic observations of ventral scales studied by transmitted light. Some were mounted in glycerine jelly to preserve the alcohol-soluble pigments; others were mounted in Clarite following dehydration and xylol clearing.

In glycerine mounts the numerous leucophores appear slightly opaque (Fig. 2) because of their content of guanin crystals and may be easily recognized by their relative numbers, irregular (dendritic) shape and their size. Other cells, less numerous, flattened, and smoother in contour, almost round (in fact having the same shape as those which reacted positively to "Dopa"), could be seen scattered among the leucophores. They are believed to be melanoblasts and are best seen when the iris diaphragm is partly closed. Young melanophores, containing few melanin granules, are of much the same appearance but usually are given a slightly more irregular form by the extensions of simple processes. In studying scales arranged in series progressing from those having no melanophores to scales possessing numerous melanin-containing cells there appears to be a direct correlation between the increase of melanin contained in the cell and the complexity of the processes. A coincidence observed so regularly that it should not be overlooked was that wherever several melanophores were growing in a group the absence of leucophores in that spot was strikingly obvious (Figs. 9 and 10). Viewed with reflected light this was even more easily seen. This suggests that in some way a substitution of melanophores for leucophores may take place or that conditions in the tissues favoring the generation of new melanophores may also be responsible for the degeneration of leucophores. Can it be that leucophores change into melanophores? The very existence on the dorsal surface of structures which apparently

contain both melanin and reflecting material (probably guanin) is evidence supporting the idea that two pigments may occur within a single cell (melanoleucophore—Figs. 4 and 5).

It was noted also that in scales possessing many melanophores the cells appeared to be larger and more complex with more numerous, irregular processes in contrast with other scales which had perhaps a half dozen or less melanophores usually of uniform small size and simple pattern, apparently less highly differentiated. To a certain extent the melanophores on a particular scale tend to differentiate more or less synchronously. The way in which experimental pigmentation first appears on the ventral surface of the flounder seems to be in harmony with this and with the evidence gained in the "Dopa" tests. In addition, occasional cells containing some yellow pigment (xanthophores) were seen.

Clarite mounts showed essentially the same picture except that no xanthophores were detected. The leucophores were much more transparent but could be recognized by reducing the light. The smaller round cells were also visible.

Observations with Reflected Light

Glycerine mounts viewed with reflected light showed the leucophores in clear relief but to the disadvantage of the other cells present. However, in cases where some melanophores had developed among the leucophores, the negative outline of the melanin-containing cells could be followed, aided somewhat by the absence of leucophores at that site (see previous page and Figs. 9 and 10). Now and then xanthophores were observed by reflected light.

The scales cleared and mounted in Clarite were less instructive when viewed with reflected light. Because their relative transparency reduced the clarity of the reflected image, they supplied little additional information.

Observations Concerning Regenerating Scales

In areas of injury on the ventral surface where scales had been scraped away, the newly regenerated ones appeared darkly melaninated if the fish was maintained in a physiological and experimental condition favorable to the development of ventral pigment. Such scales are black with melanophores as they appear (Fig. 7). However, if the injured flounders are white-adapted or on a pale natural background with normally alternating night and day (not excessive illumination), the regenerating ventral scales will be white with leucophores and possess no

PLATE I

Explanation of Figures

Fig. 1. White ventral surface of a freshly caught summer flounder. Note that the scales are normally covered with leucophores (containing guanin) but that melanophores fail to develop on this surface. About ⅙ natural size.

Fig. 2. Photomicrograph of a scale (mounted in glycerine) plucked from the ventral surface of a normal summer flounder. This scale possessed no melanophores. The numerous gray-appearing cells are leucophores which appear slightly opaque when photographed with transmitted light. About 20 ×.

Fig. 3. Photomicrograph of a scale which reacted positively to the "Dopa" treatment. The densely opaque cells which have deposited dopa-melanin are interpreted to be melanoblasts. Before treatment this scale appeared similar to that in Fig. 2. About 20 ×.

Fig. 4. Photomicrograph (*transmitted* light) of a small area of the tip of a scale plucked from the center of a white "excitation spot" on the dorsal surface of a black-adapted flounder. The melanophores are numerous even in this white area but are only slightly dispersed and well concealed by guanin crystals, as will be seen in Fig. 5 taken with reflected light. Cell "x" is the same structure marked for purposes of orientation in Figs. 4 and 5. About 100 ×.

Fig. 5. The same area as shown in Fig. 4. This photograph was made with *reflected* light, however, Note that the total area appears relatively white as it would on the fish in reflected light even though the scale is heavily melaninated. The reflecting guanin appears to be within the bounds of the melanophores because the cells retain a constant size and shape when viewed by reflected and transmitted light. Such a structure is referred to as a "melanoleucophore." Compare Figs. 4 and 5 cell for cell. About 100 ×.

Fig. 6. Ventral view of summer flounder blinded immediately after capture and continuously illuminated ventrally (direct light). Although this fish was illuminated only 18 days, its melanination is nearly as extensive as on the flounder shown in Fig. 7. This is due to the greater efficiency of direct illumination. One-sixth natural size.

Fig. 7. An area of ventral surface adjacent to the pectoral fin. The flounder was black-adapted, blinded, and illuminated continuously 74 days in a white tank. Widespread melanophore formation has occurred but pigmentation is blackest where regenerated scales have grown in an injured area from which the scales had been scraped. One-third natural size.

Fig. 8. Photomicrograph of the tip of a dorsal scale plucked from one of the white "excitation spots" of a black-adapted flounder. The numerous melanophores are only slightly distended during excitation despite the fact that the fish had been black-adapted several days. Scales possessing comparable melanination are commonly found in the darker pigmented areas ventrally. About 50 ×.

Fig. 9. Photomicrograph of part of a scale plucked from a lightly melaninated area of the ventral surface of a summer flounder black-adapted 7 days, blinded and placed in a white tank constantly illuminated from overhead for 12 days. The photograph was taken with *transmitted* light. The few young melanophores present appear in distinct contrast to the numerous leucophores which cover nearly the entire scale surface. Note that the leucophores are absent from the newly melaninated area. Melanophore "a" serves as a point of reference and orientation in Figs. 9 and 10. About 40 ×.

Fig. 10. Same area shown in Fig. 9. Photographed with *reflected* light, the numerous leucophores appear white as on the lower surface of the normal flounder. They contain no melanin and are true leucophores. The melanophores are visible only because they reflect the least light and appear black in contrast with the rest of the scale surface. Compare Figs. 9 and 10. About 40 ×.

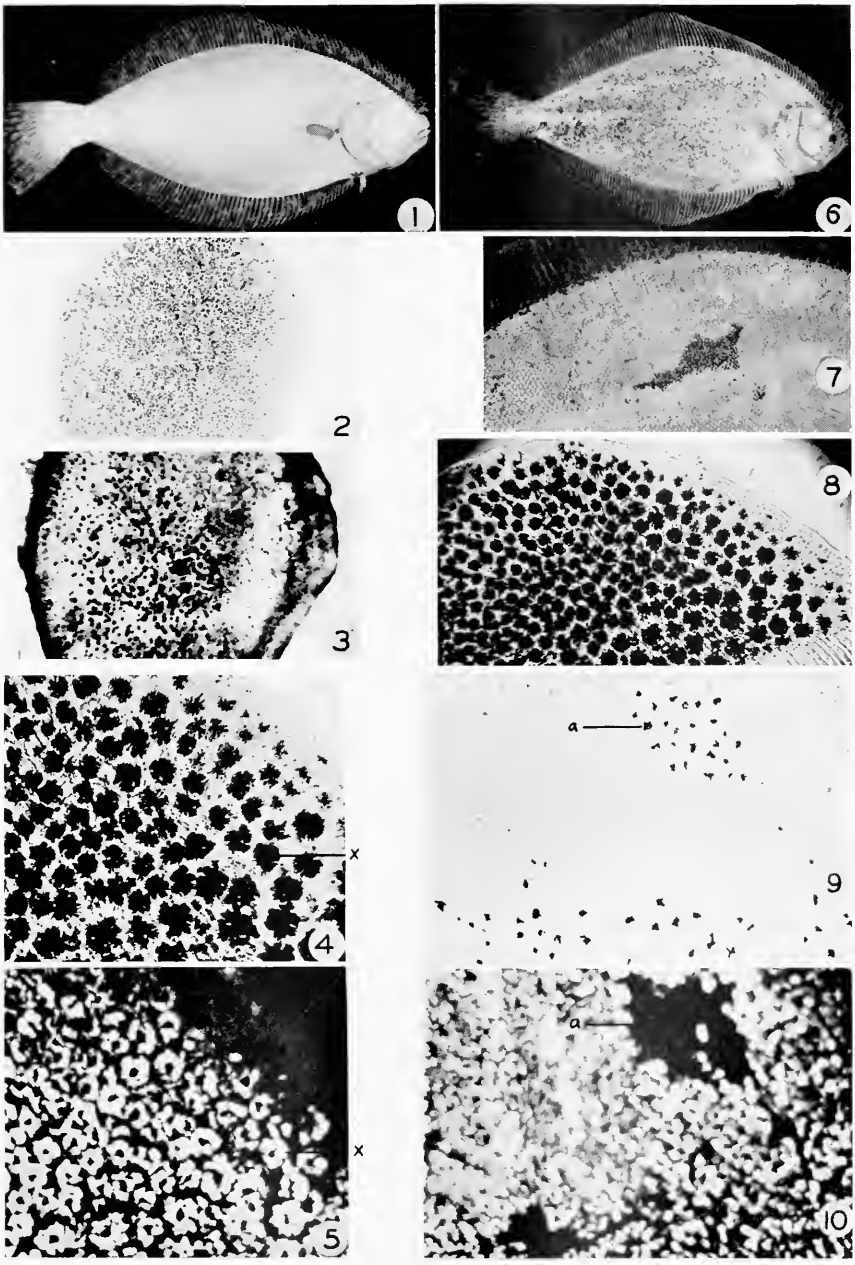

PLATE I

melanophores. On the other hand, if the dark flounders are injured, then placed immediately in the apparatus providing ventral illumination, the scales which regenerate appear as some of the darkest on the entire ventral surface. Although the reason for this is not definitely known, it may be that the numerous leucophores on the white scales tend to mask the developing melanophores during their early stages. The prompt appearance of melanin-containing cells in areas of repair far removed from normally pigmented regions lends further support to the *in situ* origin of ventral melanophores and makes the possibility of migration from previously pigmented areas seem very improbable.

Discussion

That melanophores may be grown under proper experimental conditions on surfaces naturally white and non-melaninated is now well established. The conditions favoring such growth are also known (Osborn, 1939, 1940*a*, *b*, and *c*) although many details await further investigations. The experimental requirements are two-fold: (1) That the surface in question must receive light either directly or by reflection and (2) that the physiological condition (nervous and hormonal factors) of the fish must be such that the internal environment of the normally pigmented cells favors dispersion [5] of the melanin granules (physiological darkening). Odiorne (1937) concluded that this condition also favored the slower morphological darkening in *Fundulus*. Similar findings have been reported in the lower vertebrates by Vilter (1931), Sumner and Wells (1933), Sumner and Fox (1935), Sumner and Doudoroff (1937), Sumner (1939, 1940*a* and *b*), Osborn (1939 and 1940*c*), and Dawes (1941).

The possible source of experimentally developed melanophores attracted the attention of Cunningham (1893), who saw no pigment migrating from the upper surface and so from negative evidence concluded the cells developed *in situ*.

The positive evidence presented here although partly circumstantial supports the view that these melanophores develop where they are first observed. The evidence gained from the direct observation (using both transmitted and reflected light) of small cells whose appearance and distribution agree in general with the picture seen in positive "Dopa" preparations requires no further comment. Likewise the observations concerned with the melanination of regenerated scales strongly favor

[5] That the degree of dispersion need not be maximal is shown by the fact that considerable ventral pigment may be grown on an animal whose dorsal surface is intermediate in shade. It is important to emphasize that the fish should not be in the pale phase.

the *in situ* origin of these melanophores. Furthermore, the fact that evidence gained in using the "Dopa" reaction as an indicator fits well into the other findings suggests that in this instance the reaction is significant and reliable. It is fully appreciated, however, that a positive "Dopa" reaction because of its non-specificity may in itself be of limited value or may prove to be misleading unless supported by evidence from other sources. In these experiments the "Dopa" reaction was used only to test for the presence of positive cells, thus avoiding the necessity of interpreting the implications of the chemical reaction. Because this problem is highly controversial at present some further discussion may be appropriate. That the positive "Dopa" test need not necessarily indicate the presence of a specific oxidizing enzyme ("dopa" oxidase) in the cell has been suggested by Figge (1940), who found that "Dopa" would react with other substances under test tube conditions to produce a black deposit. However, a characteristic feature of a melanoblast is that it contains some substance which will cause "Dopa" to react, forming a black material. This may be formed by a substance which oxidizes the "Dopa" or which acts as a redox substance to accelerate the auto-oxidation of "Dopa" in the absence of an enzyme. This latter possibility seems rather unlikely.

Another possibility is that the cells believed to be melanoblasts on the ventral scales may contain tyrosinase which for some reason has failed to react with tyrosine to produce pigmentation. Figge (1940) suggested that a positive "Dopa" reaction might indicate the presence in a cell of tyrosinase whose oxidizing action was inhibited by a glutathione-like substance. Such substances are known to inhibit the action of tyrosinase on tyrosine but do not inhibit the action of tyrosinase on "Dopa" (Figge, 1940). Tyrosinase actually blackens "Dopa" faster than tyrosine. It is seen then, that the observations made can be explained on a theoretical basis, although we do not, of course, know precisely what happens in the cell.

The question may be asked: "If the melanoblasts are present on the ventral surface, why do they not finish differentiation normally by manufacturing pigment?" It has been demonstrated that they do differentiate to true melanin-containing melanophores when the proper conditions are supplied. One might argue that the internal environment is the same in the ventral cells as in the dorsal cells and that the external environment differs only with regard to the amount of light which normally reaches the upper and lower surfaces of the flounder respectively. If this assumption is true, the following speculation may be offered. Is it possible that in the potential melanophores of the ventral scales a

tyrosine-tyrosinase reaction is inhibited by a glutathione-like reducing agent in the absence of light and that exposure to light (experimentally) might remove the inhibiting effect of the reducing agent and allow the enzyme to oxidize the color substrate? That the above assumption is not entirely true, however, is suggested by other observations. The internal environments for the cells of the dorsal and ventral scales are presumably alike in their hormonal constituents but not necessarily so in regard to their respective innervations. This is not known. Pouchet (1876) suggested, however, that a partial atrophy of the sympathetic system may accompany the migration of the corresponding eye during metamorphosis. In view of the present findings concerning ventral pigmentation, further experiments designed to provide new information on the possibility of the degeneration of sympathetic fibers to the ventral surface are needed.

Summary

Melanophores differentiate on the normally non-melaninated ventral surface of summer flounders when two conditions are satisfied, (1) The surface must be exposed to some light source when (2) the animal is in a physiological condition favoring darkening as witnessed by the behavior of the dorsal melanophores.

The melanophores develop "*in situ*" from potential melanophores (melanoblasts) whose presence is evidenced by the positive "Dopa" reaction, by direct observations of various stages of differentiation using direct and reflected light, by studies on regenerating scales, and by additional physiological data.

Theoretical considerations of the possible reactions involved in the experimental development of ventral melanophores and speculations as to why they are normally absent from the ventral surface are presented.

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STUDIES ON THE GROWTH OF INTEGUMENTARY PIGMENT IN THE LOWER VERTEBRATES

II. The Rôle of the Hypophysis in Melanogenesis in the Common Catfish (Ameiurus melas) [1]

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In previous communications (Osborn, 1940*a*, *b*, and *c*) it was shown that smooth-skinned as well as scaly teleosts responded to experimental procedures by developing melanophores on their normally unpigmented surface. These findings confirm and extend the earlier works of Cunningham (1891, 1893); von Frisch (1911); Herbst and Ascher (1927) and others more completely listed in another paper (Osborn, 1941, in press).

In several experiments concerned with the experimental growth of melanophores it has been observed consistently that these cells developed most rapidly and abundantly when the fish was in a physiological condition which caused pigment granules in the normally existing melanophores to be maximally dispersed (Osborn, 1940*a* and *b* and 1941 in press). Odiorne (1937) suggested that "Any condition leading to the dispersion of pigment throughout the cell will, if maintained, promote the development of melanophores or insure their continued existence"; and further wrote that "The neurohumors which are instrumental in bringing about the pigmentary migrations in Fundulus also exert trophic influences upon the melanophores."

In the catfish it has been found that melanophores can be grown experimentally on the naturally white belly of the animal by directing light upon it while the pigmented dorsal surface is in the dark phase (Osborn, 1940*c*). If the fish is white-adapted, however, ventral illumination does not result in melanophore formation. It was previously shown (Osborn, 1938) that the melanophore-dispersing principle of the hypophysis plays a major rôle in producing the dark phase (physiological darkening) in the natural color changes of the animal. By removing the source of this secretion but maintaining constant all other conditions favoring the growth of melanophores on the white belly surface, it

[1] It is a pleasure to acknowledge that this investigation was aided in part by a grant from the Elizabeth Thompson Science Fund.

should be possible to determine whether a substance necessary in physiological darkening was also essential in the production of melanophores (morphological darkening). In the experiments to be reported here the fact that melanophores failed to grow experimentally in hypophysectomized catfishes indicates that a substance necessary to produce the dark phase is also indispensable to the experimental development of melanophores. This strongly suggests that morphological color change is not the result of physiological color change but rather that both are the product of a common underlying mechanism which effects the former change more slowly than the latter.

Materials and Methods

Common catfishes (*Ameiurus melas*) six to eight inches in length were kindly furnished me by Dr. T. H. Langlois, director of the Franz Theodore Stone Laboratory, Put-In-Bay, Ohio.[2] The laboratory stock was kept in muddy water at 12 to 18° C. in large gray tanks in an animal room where the illumination was of low intensity and darkness was maintained at night. Under such circumstances fishes have been kept over a year in excellent condition and have maintained normal pigmentation. The experimental fishes were kept in water at 10 to 12° C. during the first post-operative week and henceforth the temperature was maintained between 14 and 18° C. At this temperature they took food regularly: rolled oats daily and bits of liver or ground beef about once a week.

Illumination was directed to the ventral surface of the experimental fishes either by specially constructed glass-bottomed tubs with ceiling and sides black or white (Osborn, 1940*a*) or by reflection from white tubs brightly illuminated from above. Both of these light sources have been used successfully in growing ventral melanophores. In our apparatus illumination by reflection grows pigment less rapidly, however, because of the lower intensity of the light actually falling upon the lower surface of the fish.

The fishes, after having been lightly anesthetized in a dilute chloretone solution or stupefied by chilling, were totally blinded by enucleation and were hypophysectomized by the oral approach. Hypophysectomies were checked for completeness by reconstructions at the time of operating, by observing the post-operative color changes displayed by each fish and by examination at autopsy. When for any reason the operation was considered imperfect the data for that animal were discarded.

Some of the fishes were sacrificed at convenient intervals for microscopic study, others for chemical determinations. In almost all cases

[2] Courtesy of Mr. John Sullivan, Ohio Conservation Department.

TABLE I

	Animals alive 30 days after beginning of experiment	
	Number	Percentage of original
Group A	43	71.7
Group B	29	97.0
Group C	16	72.7
Group D	15	100.0

photographs were taken of living fishes but in certain instances additional records of preserved animals were made.

EXPERIMENTAL

In these experiments over a hundred catfishes were used representing four different physiological or operative conditions as follows:

Group A—60 fishes—totally blinded; hypophysectomized 12 hrs. later.
Group B—30 fishes—totally blinded.
Group C—22 fishes—hypophysectomized only.
Group D—15 fishes—unoperated controls.

Animals from each of the above groups were placed in each of five experimental tubs: four providing continuous direct ventral illumination (apparatus only slightly modified from that previously described, Osborn 1940*a*) and one having a white bottom which reflected light to the belly of the fishes. By having representative fishes from each of the groups in every tub, any possible effects of slight differences in temperature, light intensity, feeding, etc. were automatically ruled out. The

PLATE I

EXPLANATION OF FIGURES

Figures 1 and 2 are ventral views of two fishes described below (about ⅔ natural size). Figures 3 and 4 are lateral views of the same two fishes.

FIGS. 1 AND 3. A common catfish (*Ameiurus melas*) blinded and continuously illuminated ventrally by light reflected from the white bottom of the tub in which this experimental fish was kept for 125 days. Note dark shade and excessive ventral melanination.

FIGS. 2 AND 4. A catfish blinded, 12 hours later hypophysectomized and maintained 125 days in the white tub described above continuously illuminated. Note the pale shade and relative loss of pigment compared with blinded control Fig. 8. The fishes in Figs. 1 and 2; 3 and 4 were photographed and printed together.

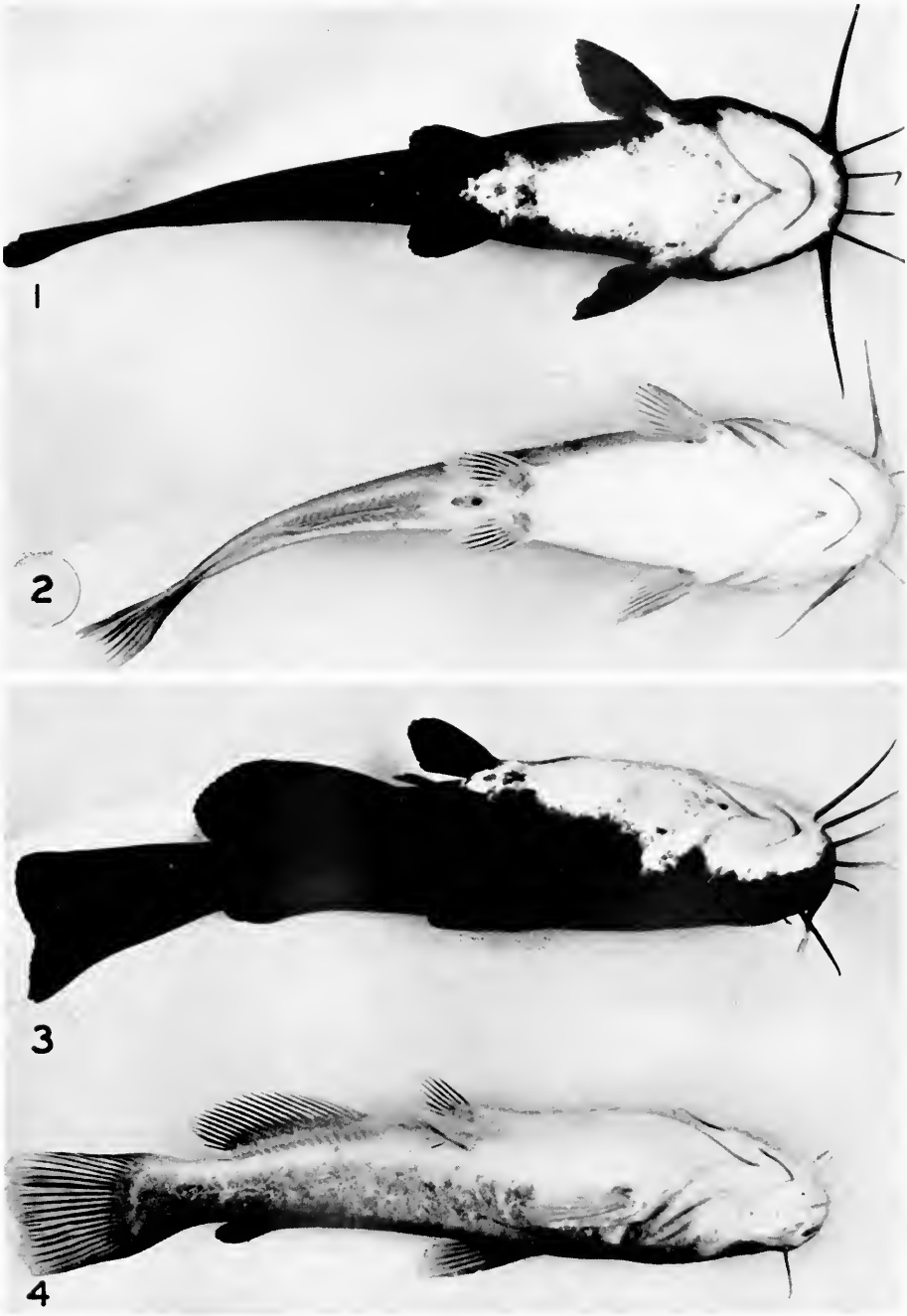

PLATE I

animals were checked twice daily for mortalities and dead or dying fishes were placed in fixative immediately and observations recorded.

Because at least a month of continuous illumination (at the intensity used) was required to develop more than a slight amount of ventral pigment in the catfish, data on fishes surviving less than 30 days were discarded. It is important, therefore, to list animals surviving the operation by 30 days. About 80 per cent of the original fishes were alive and distributed as shown in Table I.

After the first month the mortality rate decreased, presumably because the less vigorous animals succumbed earliest. After that, occasional deaths, combined with the intentional sacrifice of an animal now and then, reduced the number of experimental fishes considerably so that at the end of 180 days 51 animals (about 40 per cent) surviving in good condition were distributed in the four groups as shown in Table II.

TABLE II

	Animals alive at end of experiment—180 days	
	Number	Percentage of original
Group A	11	18.3
Group B	23	76.7
Group C	6	27.3
Group D	11	73.3

At the end of the experiment (180 days) representative animals from each group were reserved for chemical and other quantitative

PLATE II

EXPLANATION OF FIGURES

All ventral views—about ⅔ natural size.

FIG. 5. A catfish blinded and subjected to *direct* ventral illumination for 148 days. Note how the normally unpigmented white vest has become almost completely blackened with melanophores. Direct illumination grows the pigment faster than weaker reflected light. Compare with Fig. 1.

FIG. 6. A catfish (eyes intact) ventrally illuminated with direct light for 55 days. The animal remained somewhat dark-adapted to the black sides and ceiling of the tub. Note that some ventral pigment has grown, especially at the base of the anal fin. Compare with Fig. 8.

FIG. 7. A catfish blinded and ventrally illuminated (direct light) 79 days. The pigmentation is somewhat less extensive than in Fig. 5.

FIG. 8. An animal blinded and kept with the stock fishes in an unlighted tank of neutral shade 76 days. Note the dark shade resulting from blinding, but excessive pigmentation has not occurred. This fish serves as an appropriate control for some of the other animals illustrated.

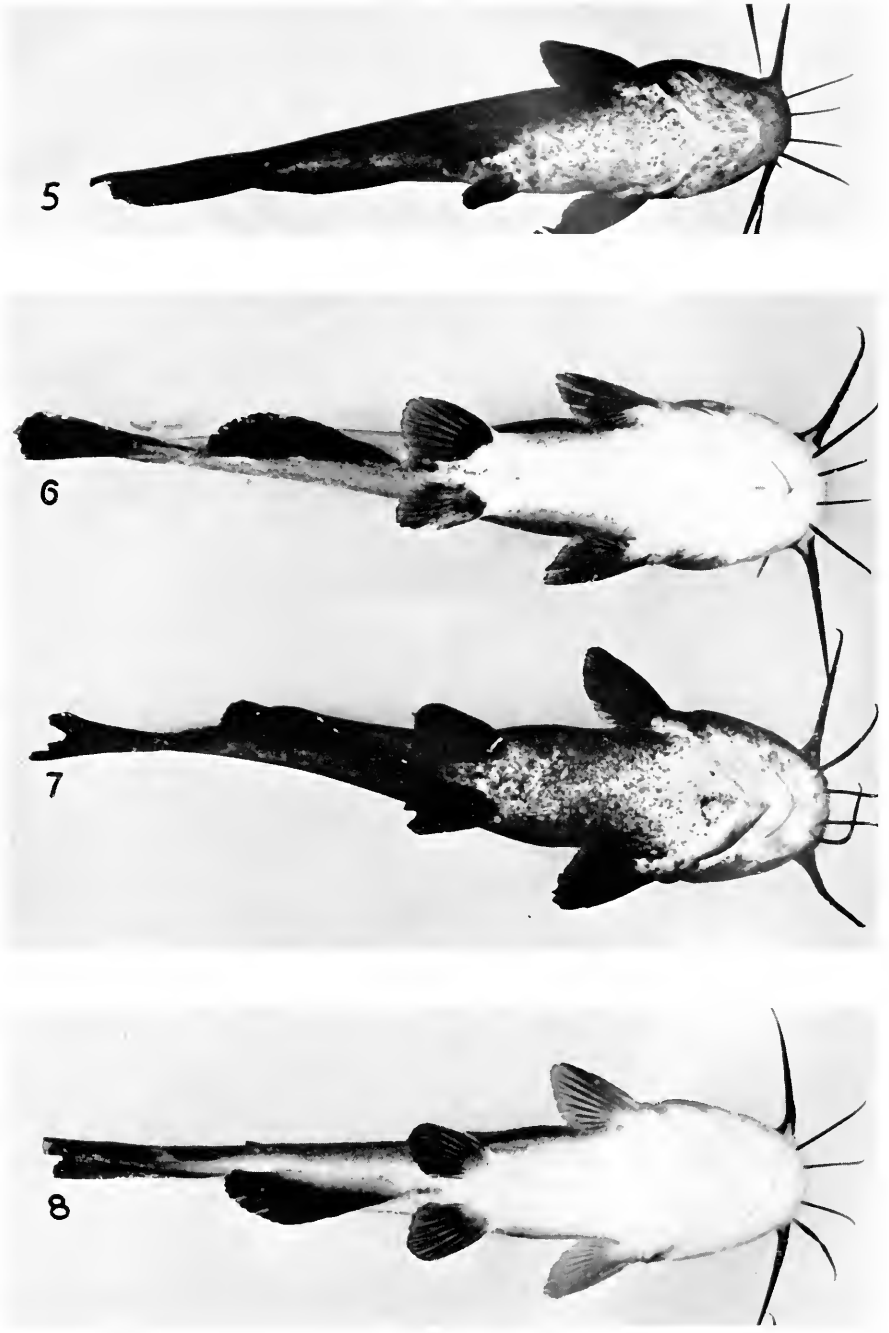

PLATE II

determinations, the results of which will be assembled in a later communication. Only qualitative results will be reported here.

Results

After prolonged treatment under the conditions described, the animals from all four experimental groups could be divided into only two categories on the basis of general macroscopic appearance.

On the one hand fishes of group B which had been blinded only (hypophysis intact) had become coal black dorsally and laterally and had developed dense ventral pigment especially around the cloacal aperture and posteriorly around the anal fin in addition to numerous spots of melanophores scattered over the normally white vest of the belly surface (Figs. 1, 3, 5 and 7). The deposition of melanin in these fishes was strikingly excessive as evidenced by the amount of black pigment which came off on one's hands when the fishes were handled for observations.[3] This never occurred in handling fishes of any of the three other experimental groups.

On the other hand, fishes of groups A (blinded and hypophysectomized), C (hypophysectomized—eyes intact) and D (unoperated) were all *pale in shade* (except those in group D in black-walled tubs) and in general not easily distinguished by original groups although in some instances those in group A seemed slightly darker than fishes in groups C and D. Whether this was a significant difference may be decided from future quantitative determinations. Of interest in our present findings is the fact that hypophysectomized animals of group A were unable to grow melanophores (Figs. 2 and 4) while those in group B, alike in all experimental details except that the hypophysis was functionally intact, grew abundant melanophores. All fishes in both groups had been totally blinded, an operation which in normal catfishes results in the pronounced darkening of the integument (Fig. 8) due to maximal dispersion of the melanin granules within the melanophores (Parker, 1934 and 1939; Abramowitz, 1936; Osborn, 1938). When animals thus blinded are hypophysectomized, however, the integument pales considerably with corresponding concentration of the melanin granules (Osborn, 1938). It appears, then, that when the pigmentary system is subjected to these two opposing influences, melanogenesis is not accelerated even though the external environment (illumination, etc.) strongly favors the growth of melanophores. Furthermore, the melanophores are not maintained normally but rather undergo gradual degeneration.

[3] This is in all probability the result of large numbers of superficial melanophores being cast off through the epidermis, a condition invariably found in catfish integuments where melanin production is going on at an accelerated rate.

Animals in groups C and D placed in white tubs remained very pale and no evidence of accelerated melanogenesis was observed although ventral illumination was continuously provided. Animals in group D were actually white-adapted normal animals (tub walls and ceiling white) while those in group C not only were white-adapted but were deprived of the hypophysis, the source of the chief melanin-dispersing factor in the normal chromatology of the catfish. Of the other fishes in groups C and D in tubs with black walls and ceiling, those in group C were very slightly darker qualitatively than corresponding fishes in white tubs while group D catfishes were rather black-adapted with a noticeable increase in pigmentation (Fig. 6).

It was noticed that the animals of groups A, C and some in D (those in white tubs) not only failed to show evidence of accelerated melanogenesis but actually appeared less heavily pigmented at the end of the experiment than stock controls.

Discussion

Two types of color change have been recognized for several years, rapid and gradual. The thesis that there is a causal relation between the phenomena of transitory and of quantitative color change referred to as "Babak's Law" recognizes as separate features the rapid color changes and those of a very gradual, less temporary type. Odiorne (1937) speaks of these as "physiological" and "morphological" color changes respectively. He found that the pigmentation of *Fundulus majalis, F. heteroclitus,* and *Ameiurus nebulosus* is "reduced through the degeneration of melanophores when these fishes are kept on white backgrounds, but tends to increase when they are kept upon black backgrounds." He also reported that "The development of pigmentation in young fishes (*Macropodus* and *Gambusia*) is retarded if they are kept on white backgrounds, but on black backgrounds the fishes become very dark." Odiorne concluded that "Morphological color changes (alterations in pigmentation) and physiological color changes (arising from pigmentary movements) are phenomena resulting from a common cause."

Other investigators (von Frisch, 1911; Vilter, 1931; Sumner and Wells, 1933; Sumner and Doudoroff, 1937; Sumner, 1939 and 1940*a* and *b*; and Dawes, 1941) have reported experiments concerning an increase or decrease in integumentary melanin. So far as the writer is aware, every case of melanin increase was associated with a condition favoring melanin dispersion in the cells, whereas decreases in melanin regularly occurred in animals maintained in the pale phase over extended

periods. These observations are in total agreement with the conclusions of Odiorne, but direct evidence to indicate that a substance active in physiological color change is also necessary for the formation of new melanophores has hitherto been lacking. The results recorded here indicate that the melanophore-dispersing substance of the pituitary gland, so important in producing the dark phase of the catfish in its normal physiology (Osborn, 1938), is also necessary for the development of new integumentary melanophores and for the maintenance of those already formed. When this substance is absent from the blood (in hypophysectomized fishes), new melanophores are not developed even when otherwise optimum conditions for their growth are maintained. This is most clearly seen in the white normally non-melaninated vest of the fish, which will become pigmented with melanophores under the conditions described in group B (Figs. 1, 3, 5 and 7), using ventral illumination. Not only did such pigmentation fail to occur in catfishes whose pituitaries had been removed, but many of the melanophores present previous to the operation underwent degeneration.

These findings suggest that the melanophore-dispersing substance circulated in the blood stream of the normal fish provides a favorable medium (internal environment) in which melanogenesis may go on. We do not yet know, of course, whether this pituitary fraction itself enters actively into the chemistry of melanin formation or whether it acts as a catalyst in some way. In this connection it is of interest to note that Fostvedt (1940) has reported that some pituitary fractions especially high in melanophore-hormone content produced marked acceleration of the oxidase system in the tyrosine-tyrosinase reaction. This was shown in hypophysectomized frogs whose legs, isolated, were incubated for specified periods of time following injection with the extract. Although this is somewhat removed from catfish chromatology it suggests, at least, how the melanophore hormone may enter into melanin formation naturally, especially in animals whose normal color change mechanism is so highly dependent upon this pituitary secretion.

Incidental to other observations, Rahn (1941) noticed in the rattlesnake that following hypophysectomy little, if any, melanin was deposited into the cells of the shedding stratum corneum. This probably indicates a failure of the melanophores to produce normal amounts of pigment in the absence of the hypophysis. Recent clinical reports by Fournier, Cervino and Conti (1941) indicate success with local injections of melanophore hormone in the treatment of vitiligo in man. Their illustrations show clearly that pigment-free patches become repigmented under administration of the hormone. This finding, together with earlier reports by With (1920), Buschke (1907) and others who

treated vitiligo successfully by stimulating the growth of pigment with light baths indicates that in the human being dual factors (*light* externally and a *hormone* internally) may coöperate in the growth and maintenance of pigment. It is interesting that similar agents are shown here to control pigment production and maintenance in a teleost.

Because facts in this field are just beginning to accumulate, anything more than speculation would be quite premature. Is it not conceivable, however, that the intermedin abundant in the mammalian hypophysis might be concerned in maintaining the degree of pigmentation peculiar to the individual and that an imbalance of this factor might be correlated with certain pathologies where active melanogenesis is characteristic?

Summary

The common catfish (*Ameiurus melas*) possesses naturally a white vest ventrally in which melanophores are only rarely found. In appropriate apparatus it is possible to grow melanophores abundantly over this naturally unpigmented area and increase the amount of pigment in other areas if the dorsal aspect (normally pigmented surfaces) of the fish is maintained in the dark phase. It is convenient, though not necessary, to continue the dark phase permanently by blinding the fish totally, a fact which "per se" indicates that the eyes are not necessary in active melanogenesis.

If the pituitary gland is removed, however, melanogenesis does not continue. In fact, melanophore degeneration sets in with the end result that the experimental fish is paler and less heavily melaninated than stock controls. This indicates that the melanophore-dispersing hormone of the pituitary gland so important in the normal color change physiology of the catfish is also indispensable to the development and maintenance of melanin in melanophores. Interpreted in another way, it suggests that morphological color change is not produced by physiological color change but rather that both are the result of a common underlying mechanism.

A possible way in which the melanophore-dispersing fraction of the pituitary may be involved in the production of melanin is discussed. It is suggested that the melanophore-dispersing hormone (intermedin) in the human hypophysis may be concerned in the production and maintenance of normal pigmentation in man.

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THE RÔLE OF ANTIFERTILIZIN IN THE FERTILIZATION OF SEA-URCHIN EGGS

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Introduction

In recent years several investigators (Frank, 1939; Tyler, 1939*a*, 1940; Southwick, 1939; Hartmann, Schartau and Wallenfels, 1940) have obtained from sperm of sea urchins and of mollusks, a substance that reacts with the fertilizin obtained from eggs, and which is therefore termed antifertilizin. The reaction is manifested by the following effects:—(1) When it is added to a solution of fertilizin, the sperm-agglutinating property is proportionately destroyed; (2) under appropriate conditions it forms a precipitate with fertilizin; (3) it agglutinates eggs of the same or closely related species; (4) it produces a precipitation membrane on the surface of the egg jelly. These four effects are evidently due to the same substance which is obtained as a sea-water extract of moderately heated or of frozen and thawed sperm.

Several other effects of sperm extracts have been described. In the keyhole limpet and in the abalone the extracts contain a lytic agent (Tyler, 1939*a*) which has the property of dissolving the membrane normally present on the unfertilized eggs of these species and which is much more heat-labile than the antifertilizin. The evidence does not as yet enable a decision to be made as to whether it is a distinct substance or a complex that is only active in combination with antifertilizin or a higher "polymer" of antifertilizin. A somewhat similar lytic action of macerated sperm on the egg membrane was reported in amphibia (Hibbard, 1928; Wintrebert, 1933) and of a sperm extract on the cell mass and membrane surrounding the egg of the rabbit (Yamane, 1935).

An agent that inhibited the activity of the spermatozoa was obtained by Southwick (1939) in the supernatant from centrifuging concentrated but not dilute sperm suspensions of the sea urchin. Identity of this agent with antifertilizin has not been established nor has the possibility been excluded that the effect is due to some simple agent such as increased acidity, CO_2, etc. A similar activity-inhibiting action has been

reported in sea urchins by Hartmann, Schartau and Wallenfels (1940) for a methyl alcohol extract of sperm that does not contain antifertilizin (the agglutinin-neutralizing agent). They also find that the extract neutralizes the stimulating effect of egg water on sperm activity and the similar action of echinochrome which they had earlier reported to be the sperm-activating agent in egg water. Since their findings with echinochrome have not been duplicated in other species (Tyler, 1939*b*; Cornman, 1941), and since they have not as yet disposed of the possibility suggested by Cornman that rise in pH might be responsible for their results, it would be desirable to have further evidence before the effect of their methanol sperm extract may be accepted without reserve.

Another effect reported by Hartmann, Schartau and Wallenfels (1940) in the sea urchin is the dissolving of the jelly coat of the egg by the action of sperm extract. They find in *Arbacia pustulosa* that addition of concentrated sperm extract or of live sperm causes the disappearance of the egg jelly and we have been able to confirm this in *Strongylocentrotus purpuratus*. But, according to our observations, this disappearance does not appear to be due to solution of the jelly. When sperm extract is added to a suspension of eggs there is formed on the surface of the jelly a precipitation membrane which, in concentrated extract, gradually increases in thickness and contracts until it reaches the surface of the egg. This precipitation membrane is evidently formed by interaction of the antifertilizin in the sperm extract with the jelly. The disappearance of the latter in concentrated extracts is most simply attributable to its incorporation in the precipitation membrane and to the considerably smaller volume it occupies in precipitated rather than in gel form. As the precipitation membrane contracts the egg may, particularly when disturbed, break out of it. When undisturbed it may contract to the surface of the egg from which it is then not readily distinguishable. The disappearance of the jelly under the influence of concentrated suspensions of live sperm is likewise attributable to combination with the antifertilizin on the sperm. There does not, then, appear to be, as yet, any necessity for the assumption of a jelly-dissolving agent in the sperm extract.

In the present work the term antifertilizin is applied to that substance derived from sperm that produces the effects listed in the first paragraph. A similar antifertilizin has been obtained from eggs (Tyler, 1940), but it will not enter into the present account. The principal question at issue here is whether or not the antifertilizin of sperm is concerned in the fertilization reaction. Several facts strongly favor the presumption that it is intimately involved. In the first place it is tissue

specific, being obtainable from no other tissues (Frank, 1939). It is, however, not very highly species-specific, since cross-reactions are obtained between species that do not cross-fertilize (Hartmann, et al., 1940). This would mean that it is not primarily responsible for the species-specificity of fertilization, but this does not exclude the possibility that it is an integral part of the fertilization process. Another fact favoring its involvement is that it is evidently present on the surface of the spermatozoön. Since, in solution, it reacts with fertilizin, it most likely is the substance on the spermatozoön that reacts in the agglutination of the sperm and therefore must form at least a part of the surface. Furthermore, fertilizin has been shown (Tyler, 1941) to serve as an aid to fertilization and may possibly be an essential agent in the process. Antifertilizin, since it reacts with it, would then be expected to have a complementary rôle.

For a direct test of the significance of antifertilizin, it would be desirable to remove it completely or partially from the sperm by some non-injurious method and to examine the fertilizing capacity of the treated sperm. We have been able, in the experiments reported here, to remove antifertilizin partially without appreciable damage to the sperm. This, as the results show, causes a considerable impairment in the fertilizing capacity of the sperm.

Materials and Methods

Two species of sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus anamesus*, were employed in these experiments. Sperm and egg suspensions were prepared by removing the gonads to sea water and straining the shed sex cells through bolting cloth of appropriate mesh. The concentration of the sperm suspension was usually determined from the increase in volume after removal of the remains of the testes and is expressed as the percentage content of "dry" sperm.

The antifertilizin concentrations in the extracts were determined roughly by the intensity of the egg agglutination reaction and more accurately by the amount required to neutralize one unit (as defined by Tyler and Fox, 1940) of fertilizin (sperm agglutinin). In all the tests the pH of the solutions was checked and adjusted where necessary by means of the glass electrode.

The respiratory rate of the sperm was employed as an index of the extent of damage produced by the various treatments. The measurements were made in the Barcroft-Warburg apparatus with the cylindrical type of vessel previously described (Tyler and Humason, 1937). To

avoid possible effects of CO_2 and variation in pH, glycylglycine (Tyler and Horowitz, 1937) was used as a buffer in carbonate-free sea water.

Removal of Antifertilizin

We found that antifertilizin could be removed from the sperm by slight acidification of the suspension and also by mild warming. The antifertilizin is obtained in the supernatant after centrifugation of an acidified sperm suspension but not in that of the control. When highly concentrated control sperm suspensions are centrifuged, particularly after aging, some antifertilizin may be obtained in the supernatant, as Southwick (1939) reported. This may mean that antifertilizin normally goes slowly into solution or that centrifugation of the concentrated suspensions involves some damage and consequent liberation of antifertilizin.

Antifertilizin is obtained from sperm suspensions acidified to pH 6 or lower. The more acid suspensions yield the more concentrated solutions. One experiment with *Strongylocentrotus* sperm may be cited. Samples of a 10 per cent suspension were acidified to pH 6, 5.6, 5.1, 4.5 and 3.5. After one hour the suspensions were brought back to the control pH (7.9) and centrifuged. The control supernatant was clear while those from the acidified suspensions were increasingly opalescent. Tested on eggs the control showed no reaction while the supernatants from the acidified samples gave precipitation membranes and agglutination which increased with increase in the degree of acidity to which the samples had been exposed. Tests of their ability to neutralize fertilizin gave the following approximate titres for the antifertilizin concentration in the supernatants of the acidified samples: ¼, ½, 1, 4 and 32 units respectively. The spermatozoa were all immotile in the sample that had been exposed to pH 3.5 and partly so in the pH 4.5 sample, while those exposed to the higher pH's showed considerable activity.

These results restricted then the investigation of the treatment required for the impairment of fertilizing capacity to the range between pH 5 and pH 6. A number of tests were run at various pH's within this range and with various times of exposure. All of these showed a considerable reduction in the fertilizing capacity of the treated sperm. Similar results were obtained by heating the sperm at 30° to 33° C. for 5 to 10 minutes. The data need not be presented here since only that part which was obtained along with the respiration measurements is of particular significance. In practically all of these tests the treated sperm were found to be quite active, although in general not as active as the controls. However, differences in activity of spermatozoa are hard to

estimate by direct observation. A more objective and quantitative method consists in measurement of the respiratory rate.

Fertilizing Capacity and Respiratory Rate of Antifertilizin-Poor Sperm

Determinations were made, therefore, of the rate of oxygen uptake of the treated and control sperm along with tests of their respective fertilizing capacities. The results of five experiments are presented in Table I. Heat treatment was employed in one of these and acidification in the rest. The measurements were made in duplicate in each experiment, and both treated and control sperm were samples of the same original suspension. The control oxygen consumption values vary rather considerably in the different experiments. This variation is probably due to a number of factors such as error in initial determination of sperm concentration, variation in original condition of sperm, in its aging, etc. For the present purposes, however, this variation is of no particular significance, since comparison of treated and control sperm is made in each experiment. The duplicate runs in each experiment are in close agreement, which is to be expected since sperm suspensions can be quite accurately sampled and since the spermatozoa respire at a sufficiently high rate to make the instrumental errors relatively small.

In none of the experiments listed in Table I was the respiration of the treated sperm equal to that of the control. The highest values were 80 per cent of the control in experiments 1 and 5 and the lowest value was 25 per cent of the control in experiment 4. The treatment is, then, not entirely non-injurious to the sperm. However, a considerably greater impairment of fertilizing power results from the treatment. The fertilizing capacity of the treated sperm is listed in the last column of the table in terms of the amount required to give the same percentage fertilization, between 1 and 99 per cent, as is given by one part of the control sperm. These values are obtained from the results of inseminating samples of the same batch of eggs with serial dilutions of the control and treated sperm taken from the manometer vessels. The two figures for each experiment cover the range of variation. Thus, in the first experiment, the amount of treated sperm required to give the same percentage fertilization as the control is four to eight times the amount of the control sperm. For comparison, the next to the last column of the table gives the calculated amount of treated sperm that would have the same respiratory rate as one part of control sperm. This value is, in each experiment, considerably less than the value for the amount of sperm having a fertilizing capacity equal to one part of control sperm.

TABLE I

Respiratory rate and fertilizing capacity of sea-urchin sperm after antifertilizin-liberating treatments.

Experiment	Treatment	O_2 consumption (mm.³/hr./cc. of 1 per cent sperm)		Amount of treated sperm equivalent in resp. rate to 1 part of control sperm	Amount of treated sperm equivalent in fertilizing capacity to 1 part of control sperm
		Treated sperm	Control sperm		
1. *Strongylocentrotus*	1½ hrs., at pH 5.3	14.3, 13.2	16.5, 17.9	1.25 parts	4 to 8 parts
2. "	1 hr. at pH 5.1	2.46, 2.58	9.17, 9.47	3.7 parts	64 to 128 parts
3. "	1 hr. at pH 5.5	8.12, 7.36	11.51, 10.72	1.44 parts	16 to 32 parts
4. "	10 min. at 32° C.	3.10, 3.35	13.19, 13.24	4.0 parts	>128 parts
5. *Lytechinus*	1 hr. at pH 5.4	0.54, 0.58	0.74, 0.67	1.26 parts	16 to 32 parts

In other words, there is, as a result of the treatment in each case, a very much greater reduction in the fertilizing power than in the respiratory rate.

It is evident, too, from the data that a considerable impairment of fertilizing power would be obtained following a treatment that resulted in no reduction in respiratory rate. That we have not, as yet, succeeded in finding the proper treatment which would give that result is not surprising in view of the variability of the sperm in the different experiments and the fact that the difference is rather small between treatments giving no effect and those giving a definite reduction in fertilizing capacity. The present results, however, suffice to show that an impairment of fertilizing power can be obtained that is disproportionately great when compared with the respiration of the sperm. This impairment cannot, then, be accounted for by a decrease in activity of the spermatozoa. It might possibly be interpreted in a rather complicated manner by the supposition that a corresponding fraction of the sperm are rendered non-respiring and non-fertilizing while the remainder have an increased respiratory rate. This would mean that the effect on the individual spermatozoa would be all or none and that mild treatment would have a stimulating effect on the respiration of the suspension. There is no evidence for this. The most reasonable interpretation is that the impairment of fertilizing capacity is correlated with the loss of antifertilizin which was shown to result from the treatment.

In the experiments described here antifertilizin is present in solution in the treated sperm suspension. To determine whether its presence might affect the results, antifertilizin was added to untreated sperm in the same or slightly greater amounts. This was found to have no effect on the fertilizing capacity of the sperm. On the other hand, when concentrated antifertilizin solutions are employed an inhibition of fertilization can be obtained, as Frank (1939) and Hartmann, Schartau and Wallenfels (1940) have shown. This inhibition occurs more readily when the eggs are first treated and is evidently due to the presence of the precipitation membrane that forms on the surface of the jelly. When this membrane is incomplete or torn the egg can be fertilized, as was previously reported in the case of treatment with the antifertilizin obtained from eggs (Tyler, 1940).

Antigenicity of Antifertilizin and Action of Antisera

In order to obtain further information on the location of antifertilizin and on its rôle in fertilization, attempts were made to produce antibodies to it. Preliminary immunization experiments showed that high

titer agglutinins could be obtained in rabbits by the injection of whole sperm of the sea-urchin. Immunization with antifertilizin solutions likewise was found to induce the formation of specific agglutinins for the whole sperm as well as precipitins for the antigen in solution.

Antisera were produced against *Strongylocentrotus* and *Lytechinus* antifertilizin. The procedure and results in one experiment with *Lytechinus* follow. A solution of antifertilizin was prepared by extraction of a 25 per cent sperm suspension at pH 4.5 for two hours. The content of organic solid was determined on a sample that had been dialyzed against distilled water and was found to be between 15 and 20 mg. per cent. The rabbit was given seven intravenous injections totaling 23 cc. within a period of two weeks and was bled two weeks after the last injection. The antiserum showed by the ring test a precipitin titer of 8. Tested on a one per sperm suspension it showed an agglutinin titer of 512.

The production of agglutinins by injection of antifertilizin means not only that the substance is antigenic but is probably a surface antigen of the sperm. An examination of the agglutinates shows that the spermatozoa are stuck by their tails as well as by their heads. The antifertilizin, therefore, does not appear to be restricted to a particular location on the surface of the spermatozoön. It should also be noted here that extraction at pH 4.5 removes only a small part of the antifertilizin from the sperm since subsequent freezing and thawing or brief heating of the residue yields at least ten times the amount obtained in the acid extract. Also the acid-treated sperm are still agglutinable by antisera and by egg water.

The antigenicity of antifertilizin supports the view that it is a protein. Other evidence (to be presented in detail later) consists in its non-dialyzability, precipitation with $(NH_4)_2SO_4$, inactivation by heat and acidity, and the fact that it gives the common (xanthoproteic, Millon's and biuret) color tests.

The effect of the antiserum on fertilization was examined by insemination of eggs in its presence. Controls were run with normal rabbit serum. The sera were adjusted to sea-water salinity by the addition of an equal volume of concentrated (1.73 ×) sea water, and equal volumes of egg and sperm suspensions were added. In all cases where the sperm was diluted to the minimum for 100 per cent fertilization in the controls, no fertilization was obtained in the antiserum. With the dilutions of sperm employed, agglutination is greatly retarded and may even fail to occur in the antiserum. The spermatozoa have not then, to any great extent, been rendered inaccessible to the eggs by incorporation in

agglutinates. The inhibition of fertilization may therefore be considered to be due to the neutralization of antifertilizin on the sperm by its antibody in the antiserum.

Discussion

The results presented here show that antifertilizin is involved in the fertilization process. In order to decide whether or not it has an indispensable rôle, one would like to have some more direct evidence such as the complete and reversible removal of antifertilizin might supply. But complete extraction without destruction of the sperm has not as yet been accomplished. From the present evidence it is reasonable to regard antifertilizin as involved in an initial step that facilitates fertilization but which may or may not be an essential part of the process. This initial step is evidently the reaction with fertilizin. In a previous article (Tyler, 1941), it has been shown that the presence of fertilizin on the egg serves as an aid to fertilization. Antifertilizin may, then, be considered to have a similar rôle in the case of the spermatozoön. For this purpose it is not effective when present in solution but only on the spermatozoön. Partial removal of the antifertilizin or its neutralization by means of an antiserum or by means of fertilizin results in a decrease or even complete suppression of the fertilizing power of the sperm. As an interpretation for the fertilization-facilitating action of fertilizin (Tyler, 1941) it was suggested that, in the form of a gel around the egg, it has a few superficial combining groups available which serve as the initial trap for the sperm but which do not neutralize all of the reacting groups (antifertilizin) on the sperm before the latter has reached the surface of the egg. On this basis the decrease in fertilizing power of the treated sperm may be interpreted to mean that, with fewer reacting groups available, there is more likelihood that they will all be neutralized before the spermatozoa reach the egg surface.

Summary

1. Acidification of sea-urchin sperm suspensions to below pH 6 or brief heating above 30° C. liberates into the solution the substance termed antifertilizin which is defined by four manifestations of its reaction with fertilizin; (a) neutralization and (b) precipitation of the latter, (c) agglutination of eggs, (d) formation of precipitation membrane on egg jelly.

2. The treatment results in a marked decrease in the fertilizing power even when the time and intensity of exposure are not sufficient to immobilize the sperm.

3. The rate of oxygen consumption of sperm, that had been exposed to mild acid- or heat-treatment, was found to be very little affected in comparison with the effect on the fertilizing power. Short extrapolation permits the conclusion to be drawn that a considerable reduction in fertilizing capacity can be obtained with no reduction of activity of the spermatozoa.

4. Injection of antifertilizin solutions into rabbits results in the production of an agglutinin for the intact sperm. This shows that the substance is a complete antigen and supports the views that it is a protein and a component of the surface of the spermatozoön.

5. Fertilization is inhibited by antisera to antifertilizin.

6. Antifertilizin is considered to be concerned in an initial (perhaps essential) step in the union of the gametes whereby the spermatozoön is entrapped by the complementary, specific reacting substance, fertilizin, on the egg; and the above inhibition experiments are interpreted on the basis of a decrease in the number of reacting groups available on the spermatozoön.

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ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XVIII. Esterases in the Grasshopper Egg [1]

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The respiratory quotients obtained for the grasshopper egg during all except the first few days of its development are characteristically those of an organism metabolizing fat (Bodine, 1929; Boell, 1935). Slifer (1930) has shown that the amount of fat in the egg measured in terms of fatty acid after saponification decreases during the phases of active development. The determination of the amounts of "lipoidal" substance that form the centripetal layer when a saline extract of these eggs is centrifuged also demonstrates a decrease in volume during embryonic growth (Bodine et al., 1939). Further, the potency of this "lipoidal" layer to activate the proenzyme, protyrosinase, varies in a different fashion than its change in volume (Bodine et al., 1939). These facts add an interest to the study of the types and activities of lipolytic enzymes present in the egg of the grasshopper (*Melanoplus differentialis*) during its embryogeny.

Materials and Methods

Grasshopper eggs were collected daily and kept at 25° C. either in the pods or separated upon damp sand within covered glass dishes. Under these conditions the eggs go into diapause within a month (Slifer, 1931). This block in development was interrupted by keeping the eggs at 5° C. for three months and then transferring them to 25° C., at which temperature they hatched in 18 days. The eggs for experiments were washed, sorted, and sterilized with 70 per cent alcohol for ten minutes (eggs 0 and 5 days of age were not treated with alcohol), rinsed and ground in a glass mortar. The ground eggs were made up to designated volume in a glycine-NaOH buffer mixture. This egg brei was centrifuged and the lipoidal or centripetal layer removed, as were the shell fragments (*A* and *C* layer of Bodine and Allen, Fig. 1, 1938). The remainder was made up to volume with the buffer mixture. Removal of the *A* and *C* layers did not alter the enzyme activity.

[1] Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

Amounts of the extract were added to a 50 cc. Erlenmeyer flask containing substrate and allowed to stand at 25° C. unless otherwise noted. Varying concentrations of enzyme and substrate were used, the total volume of the reaction mixture being 6 cc. After a period of time, the reaction was stopped with 25 cc. of a 2 per cent phenol solution and the mixture titrated with 0.05 N HCl until methyl red, used as an indicator, turned pink. Although the H ion concentration changed during the experiments, the addition of the phenol in buffer brought the pH back to the alkaline side and the HCl titre then was a measure of the NaOH neutralized during the reaction and was equivalent to the acid formed. The amount of acid thus formed is considered a measure of the rate of hydrolysis and an index of the amount of enzyme present. The equivalents of acid produced are not strictly rate values in the case of tributyrinase (Bodansky, 1937). Because of the difficulty in determining how much of the substrate was properly emulsified, a more accurate measure of rate was not practicable. Controls were duplicates of the experimentals with the exception that the enzyme extract was heated at 100° C. for 5 minutes. Reaction mixtures containing no substrate or enzyme were also tested and gave values equal to those of the control. Shaking the flasks during the reaction period did not change the rate of the hydrolysis.

The buffer mixture contained 0.1 N glycine and 0.1 N NaOH in the ratio of 9 to 1, 15 per cent glycerol, and enough NaCl to make the solution 0.17 M with respect to NaCl. The addition of salt was necessary to prevent precipitation of proteins in the extract. The phenol was dissolved in the glycine-NaOH buffer and was never used after it had acquired a brownish tinge. The methyl butyrate (Eastman Kodak), 2 per cent by weight, and the tributyrin (Eastman Kodak) and olive oil, 4 per cent by weight, were made up in the buffer containing glycerol and NaCl. Previous workers have experienced difficulty in making up tributyrin solutions which gave consistent results. Seventy milligrams of a commercial dispersing agent (Daxad No. 11) [2] per 100 cc. of solution will stabilize a tributyrin emulsion.

The method was checked using known amounts of butyric acid in place of the lipid in the protocol and the probable errors of the means of ten determinations at four concentrations between 0 and 2×10^{-4} M butyric acid were less than 2 per cent of the mean in every instance. The amount of hydrolysis of an excess of methyl butyrate (3 cc. of 2 per cent) increases linearly with extracts of one to thirty diapause eggs. The amount of acid produced by one cc. of an extract (20 eggs per cc.) was linear with time for 4 hours. When tributyrin was used as a sub-

[2] Furnished by Dewey and Almy Chemical Co.

strate, an extract of 2 eggs (diapause) would produce as much acid in 2 hours as an extract of 30 eggs would produce from methyl butyrate. The amount of acid produced from 3 cc. of 4 per cent tributyrin was proportional to the concentration between 1 to 5 eggs per cc. The reaction on tributyrin was linear with time only for the first hour. In making the following determinations, 1 cc. of an extract containing 20 eggs per cc. with 3 cc. of 2 per cent methyl butyrate in a reaction period of 2 hours and 1 cc. of an extract containing 2 eggs per cc. with 3 cc. of 4 per cent tributyrin in a reaction period of one hour at 25° C. were used as test reactions.

In a number of experiments direct titrations of reaction mixtures were made with 0.05 N NaOH to determine the extent of hydrolysis. In these the pH was first adjusted by the addition of acid or alkali and titrations made to maintain this H-ion concentration. A Leeds and Northrop pH meter with a glass electrode was used in these titrations. The time course of the reactions under these conditions at steady pH values between 4.5 and 8.0 was similar to that when the method described above was used.

Experimental

Enzymes are present in the grasshopper egg which will catalyze the hydrolysis of methyl butyrate and tributyrin but not olive oil. The enzymes are designated as methyl butyrinase and tributyrinase respectively in the following discussion although other substrates may be attacked by these enzymes. According to the nomenclature of Oppenheimer (1936), both are esterases; the one more specifically a lipase since it splits a glycerol ester of the fatty acid. The amounts of the two lipolytic enzymes vary independently during the development of the grasshopper egg (Fig. 1).[3] The methyl butyrinase activity is high at the start of development and then drops markedly between the tenth and fifteenth day. A steady level is then maintained during the diapause or inactive stage. Upon resumption of development a slow decline in activity takes place. Tributyrinase action, however, remains at the same level during prediapause and diapause and then drops rapidly in post-diapause development. An extract from a single grinding was used on both substrates in each of the ten determinations represented by the averages in the figure.

Two types of experiments were used to determine the relative amounts of the lipolytic enzymes being studied in the embryo and yolk

[3] In preliminary work (Carlson, 1940) the enzyme extracts used were so concentrated that the changes in activity were obscured.

constituents of the egg. Early prediapause (6-day) eggs were irradiated at 1000 roentgens, which is known to prevent the embryo from developing but to have no visible effect on other constituents of the egg (Evans, 1936).[4] The oxygen consumption of eggs treated in this manner decreases until the time of diapause. The O_2 uptake is low during diapause, and when the diapause is broken the oxygen uptake increases for the first two days and then remains constant (Bodine,

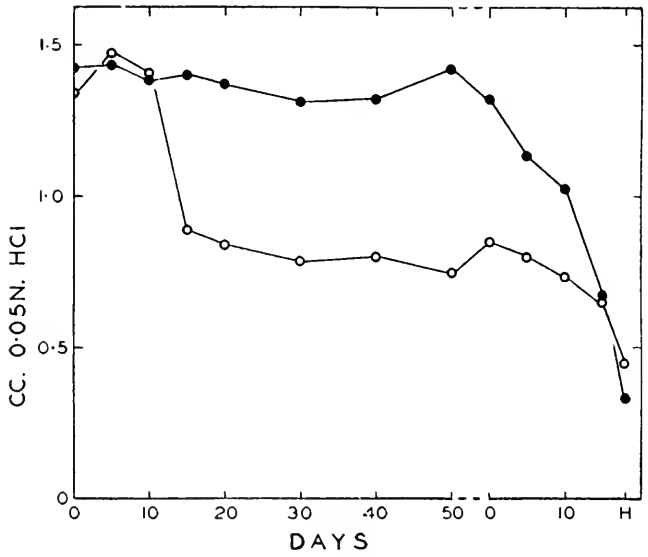

Fig. 1. Average esterase activity of five lots of eggs at each of the developmental ages shown. Ordinate, the equivalents of acid produced by hydrolysis of the esters in cc. of 0.05 N HCl; abscissa, time in days at 25° C. since laying followed by the time in days at 25° C. after termination of the diapause by exposure to 5° C. for three months. Open circles, the activity of an extract of 20 eggs in two hours with methyl butyrate; closed circles, that of an extract of two eggs in one hour with tributyrin as a substrate.

Carlson, and Ray, 1940). No significant difference could be shown between the enzyme content of the irradiated eggs and that of the controls. Determinations were carried out for 30 days after the irradiation.

In postdiapause, the embryos could be dissected from the egg and determinations made of the lipase content of the embryo and other egg constituents. The dissections were carried out in the buffer mixture. The embryos were freed from as much adhering yolk as possible and transferred with a minimum of fluid to a mortar and ground with a

[4] Dr. T. C. Evans irradiated the eggs for the author.

small amount of sand. The remaining yolk and shells were also ground and used with the dissection fluid in the determinations. There was some difficulty in freeing the embryos of yolk, but in all cases this was done as completely as possible. The amounts of enzyme in embryo plus yolk, etc. were always less than those of the whole eggs. The lipolytic enzymes seem to be associated with the yolk or its derivatives until just previous to hatching (Table I). The yolk removed in later stages of development usually included parts of the gut that could not be adequately separated. The presence of some esterase in the 5-day post-diapause embryos is attributed to the fact that the yolk and embryos were especially hard to separate at this stage.

Although the time course of reactions was similar at different pH values, the extent of hydrolysis of tributyrin was markedly affected.

TABLE I

Days Post-diapause	Embryo	Yolk etc.	Substrate
	Per cent	*Per cent*	
0	0	100	Tributyrin
5	19.3	80.7	
10	0	100	
15	0	100	
18	100	0	
0	0	100	Methyl butyrate
5	20.3	79.7	
10	8.7	91.3	
15	0	100	
18	100	0	

Reactions were carried out at pH 4.5, 5.0, 6.0, 7.0, and 7.5 by titrating frequently with 0.05 N NaOH. In Fig. 2, curve *A* shows the total amount of alkali used in this procedure over a one-hour period with the enzyme from two eggs reacting with 3 cc. of 4 per cent tributyrin. Similarly, curve *B* shows the result of experiments using the extract of 20 eggs with 3 cc. of 2 per cent methyl butyrate. The pH for maximum tributyrinase activity is at 6 while the H-ion concentration affects the methyl butyrinase activity to a lesser degree. This dissimilarity in the effect of pH on the activity of the enzymes studied is one of several differences noted. No explanation of the difference in the values for the rate of methyl butyrate hydrolysis when the pH is kept constant and when it is allowed to change is at hand.

The effect of heat treatment on the enzyme extract as well as its effect on the amount of hydrolysis was determined. In the former case

the lipolytic activity of the extract was affected differently for the two substrates. The activities of extracts were tested at 25° C. after ten-minute exposures to temperatures between 25 and 85° C. The ability to split methyl butyrate was diminished by temperatures higher than 55° C. while tributyrinase activity was unchanged after exposures to 65° C. (Fig. 3).

When the reaction mixtures were kept at temperatures varying from 0° to 45° C., the amount of hydrolysis of the two substrates differed in

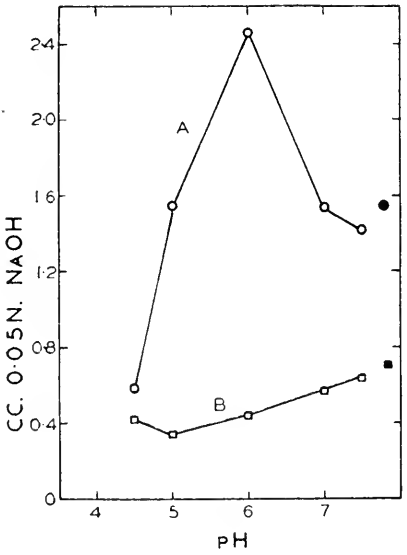

FIG. 2. The amount of hydrolysis at various H-ion concentrations. The reactions were kept at the pH noted by continuous titrations with 0.05 N NaOH. ○, the hydrolysis of tributyrin by an extract from two eggs over a period of one hour; □, methyl butyrate split by an extract of 20 eggs at the end of 2 hours. Solid symbols represent amount of acid formed when the mixture was allowed to react over the total time. Four to seven experiments averaged in each point. Reactions at room temperature.

a striking manner. The hydrolysis of methyl butyrate increased with temperatures up to 45° C. (Fig. 4*A*) and between 0° and 35° a μ value of 5700 calories was obtained (Fig. 4*B*). The Q_{10} over the corresponding range averaged 1.4. The tributyrinase activity showed a maximum at 25° C. with a decrease on either side of this temperature (Fig. 4*A*). The Q_{10} value between 5 and 15° C. is 1.97; between 15 and 25°, 1.47, using the amount of acid produced per unit time as a rate value. The values shown in the figure were obtained using an extract made in the

following manner: the eggs were ground and diluted to a volume so that the concentration was 40 eggs per cc. When this was allowed to stand a considerable precipitate was formed. This was centrifuged off and the supernatant fluid diluted to a volume corresponding to 20 eggs per cc. This resulting extract still retained its tributyrinase activity, but the methyl butyrinase reaction was reduced to one-sixth to one-fifth of that of an extract prepared in the usual manner. Falk and Sugiura (1915) were able to separate esterase and lipase materials in the castor bean, the one soluble in distilled water, the other in NaCl solution. The temperature relationship to activity of the enzyme is similar to that reported by Fiessinger and Gajdos (1936) working with an extract of the larva of *Galleria mellonella*. Their extract showed maximum activ-

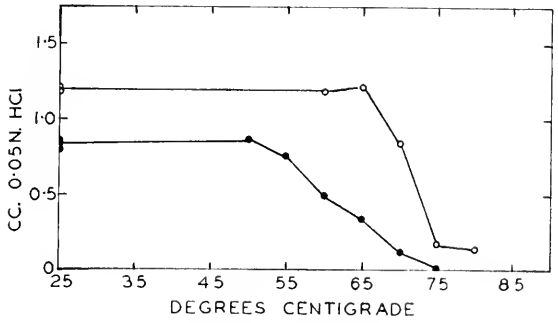

FIG. 3. The effect of temperature on the activity of the enzyme extract. Ordinate, equivalents of acid produced in cc. of 0.05 N HCl in one hour for tributyrinase and two hours for methyl butyrinase at 25° C.; abscissa, temperature in °C. to which the extract was exposed for 10 minutes. Closed circles, the hydrolysis of methyl butyrate; open circles, the hydrolysis of tributyrin. Methyl butyrinase from 20 eggs; tributyrinase from 2 eggs.

ity between 18° and 25° C. and declined at temperatures above or below this range.

Most observations concerning esterase activity indicate that the calcium ion, sodium oleate and albumin accelerate the activity of the enzymes. This is not found to be the case in extracts of the grasshopper egg. Sodium oleate reduces the lipolytic activity of the preparations used in these experiments. Calcium chloride has no effect on the enzyme but counteracts in part the effect of sodium oleate (Table II). Neither of these substances has any effect in stabilizing the pH. Albumin was not used since the extract was rich in protein. Attempts to show hydrolysis of olive oil with addition of sodium oleate and the calcium ion to the egg extracts at 25° C. and 35° C. were without success.

Various esterases are affected differently by such compounds as

phenol, quinine, atoxyl and sodium fluoride (Falk, 1924; Oppenheimer, 1936). Curiously, extracts from pancreas, liver and kidney are inhibited in their action on tributyrin in a diverse manner by quinine and atoxyl (Falk, 1924). The effect of 0.5 per cent phenol, NaF and quinine were tested on the esterases obtained from the grasshopper egg.

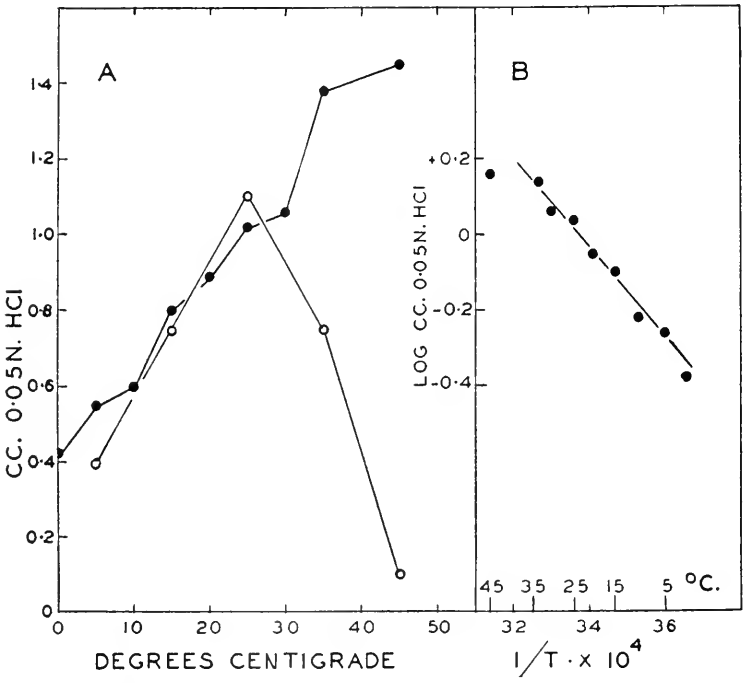

FIG. 4. The amount of hydrolysis of methyl butyrate and tributyrin at different temperatures. In *A*, the ordinate gives the equivalents in cc. of 0.05 N HCl; the abscissa, the temperature at which the reaction took place. ○, tributyrinase reaction using an extract of 2 eggs with 4 per cent tributyrin for 1 hour; ●, methyl butyrinase from 20 eggs reacting with 3 cc. of 2 per cent methyl butyrate for 2 hours. *B* shows the data for methyl butyrinase plotted as log concentration of HCl, ordinate, versus the reciprocal of the absolute temperature × 10^4, abscissa. The points are average values of ten determinations at each temperature. The straight line in *B* is fitted by the method of least squares. The μ value between 0 and 35° C. is approximately 5700 calories. For further description see text.

The results are summarized in Table III. Both methyl butyrinase and tributyrinase are inhibited by quinine and NaF. Only tributyrinase is inhibited by the 0.5 per cent phenol; methyl butyrinase activity is stimulated. Two per cent phenol will completely block both reactions. Fiessinger and Gajdos (1936), in studies on the esterase obtained from the larva of *Galleria mellonella*, found their enzyme extract unaffected by

phenol and quinine and strongly inhibited by NaF in the same concentrations as noted above with tributyrin as a substrate.

Discussion

The expectation that the grasshopper egg contains an enzyme capable of hydrolyzing triglycerides of higher fatty acids was perhaps based on a fortuitous assumption. The presence of such an enzyme in an animal metabolizing fat as the R.Q. indicates (Bodine, 1929; Boell, 1935) and consuming 60.3 per cent of its initial store of fats during development (Slifer, 1930) seemed highly probable. No evidence for this enzyme could be elicited using the methods described. The activity

Table II

	0.05N HCl (in cc.)			
Control	Control + NaOl 0.2 cc.–0.4%	Control + NaOl + $CaCl_2$ 0.4 cc.–2%	Control + $CaCl_2$	Substrate
1.19	0.03	0.21	0.74	Tributyrin
0.52	0.06	0.23	0.33	Methyl butyrate

Table III

	0.05N HCl (in cc.)			
Control	0.5% Phenol	0.5% NaF	0.5% Quinine HCl	Substrate
1.06	0.53	0.50	0.19	Tributyrin
0.77	1.64	0.19	0.42	Methyl butyrate

on the esters of the lower fatty acid (butyric) was, however, quite high during early stages of development. A summary of the data concerning the lipids of the grasshopper eggs is of interest. The fat in the egg of *Melanoplus differentialis* is liquid at room temperature (fusion point, 26.2° C.); in *Chortophaga viridifasciata* the fat is solid (fusion point, 39.4° C.) (Slifer, 1930). The former insect spends the winter as an egg, the latter as a nymph. The iodine number of the fats is the same in both animals (135 to 140) (Slifer, 1932). The low melting point in the winter eggs may possibly be due to the higher proportion of short chain fatty acids. This is the explanation of the liquid fat of the aphid, *Pemphigus*, which contains glycerides of butyric, caprylic and lauric acids (Timon-David, 1927–28). The presence of monoesters

rather than glycerol triesters might give similar results. The data concerning the enzymes present in the grasshopper egg lend credence to the assumption that the lower fatty acids are present in the egg. Slifer (1930) has shown that the total fat (measured after saponification by a method for higher fatty acids) decreases only slightly during prediapause (9.7 per cent), yet the volume of the lipoidal layer as measured by Bodine et al. (1939) decreases 32.5 per cent in the same period. Slifer (1930) found a loss of 50 per cent in postdiapause, the volume determinations, 42.5 per cent. The amount of fatty acids in a diapause egg is approximately 8 per cent of the wet weight of the egg (Slifer, 1930) as compared to an amount of lipid equal to 3½ per cent of the wet weight of the egg obtained by the centrifuge separation. The fat obtained by this latter method is a mixture of esters (probably glycerol) which contains C_{12} to C_{18} fatty acids (Allen, T. H., personal communication). Experiments to determine the hydrolysis of the lipid separated by centrifuging and also lipids extracted from the egg brei with petrol ether showed demonstrable amounts of hydrolysis after a 24-hour period only in the case of the latter. This might well be due to the existence of esters and acids in equilibrium.

The relative amounts of hydrolysis in these two enzymatic reactions cannot be quantitatively compared with the activity of esterases from other sources. In general it seems evident that the enzymes are relatively concentrated in the grasshopper egg, since experiments described with other esterases involve periods of four hours and upwards at 37° C. to produce enough acid to be measured. Fiessinger and Gajdos (1936) found that the tributyrinase from the larva of *Galleria mellonella* was much more active than that from human blood serum (ca. 10 times). They also could demonstrate no reaction with olive oil as a substrate.

The two lipolytic enzymes possess strikingly different physical and chemical properties as evidenced by the independent change in potency during development, the inactivation by heat, the effect of temperature on the rate of hydrolysis, the possibility of separating the two enzymes, and the difference in effect of the inhibitors used. Curiously, the tributyrinase, *per se,* is less sensitive to heat treatment than methyl butyrinase yet more susceptible to temperature in the presence of its substrate. This may be due to a reversal of the heat inactivation in the former case.

The evidence indicates the lipolytic enzymes in the grasshopper are present in greatest quantities at the time the egg is laid. From these high levels at the time of least differentiation in the egg the enzymes decrease in amount during development or differentiation (Fig. 1). A change in the amounts of esterase in the egg of the trout (*Salmo fario*) was observed by Falk and co-workers in a careful and detailed study of

this material. Methyl butyrate was not hydrolyzed by the esterase from the unfertilized egg, but the hydrolysis was accomplished by eggs 35 or more days after fertilization. Methyl and ethyl acetates were easily hydrolyzed by the egg but steadily less so as development proceeded; ethyl butyrate showed a reverse effect. The value of esterase action generally was high in immature eggs, small in mature eggs, increasing with development (see Needham, 1931, for summary). In the work of Falk et al. cited here no data are given for the esters of the long chain fatty acids. In the grasshopper egg the decline in the activity of the monobutyrinase after the tenth day of prediapause development occurs somewhat later than the decline in potency of the natural activator (presumably a lipid) of protyrosinase (Bodine et al., 1939). It is possible that some of the substances serving as activators are monoesters of fatty acids and that these are utilized rapidly in early development. Subsequent to this period the amount of monobutyrinase falls. However, the explanation of this effect suggested by Bodine and Carlson (1940) seems more tenable. The decline in the amounts of both enzymes studied during post-diapause development seems correlated with the rapid disappearance of yolk. The possibility that these enzymes may be found in the serosa has not been excluded in these experiments, yet the major part seems to be contained in the yolk and probably is incorporated into the midgut after its absorption. This conforms to the evidence of Stuart (1935) that the yolk cells become part of the midgut just previous to hatching. The cells of the intestinal tract then "inherit" these enzymes from the yolk. Other hydrolytic enzymes may come to be in the gut of the adult in a similar manner.

Summary

1. Glycerol extracts of the grasshopper egg (*Melanoplus differentialis*) have been tested for hydrolytic activity on methyl butyrate, tributyrin and olive oil during various stages in the development of the egg. The ability to hydrolyze methyl butyrate is high when the egg is laid; this value declines between the tenth and fifteenth day of development, remains constant during diapause and slowly declines again during the post-diapause period. The action of extracts on tributyrin is much stronger, remains constant from the time of laying until the cessation of the diapause and then declines markedly. No action on olive oil could be demonstrated.

2. Optimum activity in hydrolysis of tributyrin is at pH 6; the activity of the enzyme reacting with methyl butyrate is only slightly affected by changes in the H-ion concentration.

3. Temperature affected the methyl butyrinase and tributyrinase activity in a different manner. Exposure to temperatures above 55° C. depressed the activity of the former while the activity of the latter persisted to 65° C.

4. The hydrolytic action on tributyrin increased with temperature between 5° and 25° C. and declined at higher temperatures. Methyl butyrinase activity increased with temperature between 0° and 45° C.

5. The esterases seemed to be associated with the yolk until just before hatching.

6. The effect of sodium oleate, calcium ion and various inhibitors of lipolytic enzymes on the extracts used were determined.

The author wishes to express his appreciation to Professor J. H. Bodine for his helpful advice and criticism.

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ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XIX. Protyrosinase and Morphological Integrity of Grasshopper Eggs [1]

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Although protyrosinase has been found in extracts of grasshopper eggs, no evidence for its existence within the intact egg has been presented. In view of the possibility that the very process of extraction might inactivate the enzyme, it seems desirable to examine the relation of protyrosinase to morphological integrity. It should be possible to perform such a test by subjecting eggs to one of those treatments which cause the activation of extracted protyrosinase. An increased rate of oxygen uptake and the appearance of melanin in the intact egg should then indicate that protyrosinase had been present before its transition into tyrosinase. This paper deals with results of experiments showing the occurrence of protyrosinase within the intact egg of a grasshopper, *Melanoplus differentialis* (Thomas).

The data which are graphically illustrated in the accompanying figure were obtained from recordings of a Warburg apparatus operated at 24.9° C. The time course of oxygen uptake was plotted for groups of 100 intact eggs which had just previously been heated for five minutes in water kept at certain indicated temperatures. The rates of oxygen uptake of diapause eggs heated between 62° to 85° C. remained constant through the first 100 cu.mm. but declined as a limiting volume of 225 to 230 cu.mm. was approached. However, the rates of oxygen uptake of eggs which had been exposed to temperatures below 50° C. were constant. Relative values for the velocity of oxygen uptake may thus be given by the reciprocal of the time in minutes for the utilization of the initial 100 cu.mm. of oxygen. When these values are compared, a complex temperature effect is found (see figure). It is proposed to interpret this effect according to the properties and occurrence of protyrosinase.

If an egg extract containing protyrosinase is heated for five minutes at temperatures between 60° and 85° C., a tyrosinase is formed (Bodine

[1] Aided by a grant from the Rockefeller Foundation for work in cellular physiology.

and Allen, 1938). Heating seems to affect the stability of both protyrosinase and tyrosinase. With ascending temperature the former is activated, while the latter is destroyed. Consequently, the tyrosinase activity of an extract increases from 60° to 75° but declines from 75°

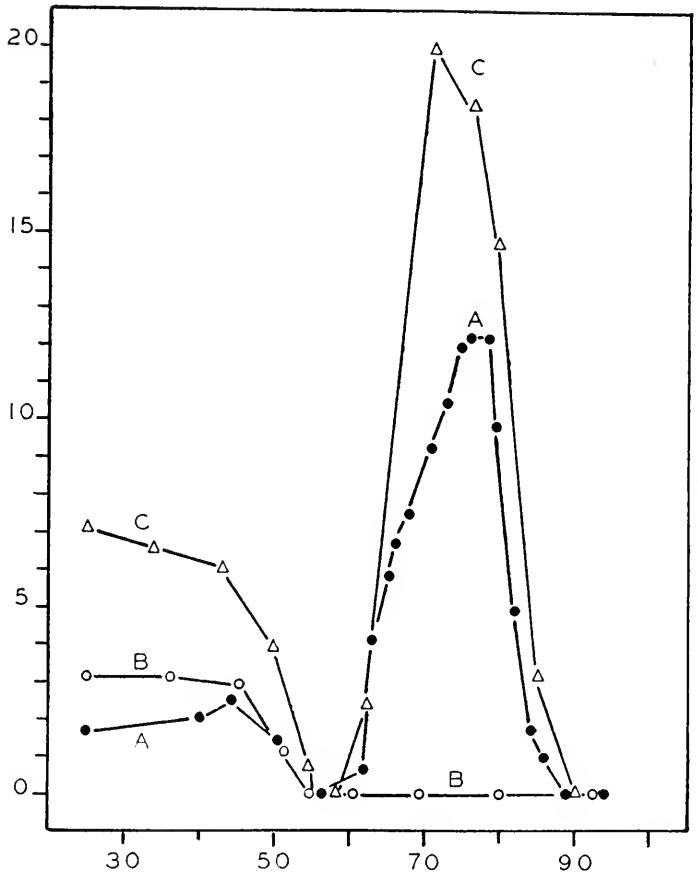

FIG. 1. The effect of heat treatment on the oxygen uptake of grasshopper eggs in various stages of development. Ordinate, reciprocal of the time in minutes for the uptake of 100 cu.mm. of oxygen multiplied by 1000; abscissa, temperatures in °C. to which eggs were exposed for five minutes. Curve *B*, 7-day eggs (prediapause); curve *A*, 60-day eggs (diapause); curve *C*, eggs 3 days post-diapause.

to 90°. A similar differential effect of heat is found for the velocity of oxygen uptake of intact diapause or post diapause eggs (see figure, curve *A* and *C*). Since protyrosinase and a naturally occurring substrate can be extracted from eggs of these stages (Bodine, Allen, and

Boell, 1937), it appears that the increased velocity of oxygen uptake of the intact egg must be due to the heat-induced enzymic oxidation of the native substrate. Curve *C* is presumably higher than curve *A,* because in post diapause there is more native substrate than in diapause (Bodine, Allen, and Boell, 1937).

The latter interpretation also seems to be supported by the eventual formation of melanin, by the low value for the "respiratory, quotient," and by the sensitivity to cyanide. Diapause eggs, which six hours previously had been heated between 62° to 84° C., changed from a pale lemon yellow to a dark olive-green color. Upon dissection it seemed that the darker color was due to the presence of a brown pigment—melanin—located in the "liquid-filled space" (Slifer, 1937) between the serosa and cuticle. Similar eggs heated below 62° and above 84° C. remained a lemon yellow, because their protyrosinase supposedly had either not been activated or else had been destroyed. From measurements of the oxygen uptake and carbon dioxide production performed according to the indirect method of Warburg (Dixon, 1934), an R.Q. of 0.1 to 0.2 was found for eggs that had been heated at 75°. Such a value is to be expected during the production of melanin (Raper, 1928). Potassium cyanide in a concentration of 0.01 M abolished the oxygen uptake produced by heat activation. These properties are usually considered to be characteristic of a tyrosinase reaction.

Since protyrosinase has not been found in extracts of eggs younger than eight to nine days of age (Bodine, Allen, and Boell, 1937), one should not expect an increased velocity of oxygen uptake for seven-day eggs that have been exposed to those various degrees of heat sufficient for activating protyrosinase. The occurrence of such a phenomenon would serve essentially as a control experiment for the heat treatment of those eggs containing protyrosinase (see figure, curve *B*). The respiratory processes of prediapause and diapause eggs are evidently susceptible to the effects of heat. Perhaps the normally working respiratory enzymes are destroyed at 56° C. (see figure). If such be the case, it may then be supposed that the portion of the curve for diapause or post-diapause eggs between 62° and 85° C. pertains entirely to the activation of protyrosinase and the destruction of tyrosinase.

The addition of an activator followed by the formation of an enzyme presumably should indicate through cause and effect relations that a proenzyme had once been present. It therefore seems that heat treatment has demonstrated the occurrence of protyrosinase as a constituent of diapause and post-diapause grasshopper eggs. This demonstration of protyrosinase seems to be independent of the trituration and dilution

inherent to an extraction process. Thus it appears that protyrosinase exists within the intact grasshopper egg and that this protyrosinase does not lose characteristic properties as a result of extraction. Moreover, these deductions on the occurrence of the inactive rather than the active enzyme would lead to the conclusion that oxidations coupled with a tyrosinase reaction (Allen and Bodine, 1940) can hardly be expected to complement the respiratory processes of these eggs. Although extracted protyrosinase can be activated by an oil native to these eggs (Bodine, Allen, and Boell, 1937), this lipide is probably bound to various proteins or isolated in such a way that it is inaccessible to the protyrosinase of intact eggs (Bodine and Carlson, 1940).

Summary

Protyrosinase occurs in the intact egg of the grasshopper, *Melanoplus differentialis,* and shows properties similar to those for extracts prepared by trituration of the eggs. Moreover, it seems that protyrosinase, as a naturally occurring entity, is not an artefact produced by extraction procedures.

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THE FOUNDING OF ANT COLONIES

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The normal method of founding colonies among the more typical of formicine species is generally understood to be as follows. The males and females swarm on a given day and copulate in the air, the males subsequently dying. Each female then descends to earth, tears off her wings, and finds a suitable spot for her colony. According to Wheeler [1]: "In her cloistered seclusion the queen now passes days, weeks, or even months, waiting for the eggs to mature in her ovaries. When these eggs have reached their full volume at the expense of her fat-body and degenerating wing-muscles, they are laid, after having been fertilized with a few of the many thousand spermatozoa stored up in her spermatheca during the nuptial flight. The queen nurses them in a little packet till they hatch as minute larvae. These she feeds with a salivary secretion derived by metabolism from the same source as the eggs, namely, from her fat-body and wing-muscles. The larvae grow slowly, pupate prematurely and hatch as unusually small but otherwise normal workers. In some species it takes fully ten months to bring such a brood of minim workers to maturity, and during all this time the queen takes no nourishment, but merely draws on her reserve tissues. As soon as the workers mature, they break through the soil and thereby make an entrance to the nest and establish a communication with the outside world. They enlarge the original chamber and continue the excavation in the form of galleries. They go forth in search of food and share it with their exhausted mother, who now exhibits a further and final change in her behavior. She becomes so exceedingly timid and sensitive to the light that she hastens to conceal herself on the slightest disturbance to the nest. She soon becomes utterly indifferent to her progeny, leaving them entirely to the care of the workers, while she limits her activities to laying eggs and imbibing liquid food from the tongues of her attendants." To this general picture I wish to suggest three types of modification; as to fasting, hazards to the colony, and coöperative founding.

Experiments have shown that queens can live for ten months or more without food, and bring up young in the meanwhile. Experiments have even been made with the precaution of furnishing nothing but distilled water. But it does not follow that because ants are able to fast

[1] William Morton Wheeler, *Ants*, Columbia University Press, 1910, p. 185.

for long periods that they regularly do so, any more than the fact that these same ants can withstand immersion implies that they regularly stay under water for any considerable duration: certainly no one supposes that in nature the queens abide by a diet of distilled water. And there are a number of reasons to believe that queens regularly leave their incipient nests to look for food.

For many years I have kept nests of ants, principally small ones developed from queens taken during swarms or from incipient nests less than a year old. In searching for incipient nests it has been my experience that I find as many queens wandering at large as I find queens in their nests. This does not mean, clearly, that half the queens of incipient nests are away from them at any given time, since the nests may be very hard to find. One day this June, for instance, I spent a few hours in a wooded area looking for young nests under loose bark. Appropriate trees were rare, and most of them were preëmpted by well-established colonies, so that I failed to find a single incipient nest. But there may have been several so well hidden that I did not find them, and there were undoubtedly hundreds of such nests in the soil, where I was not concerned to look for them. If any of these queens left her nest, however, the chances were good that I would spot her, and I did in fact so find a queen of an earth-nesting species that had probably swarmed the previous fall. This and many other similar experiences serve to convince me that it is by no means rare for a queen to leave her nest.

What is the purpose of these excursions? For several days after a swarm queens may be observed in decreasing numbers. Some of these may be late swarmers, but not many, as winged females are not found in comparable numbers. Here the reason is undoubtedly the search for more suitable quarters. During the remainder of the year, queens are less commonly observed, and the motive of their wandering is hunger. Invariably, when these queens are put in artificial nests, their first act is to eat heartily. The queen referred to in the previous paragraph spent more than an hour and a half continuously imbibing honey.

It may well be that many queens, attempting to found a nest on the starvation basis heretofore described, fail, and after consuming all their progeny wander forth to be detected by the myrmecologist. This is directly suggested by the fact that in several recorded experiments where queens failed to rear colonies, they eventually sought to escape. There is also the possibility, more significant if true, that queens may leave their incipient colonies in a perfectly healthy state while they forage for food. In artificial nests they frequently leave their brood to obtain honey or other foodstuffs some distance away within the nest. It should

also be mentioned that a few queens in artificial nests show no interest in food made available to them. In only one case in my experience, however, has the queen died, and as this occurred with a species with which I have been uniformly unsuccessful, the single case of the queen that died without eating is hardly significant in view of the two score that ate and died too. The situation here is artificial, in that no barriers are placed between the queen and the food, and she does not have to tunnel out or tear down a wall as would usually be the case in nature.

One observation of mine, however, throws direct light on this situation. In September, 1940, while waiting for the ferry at Hadlyme, Connecticut, I removed the sole piece of bark from an old fallen log and discovered underneath a typical incipient nest of *Camponotus pennsylvanicus* containing three pupae besides some eggs and small larvae. The queen was absent, but I soon saw her hurrying in a straight line for her nest. She was about two yards away when I first noticed her. Here we have a case where the queen, without the intervention of any artificial circumstances, was absent from her nest, possibly in the search for food, while that nest was in a perfectly healthy condition. On the other hand, a few queens in my own nests have failed to eat food provided within the nest, but these queens have been unsuccessful in every case.

Likewise, one occasionally finds incipient nests with such a considerable quantity of young that it is extremely unlikely that the queens have existed on a starvation basis. For example, in a very populous stump in Arlington, Vermont, I found a number of isolated queens of *Camponotus noveboracensis* that had undoubtedly swarmed the same year. In a few cases I was able to examine the cavity carefully and to take a census that was accurate as to pupae and larvae, although possibly incomplete in the count of eggs. One queen had four pupae, two larvae, and three eggs; another three pupae, three larvae of pupal size, and five eggs; a third three pupae, two large larvae, and fifteen eggs. Pupae and larvae about to pupate have consumed all the food that they eat before they emerge as adults; consequently these queens had provided the food for four, six, and five workers respectively, and had considerable numbers of other young as well. The queen of *Camponotus pennsylvanicus* previously mentioned as having been taken while returning from a foraging trip had three pupae besides a considerable number of small larvae and eggs.

If starvation is less of a hazard to ant queens than has been generally supposed, there is nevertheless a terrific mortality from other causes even when the queen survives the day of the swarm. Many queens must be killed while foraging, or have their nests invaded by other ants

or hostile insects; some succumb to parasites or to fungoid growths; and many must find themselves in such an unfavorable environment that their callow offspring, on emerging, find insufficient food or are killed or captured by other ants.

Besides these obvious perils, experience with a large number of incipient colonies in my artificial nests has indicated the importance of a number of other factors, an importance which seems to me not inferior to that of obtaining food. These factors are the following:

1. There is a very considerable mortality rate among queens during the first few weeks after swarming. This I estimate at 20 per cent, a figure which has held true from species to species, year in and year out, despite the best of laboratory care and even when the queens are adopted by workers of their own species or are given callow young immediately.

2. Some queens fail to take necessary sanitary precautions, fouling their nests and allowing mold to destroy their young and themselves. This is one of the many faults I have noticed in *Prenolepsis* queens.

3. Some queens fail to lay eggs at all; or, having laid some, cease to do so. Unless ovipositing recommences within a month or two this has always led, in my experience, to the death of the queens. Six queens of *Formica subsericea* were taken by me on August 8, 1940. One of them died in the first few weeks, leaving five, of which one failed to lay eggs. She died on January 16, 1941. Another queen of this group experienced difficulty in laying an egg; and on February 5 I observed her bent double for over ten minutes, an egg occasionally visible in her cloacal orifice. Later, however, she became normal in her egg-laying. A third queen of this group was badly mauled by the others on January 2, but was successful with her colony until she ceased laying eggs about February 20. She died on April 18. Of seven *Camponotus novebora-censis* queens taken on July 6, 1941, and previously mentioned in this article, one died on July 11 for no apparent reason, and a second, failing to lay any eggs after being taken, died on July 24.

4. Sometimes queens neglect to collect their eggs, allowing them to die and become moldy wherever dropped. *Prenolepsis* is particularly prone to do this. A similar fault is to drop eggs into crevices whence the queen is later unable to extract them. *Prenolepsis* is again a frequent offender in this way, and I have observed it not infrequently with *Cremastogaster*. Of course, any ant may occasionally lose an egg in a crack, if one exists in the vicinity. A curious incident occurred, however, with one queen of *Formica subsericea,* who developed a mania for hiding her eggs. A number were shoved as far as possible under the rim of a bowl, where she could not extricate them, and others were

hidden individually in wet cotton, where they were forgotten. When moved to another nest and given a worker, this queen became perfectly normal and successful.

5. Some queens eat all their progeny regardless of the presence of other food. In mid-August, 1923, a period when other queens had many larvae, pupae, and even callow young, I took a queen of *Camponotus pennsylvanicus* without young. On July 26, 1941, I took a *Camponotus noveboracensis* queen with only one medium-sized larva. On the twenty-seventh the larva had disappeared and an egg was present, although there was honey and meat in her cell. By July 31 the egg, together with a second laid subsequently, had disappeared; and for the next two weeks eggs were eaten shortly after being laid. A queen of *Formica subsericea* laid four eggs on November 26, 1940. One was eaten that evening. Another was laid on the twenty-seventh, and one eaten November 28. Two more were laid November 29, but all were eaten by December 12. While this article was being written, two affiliated queens of this species devoured the fifteen eggs they had collected, and have not acquired any more in the two weeks since that event.

6. That a few eggs should be eaten to further the development of the more advanced young is natural enough, but I once saw a *Camponotus pennsylvanicus* eat her most advanced larva, and on several occasions larvae have been consumed in the presence of both pupae and eggs; and once or twice pupae have been eaten in the presence of older pupae, as well as larvae and eggs. A *Camponotus ferrugineus* queen and one worker devoured three well-formed naked pupae, but saved a medium-sized larva.

7. In one nest larvae twice failed to grow, either because of some constitutional defect or because they were not fed. This instance is that of the *Camponotus pennsylvanicus* queen taken in September, 1940 and already mentioned in another connection. Of the three pupae, two developed normally and one was stillborn: the small larvae remained small, however, from September until the following June, when all but one disappeared during some affiliation experiments. In this period eggs developed normally into larvae, but failed to grow in the larval stage. The larvae appeared healthy enough, except that their skins were less shiny than normal. At the conclusion of the affiliation experiment this nest contained only the queen and one larva of the queen's first year progeny, two alien workers, and five alien males. A new lot of eggs began to be laid on June 1, just before the experiment, and these started hatching July 17. Once more, however, despite the presence of a new lot of workers, the larvae failed to grow.

8. When larvae pupate and commence to spin, the queens sometimes allow eggs and small larvae to become entangled in the spun silk of the pupating larva, usually with fatal results. This failure might be much more rare under natural conditions, where the pupating larva can be buried in earth. The same *Camponotus* queen previously mentioned invariably put her whole stock of eggs and small larvae on any one of the introduced male larvae which happened to be pupating at the moment. If these were rescued, she restored the status quo ante as soon as the human intervention was over. In fact, it is to this behavior that I ascribe the eventual death of the first-year eggs and larvae some nine months from their first appearance.

9. A not infrequent fault in incipient nests is the failure to open cocoons, though this is the fault of workers more commonly than of queens. One *Formica nitidiventris* queen lost ten out of fifteen cocoons in this way. Four were saved only because I opened them, and the fifteenth was tenderly guarded by the queen, long after its demise was evident (on close application) even to human nostrils. These four were removed to another nest, after which the sixteenth and seventeenth cocoons were successfully opened.

10. It is less easy to understand why cocoons are sometimes partially opened, and the unborn ant allowed vainly to struggle with one leg or antenna protruding, or perhaps a whole head. Yet this happens not infrequently, while the queen and sister workers pay no attention. The third worker of the same *Camponotus pennsylvanicus* queen struggled for forty hours with only its head emerged, before I took pity on it and released it.

11. When the cocoons are opened, the membranes may not be completely removed. As these dry, they contract and twist the still supple exoskeleton into awkward and useless shapes. At best this results in a curving and weakening of a leg—most commonly one of the hind ones—at worst it results in the complete incapacitation of one or more members. Some examples may be given: a *Camponotus noveboracensis* queen left her third worker hampered by membranes around both hind legs, and I removed these membranes thirty-six hours after birth. A *Formica subsericea* colony worked on the tenth callow to be born for six hours without removing all the membranes. A *Cremastogaster* queen left her first worker swathed in membranes for twenty-four hours, after which my efforts to save it were unsuccessful. In a colony that contained three queens of *Formica subsericea,* fighting among the queens prevented proper attention to the young, and several died; one had badly deformed antennae; and two were saved only by my intervention. In the nest of *Formica nitidiventris,* already mentioned, the third worker

born (exclusive of the fifteen cocoons of early vintage) was left with membranes binding one hind leg to the gaster.

12. The infant mortality rate, among ants as among other animals, is higher than the rate at any other period. In a healthy and well-developed nest it may be negligible, with incipient nests I expect to lose between 5 and 10 per cent of callows through stillbirth and death in the first two weeks of life. This estimate does not include losses through failure to open cocoons or remove membranes.

13. The young workers, when they arrive, may be deficient in very much the same ways as the queens. One additional defect, however, is failure to forage. During the nine months that her two workers lived with the *Camponotus pennsylvanicus* queen before mentioned, they failed to seek food, but obtained it instead by regurgitation from the queen.

14. Workers may fail to keep the nest clean. In my experience this is particularly true of *Formica neocinerea*, and of slave-making ants where this species is used as slave. The related *Formica subsericea* is also addicted to this carelessness, although to a lesser extent.

15. Misdirected activity with regard to eggs is not unknown among workers. In a nest of *Lasius* which I collected in 1936, all the pupae were allowed to soak in the water compartment. As this would kill the pupae in short order, I turned out the whole nest. A few hours later one worker began putting the pupae in the water once more, but I removed them and the act was not repeated.

16. Workers may eat the young. In a nest of *Formica subsericea* containing one queen and one worker, the latter ate the eggs laid by the queen despite the presence of other food. She never molested one egg, but would eat any excess over this number. Finally, after the death of the queen, she was left alone with this one, and nursed it to medium-large size. Then she affiliated with another queen, and did not return to her infantivorous practices. In another nest of this species the two workers consumed the only young then present, which consisted of five eggs.

17. In small nests, inexperienced workers are more prone than queens to leave cocoons unopened. In larger nests, one at least of the workers will usually be successful in caring for the young, and the others take their cue from her. In nests started with nothing more than larvae and pupae, some of the latter being opened by hand, it is usually necessary to continue opening operations for some time before the workers take over. Somewhat the same conditions prevail in an incipient nest, and if the queen immediately leaves everything to the first few workers born, which she frequently does, one or two callows are apt to die or be born crippled before the workers become adept at their jobs. In one nest of

Formica subsericea the eighteenth worker to be born was left helpless in a half-opened cocoon and was relieved of her cocoon and membranes by me some ten hours after her birth.

It is interesting to notice, in passing, that when callows are being relieved of membranes, they almost invariably submit willingly to the necessary treatment. When picked up, the callow tries to escape, although its efforts are much less violent than those of a full-grown ant. But as soon as the point of a teasing needle is applied in an effort to remove the membrane, the callow remains quiet without being held until the operation is over. I have known adult ants to act in the same way when I have attempted to remove wax which adhered to them and interfered with their movements.

Another point of interest is that when a queen shows a failure in one aspect of nest-building, that queen and her progeny are very apt to show degeneracy in other ways as well. Consider the case of the *Camponotus pennsylvanicus* queen. Her first worker was the only one born successfully. The second was stillborn; the third required my aid at birth. Though they were ready enough to defend the nest, these two workers failed to aid the queen in caring for the young, or in foraging for food. And in two successive seasons, the larvae failed to grow beyond minimum size. Such a series of mishaps could not occur in nature, for any one might well be fatal, and any two would almost certainly be so.

We now pass to the third and last modification of the usual picture of colony foundation. While queens are capable of founding nests unaided, they may receive aid in doing so, and this method seems to me to be more important than has been generally realized. It is well known that certain species are temporarily or permanently parasitic; and that in other species minims accompany the queen on the marriage flight. It is also known that queens sometimes collaborate in the founding of nests and that a large colony may retain many of its own females as additional queens, but these facts have not been given their full weight. My own experience is that in the two highest subfamilies—and these include all the well-known ants of the north temperate zone—polygynous colonies are just as typical as monogynous. In some cases, indeed, the number of queens is fantastic. Windsor[2] reports that he removed forty-nine dealeated queens of *Formica neocinerea* from one spadeful of earth, and in opening a nest of *Formica sanguinea subintegra* his impression was that the queens were almost as numerous as the workers.

Furthermore, on the day of swarming and for a few days thereafter, the queens of most species are exceptionally ready to form alliances, and

[2] Reported to me by letter and to be published in "Anti-Social Behavior among Ants," *Journal of Comparative Psychology, circa* April, 1942.

any number can be put together successfully. This even applies to queens of different species, if they happen to swarm within a day or so of each other. This was forcefully demonstrated while this paper was being written, for *Lasius* and *Acanthomyops*[3] swarmed on the same day and two boys who had been asked to collect queens for me put sixty of the former and eighteen of the latter in one jar. The group was entirely peaceable, and no casualties whatever resulted from the strange mixture. The rule is not true of all species, however, nor of all queens of any species. When *Cremastogaster* swarmed this year, I collected three groups of six queens each, and one of four. In one group one queen killed all the others; in a second only one queen died, while no injuries occurred in the other two.

An instance is on record where a nest divided into two sections, which gradually separated and became distinct colonies. This process of colony formation is probably quite important: that it is so is indicated by the fact that many nests in a given area fraternize (sic) with each other.

It must happen not infrequently that colonies lose their queens and descend towards extinction. Under such circumstances the workers are exceptionally ready to adopt queens of their own or even of a related species. The same is probably true of colonies whose queens have become infertile, and possibly for colonies that have undergone other discouraging misadventures. When large colonies are deprived of their queen, their morale may be shattered and groups of workers migrate into suitable holes and shelters in the vicinity. In such cases it is possible for several groups to adopt queens and for one colony to aid in the development of several new nests. The evidence for this consists partly of the fact, easily determined by experiment, that queens and workers affiliate more readily than do queens alone or workers alone. It is also easy to show that small, queenless, or demoralized groups of workers affiliate more readily than do normal colonies, and this principle has been successfully applied by me in artificially inducing affiliation. On several occasions, indeed, such groups have actively sought affiliation, as when a group of about two hundred queenless workers of *Tetramorium caespitum* forced their way into an alien colony of fifty workers and several females and affiliated with them. Third, I have noticed in both natural and artificial nests that when deprived of a queen and of young the workers exhibit a tendency to wander, and to congregate in small groups of from five to fifty. And lastly, the well-known behavior of many of the permanent parasites demonstrates that queens can get themselves accepted by another colony.

[3] *Acanthomyops murphyi*, determination by William S. Creighton. The *Lasius* were principally *L. americanus*, with two *mixtus* and one *nearcticus*.

We now come to the last method by which queens may receive aid in founding a colony. In nature, worker ants must occasionally wander so far that they are unable to find their way home, and others must be carried away by wind or water, or be transferred to new areas by clinging to birds, animals, or to human transport. Solitary workers will die in a day or so at most, but I seriously doubt whether the majority of strays die in this manner. It appears to me, on the contrary, that most of these strays will enter other formicaries and there be killed or adopted, largely dependent upon the size of the community entered. If an object being explored by an ant be removed to some considerable distance from her nest, the typical behavior may be readily observed. At first the worker shows the normal exploratory behavior: she examines crevices for food, and will capture any available. If she meets an alien ant she avoids her, but without any appearance of panic. Sooner or later she apparently becomes aware that she is lost: her movements are now more rapid, she usually ignores food (except for an unusually luscious tidbit such as a drop of honey), and she exhibits fright in contact with alien ants. If she is put into another nest during this period she tries to escape but rarely fights back if attacked. Still later another change may be noted: the ant apparently abandons hope of finding her own nest, and there are two conditions frequently met. In one the worker becomes quiescent, with only a quivering of an antenna or leg to indicate life, and dies within a day—sometimes within an hour. In the second typical condition the worker becomes interested in other ants and will actively seek alliances with them. In either case the worker forms alliances readily, although only passively in the first type. Even after making alliances, however, workers of the first type occasionally die or wander off in a few days. It is quite clear that these stray workers, who cannot be excessively rare, form an accessible auxiliary to queens who are founding nests.

I have observed a related type of behavior in—so far—only a single species. Almost any worker of *Camponotus noveboracensis* will affiliate with any solitary queen if she wanders or is put into the latter's nest. On several occasions I have seen such workers feed the queen and care for the young for a day or so, and then seek to leave. This raises the question whether this temporary affiliation can occur at all frequently in nature. Somewhat similar behavior has been previously reported of *Acanthomyops,* which are said to feed alien workers that enter the nest. I have been unable to verify this behavior. If either type occurs, it would almost amount to a confederation of a whole species for mutual aid, a condition previously unknown in the insect world, and infrequent elsewhere.

STUDIES ON EXPERIMENTAL HAPLOIDY IN SALAMANDER LARVAE

II. Cytological Studies on Androgenetic Eggs of Triturus viridescens

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Introduction

One of the outstanding features of the experiments on androgenesis with eggs of salamanders has been the high rate of mortality during cleavage and gastrulation (Fankhauser, 1934*a*; Kaylor, 1937). However, there have been surprisingly few investigations on the cytology of failure of development in these early stages of androgenetic development. The most extensive observations have been those of Fankhauser (1934, *a* and *b*) on androgenetic egg fragments of *Triton palmatus* and more recently of Fankhauser and Moore (1941) on androgenetic eggs of *Triturus viridescens*. There have been a number of cytological studies on parthenogenesis in eggs of frogs (review of literature, Parmenter, 1933), but these have been concerned more with the rôle of the nucleus in early development of the egg (Dalcq, 1932) or with the origin of diploid and higher numbers of chromosomes (Parmenter, 1933, 1940) in cells of eggs and embryos rather than some of the factors underlying a failure of development beyond certain stages.

In view of the scarcity of studies on the cytology of early stages of androgenetic development in salamanders, the present study seemed to be indicated. It is a survey of the microscopical evidences of the causes of cessation of development in androgenetic eggs of *Triturus viridescens*. A preliminary cytological examination (Kaylor, 1939) showed that an irregular distribution of chromosomes had taken place in these eggs, as in the merogonic eggs of Fankhauser, and was probably responsible for the arrested development, since in this type of experiment no injury to the existing organization of the egg is possible.

Material and Methods

Material

During the course of experiments on androgenesis in *Triturus viridescens* (Kaylor, 1937, and later experiments not published) consider-

able material was preserved for future cytological studies. Of this material, 63 eggs which had ceased development during early cleavage, blastula or gastrula stages were selected for cytological examination. Fifty-nine of these eggs had actually completed their developmental possibilities; they were fixed either after they had remained in the same stage for 12 hours or more or at the onset of cytolysis as indicated by a beginning discoloration of some of the cells. Four of the eggs were preserved because of broken yolk membranes.

Methods

Experimental.—The technique used in obtaining these androgenetic eggs has already been described in detail (Kaylor, 1937). It consists essentially of the removal of the second maturation spindle from the egg by puncturing the polar area containing the spindle with a fine glass needle and sucking a small amount of material into a capillary pipette. The egg then develops with only the male, haploid set of chromosomes.

Fixation, Sectioning, Staining.—All eggs were fixed in Bouin's fluid, cleared from 95 per cent alcohol through wintergreen oil, and imbedded in paraffin containing about 5 per cent bayberry wax. This fixative hardens the yolk, but satisfactory sections were obtained by soaking the imbedded eggs in water for 12 to 24 hours, after the first 10 or 12 sections were cut and mounted: the method used by Fankhauser and Moore (1941). After this soaking, a complete ribbon of perfect sections was obtained. The sections were cut at 15 μ, parallel to the animal-vegetal axis. The sections were stained in Harris' acid-haemalum for the nuclear stain, eosin as a counterstain for the yolk granules, and Light green for the spindle fibers. They were then cleared from 95 per cent alcohol through pure aniline oil and mounted in an aniline-balsam mixture. The use of aniline was necessary since the use of xylene after the staining and dehydration processes always cracked the sections.

Figures 1 and 9 were drawn at a magnification of 80 and reduced to one-half in reproduction.

Observations

Observations on the Living Eggs

To review briefly the former observations on the living androgenetic eggs of *Triturus viridescens* (Kaylor, 1937), it was found first of all that although the majority of the androgenetic eggs underwent irregular cleavage and died prior to gastrulation, this abnormal cleavage was not

entirely responsible for the early cessation of development, since approximately one-half of the normally segmenting eggs failed to develop beyond the gastrula stage. Secondly, there existed no correlation between the type of cleavage of an androgenetic egg and the number of spermatozoa present in the egg at the time of operation. It was obvious, then, that a detailed cytological study of the early development of androgenetic eggs might determine the causes of the early arrested development.

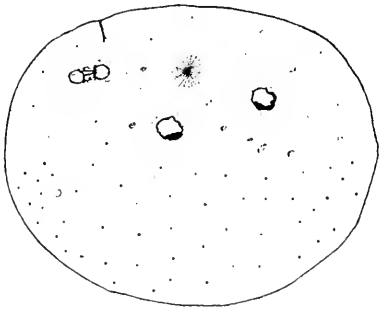


FIG. 1. Drawing of a median section of the egg 30.4e, sectioned parallel to the egg axis. All nuclei projected into this section from neighboring sections. Three degenerating sperm nuclei: two in prophase, one in telophase. One cytaster. Indication of a furrow.

Cytological Observations

The following stages of development of androgenetic eggs were examined in sections:

Stage of Development	Number of Eggs Examined
a. Irregular beginning cleavage	
1. Abortive cleavage	7
2. Early irregular cleavage	5
b. Early blastula	19
c. Late blastula	23
d. Gastrula	9
	Total 63

a. Irregular Beginning Cleavage Stages.—1. *Abortive cleavage.* Seven eggs were fixed 25 to 36 hours after operation, during which time only a few irregular, incomplete furrows had appeared on the egg surface. These furrows were still visible at the time the eggs were preserved. Surprisingly enough, in the sections there was no evidence of furrows in six of the seven eggs (Table I). One egg showed definite irregular furrowing, not connected with mitotic activity within the egg.

In each of these eggs there was evidence of early mitotic activity on the part of the sperm nuclei. The evidence is summarized in Table I. The cytological condition of each egg showed very little variation. The majority of sperm nuclei degenerated either before or after early mitotic activity. Cytasters were present in most of the eggs. Figure 1 illustrates the typical cytological condition encountered. In this particular egg, three or four sperm entrance marks were present on the egg surface at the time of operation. Three degenerating nuclei and

TABLE I

Summary of cytological conditions in abortive cleavage stages

Egg No.	No. Sperm Marks	Age	External Appearance	Cytological Condition
		hours		
26.1e	1	32	Irregular furrows at animal pole	No furrows, 2 nuclei degenerating in prophase, 1 spindle, no chromosomes
27.5e	3	36	Irregular furrows at animal pole	No furrows, 1 nucleus degenerating in prophase
28.6e	3	26	Irregular furrows at animal pole	No furrows, 3 nuclei degenerating in prophase
30.4e	3–4	26	One irregular furrow	Indication of furrows, 2 nuclei degenerating in prophase, 1 nucleus degenerating in telophase, 1 cytaster
30.5e	2	36	Irregular furrows at animal pole	No furrows, 1 cytaster, cytolysis
30.6e	4	25	Irregular furrows at animal pole	No furrows, 1 degenerating nucleus, 4 cytasters
101.1e	6	28	Irregular furrows at animal pole	No furrows, many degenerating nuclei

one cytaster were actually found in the egg; two of the nuclei were degenerating at prophase, and one at telophase.

2. *Early irregular cleavage.* Five eggs, fixed 24 to 26 hours after operation, were examined in this group. The cytological condition of each of these eggs is summarized in Table II.

From this table it is clear that the sperm nuclei in these eggs began to divide at the same or nearly the same time. One nucleus divided sooner than the others, but succeeded in forming only a few small, irregular cells. The "accessory" sperm nuclei either degenerated dur-

ing early mitosis, or continued to divide haphazardly. In any case, the presence of so many constellations in the egg does not lead to the formation of complete cleavage furrows.

Figures 2 and 3 illustrate the cytological condition of two of the most interesting eggs of this group. In the egg shown in Fig. 2, five

TABLE II

Summary of cytological conditions in early irregular cleavage stages

Egg No.	No. Sperm Marks	Age	External Appearance	Cytological Condition
		hours		
32.1e	6	26	Irregular cleavage	4–6 irregular "cells," no nuclei. In unsegmented region: 6 nuclei degenerating in prophase (monaster), 1 cytaster
34.3e	7+	26	Irregular cleavage	6–8 irregular cells, degenerating nuclei. In unsegmented region: 3 nuclei degenerating in prophase (monaster), 3 nuclei degenerating in metaphase (bipolar), 1 degenerating nucleus
61.1e	7	25	Irregular cleavage	6–8 irregular cells, degenerating nuclei. In unsegmented region: 3 nuclei degenerating in prophase (monastral), 1 nucleus degenerating in bipolar mitosis, 2 cytasters
63.1e	6	24	Irregular cleavage	4 irregular cells, mitosis in each. In unsegmented region: 5 nuclei degenerating in prophase (monastral), 9 cytasters, 1 triaster
64.2e	3	26	Irregular cleavage	many irregular cells, nuclei in majority degenerating. In unsegmented region: 4 small bipolar spindles, 7 degenerated nuclei, 3 large triastral mitoses with large number of chromosomes, 2 large tetrastral mitoses with large number of chromosomes, 10 cytasters

of the six spermatozoa entering the egg are degenerating after a beginning monastral mitosis. It is probable that the sixth sperm nucleus divided in a normal manner and was responsible for the formation of the few cells in the upper part of the egg. In four of these cells, a normal haploid mitosis is in progress. The nine cytasters scattered through the unsegmented part of the egg apparently have no connection with any of the sperm nuclei and for this reason probably originated

de novo in the cytoplasm, as they do in egg fragments of *Triton* (Fankhauser, 1934*a*). The large triaster is probably a fusion of three cytasters.

The cytological condition of the egg in Fig. 3 is much more complex. An inventory of the contents of this egg is given in Table II and in the explanation of Fig. 3. Since there were only three sperm entrance

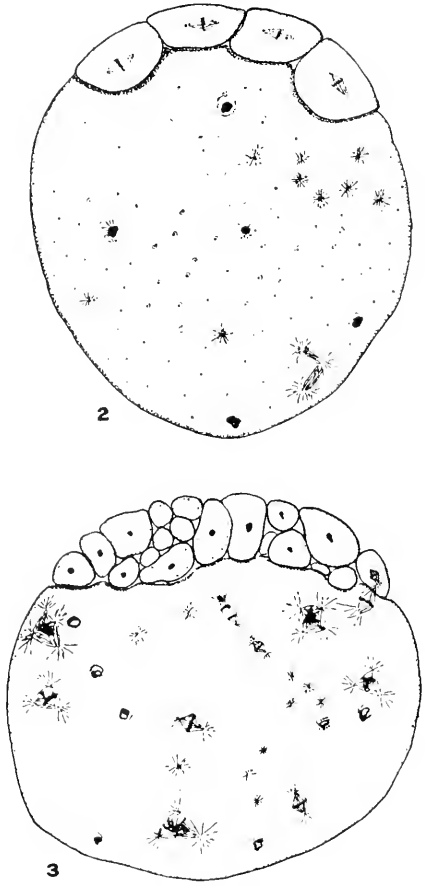
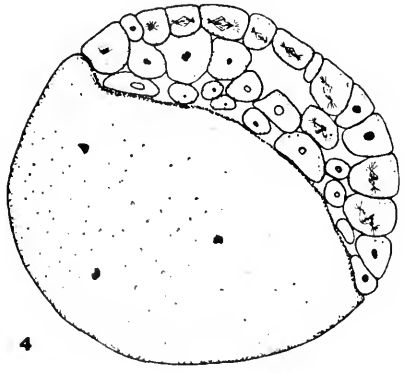


FIG. 2. Drawing of a median section of the egg 63.1e, sectioned parallel to the egg axis. All nuclei and cytasters projected into this section from neighboring sections. Irregular cells, mitosis in each. Five degenerating sperm nuclei, nine cytasters, one triaster in the unsegmented yolk region.

FIG. 3. Drawing of a median section of the egg 64.2e, sectioned parallel to the egg axis. All nuclei projected into this section from neighboring sections. Irregular cells, degenerating nuclei in most cells. In the unsegmented region: four small bipolar mitoses, seven degenerated nuclei, three large triastral mitoses and two large tetrastral mitoses with large numbers of chromosomes, ten cytasters.

marks on the living egg, it seems probable that only two sperm nuclei could have been responsible for the large number of irregular mitotic figures present, while the third sperm nucleus initiated the formation of the few small, irregular cells in the upper part of the egg. Several chromosome counts were made in the figures present in the yolk region.



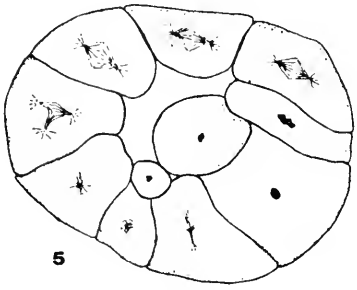


FIG. 4. Drawing of a median section of the egg 28.5e, sectioned parallel to the egg axis. Nuclei projected from neighboring sections into the cells and unsegmented region. Many cells non-nucleated, some with single asters, others with degenerating sperm nuclei in the yolk region.

FIG. 5. Drawing of a median section of the egg 30.7e, sectioned parallel to the egg axis. Nuclei projected from neighboring sections into the cells. Majority of cells non-nucleated. Fairly normal blastula.

In one normal anaphase figure, seventeen chromosomes were identified; eight at one pole and nine at the other (Fig. 10). In another anaphase, 18 chromosomes were identified, while in a nearby metaphase plate, 13 chromosomes could be counted. Several large, irregular triasters and tetrasters were in this yolk region. Large numbers of chromosomes were present in each of these figures.

b. Early Blastulae.—Nineteen eggs were fixed approximately 28 hours after operation, when they failed to develop beyond the mid-blastula stage. The most conspicuous features of the sections of these eggs were, first, that in 13 eggs a large area of the vegetative region was unsegmented. Only 6 eggs were completely segmented. Secondly, closer examination revealed a large number of abnormal mitotic figures

TABLE III

Chromosome numbers in early androgenetic blastulae

Egg No.	Total Number of Analyzable Mitotic Figures	Chromosome Numbers									
		9	10–11	11	11–12	12–14	14–16	16–18	18+	22	22+
26.9e	6				2		2				2
20.21e*	6				1	2		1			2
28.5e	2	1				1					
34.2e	25		2	3	5	6	2	2	4	1	
37Ae	11				2	1		1	1		6
52Ae	6				1	3		2			
53Ae*	6				1	1	2	2			
54.3e	12		1	3	8						
68Ae	2					2					
70.2e	11	3	3	3	2						
208.16e	8					4		2			2
00.E	18				8	3		3			4

* Preserved while still developing.

in the cells and unsegmented regions; in the 19 eggs a total of 312 mitoses of the following types were observed:

(*a*) Pluripolar mitoses.
(*b*) Monastral mitoses.
(*c*) Bipolar mitoses with degenerating chromosomes.
(*d*) Bipolar mitoses with no chromosomes.

Figure 4 is an example of an egg of the group of thirteen eggs with the undivided vegetal region. The cytology of this egg is given in detail since, although the egg does not possess all of the irregularities listed above, it is in general illustrative of the cytological condition of this group of eggs. A large sector of the roof of this blastula is composed of cells without nuclei; each cell contains a small bipolar spindle with no chromosomes. Other cells nearby contain only a single aster. In a few cells, bipolar mitoses are in progress, but in several of these figures chromosomes are showing signs of degeneration. Figure 11 il-

lustrates an anaphase spindle in one of these cells. Four chromosomes are lagging on the spindle and show definite abnormal swelling. The large unsegmented yolk region of this egg contains three nuclei which are degenerating in early prophase. The undivided yolk region of similar eggs, however, contained a larger number of abnormal figures

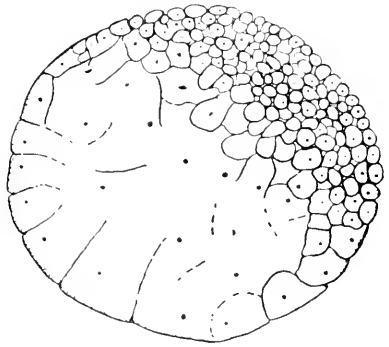


FIG. 6. Drawing of a median section of the egg 25.3e, sectioned parallel to the egg axis. Abnormal late blastula. Large areas of the yolk region unsegmented. No blastocoele.

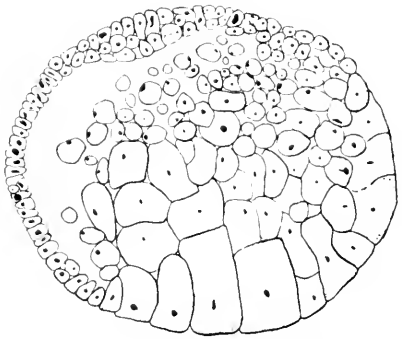


FIG. 7. Drawing of a median section of the egg 23.2e, sectioned parallel to the egg axis. Abnormal late blastula. Cells with pycnotic nuclei in segmentation cavity.

than are seen in this egg. Figures 12 and 13 show two of these mitoses.

The egg shown in Fig. 5 illustrates the typical condition in the group of six completely segmented eggs. The majority of cells contain bipolar spindles with no chromosomes; spindles similar to the one shown in Fig. 14. A few cells contain single cytasters. In other cells, the nuclei are degenerating. The small blastocoele has a few fragments

of cytoplasm containing no chromatin. The other five eggs did not have as many cells without nuclei.

Chromosome counts were possible in some of the cells of twelve of the nineteen eggs (Table III). In all but one of the eggs, the counts deviated from the haploid number (11 chromosomes) in the majority of cells. The one blastula which was haploid happened to have been fixed while still developing. It is doubtful that this egg could have reached an advanced stage of development because the cleavage was very irregular.

TABLE IV

Chromosome numbers in late androgenetic blastulae

Egg No.	Total Number of Analyzable Mitotic Figures	Chromosome Numbers									
		7–8	9–10	10–11	11	11–12	12–14	15–18	22	27+	30–33
23.2e	23	12	2				3	6			
25.3e	17	1		1	1	2	3	8	1		
25.9e	22	2	2	4	2	3	5	4			
26.5e	40	5	1	4	14	10		3	1	2	
26.6e*	40			14	15	8				2	1
27.1e*	26			3	5	5	9	4			
36.1e	19	1		4	6	6	1	1			
61.5e	11	1		3	5			2			
64.1e	11	2	7	2							
86.2e	9					1	2	4	1	1	
A.A.e	11			1	1		2	7			
3.Ae	15	1	1	4	2		2	4	1		
56.Ae	13	2	3	3	3			2			
60.Ae	12	1	1	4	1	1	2	2			

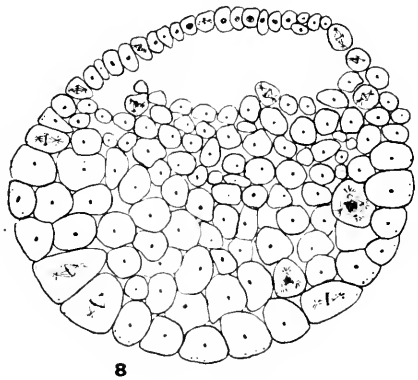
* Preserved while still developing.

c. Late Blastulae.—Of the 23 eggs fixed in the late blastula stage, only two were fixed while still developing. The following description will cover first of all the 21 eggs which had ceased development.

Although in external appearance each of these eggs resembled a normal blastula, the sections showed that all eggs were abnormal. Eighteen eggs were incompletely segmented in certain areas of the vegetal region. Only three eggs were completely segmented. All of the irregularities of mitosis observed in the earlier cleavage stages could be identified in the cells of these blastulae.

Since it would be impossible to describe the cytology of each of these eggs, the blastula shown in Fig. 6 was selected as representative of the group of 18 incompletely divided eggs. No blastocoele is present in the

egg. The upper half of the egg is composed of regularly segmented cells, while in the lower half the boundaries of many of the cells are incomplete. In sixty or more cells, the nuclear conditions were abnormal. The nuclei in the majority of these were degenerating, and in



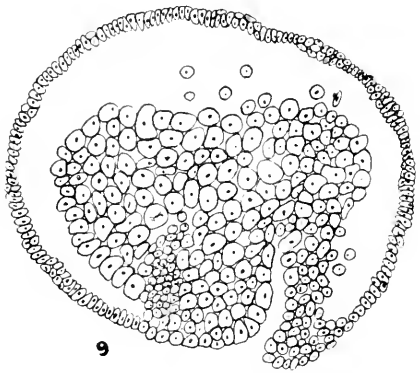


FIG. 8. Drawing of a median section of the egg 26.6e which was preserved while still developing. Sectioned parallel to the egg axis. Fairly normal late blastula. Irregular mitoses beginning. Tetrastral mitosis in cell of vegetal region at right of drawing, one triastral mitosis in nearby cell.

FIG. 9. Drawing of a median section of the egg 37.3e, sectioned parallel to the egg axis. Abortive gastrula. Incomplete invagination of the yolk. Many cells with pycnotic nuclei in the blastocoele.

other cells, mitoses, still in progress, were frequently of a monastral type. Chromosome counts in 17 cells varied from 7 to 22 in number, indicating that irregular distributions of the chromosomes had occurred earlier in the cleavage history. Several mitotic figures in this egg showed stages of chromosome elimination. Figure 15 illustrates a

metaphase figure in which all of the chromosomes have degenerated. In another cell (Fig. 16), the chromatin is completely removed from the spindle. Other mitoses were observed in which the elimination of chromosomes was occurring more gradually; a few chromosomes at a time were being lost from the spindle. This is seen in Fig. 17. At least two and probably six chromosomes are not included in the metaphase group and will remain outside the nucleus in one of the two daughter cells. A telophase in a cell from another egg (Fig. 18) shows several degenerating chromosomes near the new cell membranes. These chromosomes will not be included in the daughter nuclei.

Each of the three completely segmented eggs possessed a segmentation cavity. Figure 7 illustrates one of these blastulae. About one-

TABLE V

Chromosome numbers in androgenetic gastrulae

Egg No.	Total Number of Analyzable Mitotic Figures	Chromosome Numbers										
		7–8	9–10	10	10–11	11	11–12	12	12–14	15–17	20–21	22+
26.11e	10										7	3
31.1e	14			14								
35.1e	12					8	4					
35.2e	18				6	10	2					
35.9e	6									4	2	
37.2e	25			2	1	7	3	9	1	2		
37.3e	21	1	2	2		7	4	2		3		
75.1e	10		2	2		2		4				
76.3e	9	2	2		1				1	3		

half of the roof of this blastula is composed of a double row of cells. The vegetal region still has abnormally large cells. A number of cells with pycnotic nuclei have separated from the yolk into the blastocoele. Although abnormal mitoses were not observed, an irregular distribution of chromosomes had occurred in earlier stages of development since chromosome numbers in 23 cells varied from 7 to 16 or 18.

Even though development was at a standstill in most of these eggs, mitoses were still frequent. The chromosomes of metaphase plates could be counted accurately in 14 eggs (Table IV). From Table IV it is clear that none of these blastulae were completely haploid.

Two late blastulae were preserved because of ruptured yolk membranes. One of these, Fig. 8, is most interesting because, unlike the majority of operated eggs, its cleavage had been undelayed and perfectly normal. There had been no suspicion, therefore, that the female nucleus

was actually out of the egg. In Fig. 8 it is seen that the egg was a fairly normal blastula. The first few chromosome counts were all haploid. Then the following mitoses were observed: (*a*) two normal bipolar figures with 22 chromosomes (the diploid number); (*b*) one triatral mitosis with 33 chromosomes (Fig. 19); and (*c*) a tetrastral figure with a large number of chromosomes, presumably the tetraploid number. One other cell (Fig. 20) contained a telophase figure with fragments of chromosomes at the center of the spindle. In view of the small number of cells with slightly irregular cytological conditions, this egg could probably have developed to a more advanced stage. The other egg possessed irregular chromosome numbers in the majority of cells. For this reason it probably would not have developed farther.

d. Gastrulae.—The last group of androgenetic eggs consisted of nine eggs fixed at the end of their development in the gastrula stage. In section, all of these eggs were found to be abortive gastrulae. The process of invagination of cells into the blastocoele was incomplete. In most of these eggs yolk cells with pycnotic nuclei were accumulating in the blastocoele (Fig. 9).

Although mitoses were not frequent in these gastrulae, a few chromosome counts were made in each egg (Table V). In all but two eggs, the majority of cells were not haploid. It is interesting to note that one gastrula had only 10 chromosomes in every cell clear enough for analysis. Apparently the lack of even one chromosome may be sufficient to disturb the processes of differentiation occurring for the first time at gastrulation.

The abnormal gastrulation of the two eggs which were completely haploid is not surprising since in later stages of development, as for example the formation of the neural plate, haploid embryos frequently have serious difficulties. This was observed in an earlier report (Kaylor, 1937), and in the experiments on the androgenetic development of frog embryos (Porter, 1939).

Discussion

The cytological conditions found in these eggs explain fully the high mortality rate during cleavage and gastrulation. In eggs fixed after irregular beginning cleavage, it was observed that either none of the sperm nuclei was sufficiently active to form cleavage furrows, or, quite the opposite, all of the sperm nuclei divided at the same or nearly the same time causing incomplete and irregular cleavage of the egg. The cytological conditions were somewhat the same in eggs which ceased de-

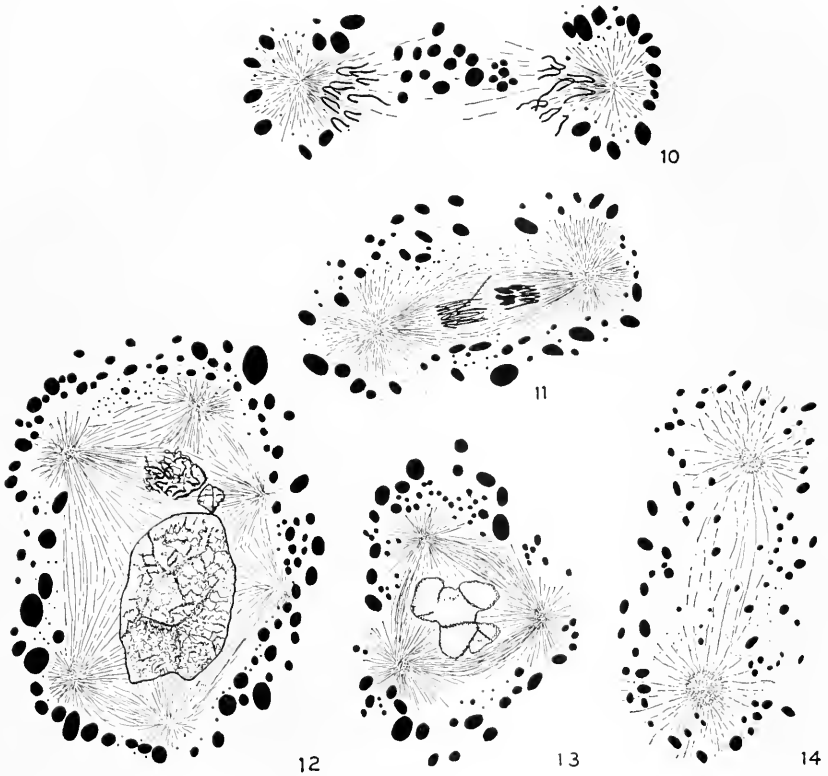


PLATE I

Explanation of Figures

Figures 10 to 14 were drawn at a magnification of 1200 and reduced to ca. 400 in reproduction.

Fig. 10. Anaphase figure in the yolk region of the egg in Text Fig. 3. Nine chromosomes at the upper pole and eight at the lower.

Fig. 11. Anaphase figure in a cell of the egg in Text Fig. 4. Four chromosomes, lagging on the spindle, show beginning degeneration.

Fig. 12. Pluripolar figure in the yolk region of the egg 37Ae. Apparently the fusion of several nuclei.

Fig. 13. Triastral figure in the yolk region of the egg 37Ae. Degenerating nucleus.

Fig. 14. Bipolar figure without chromatin in a cell of the egg 20.21e. The spindle shows a reduction in the number of spindle fibers.

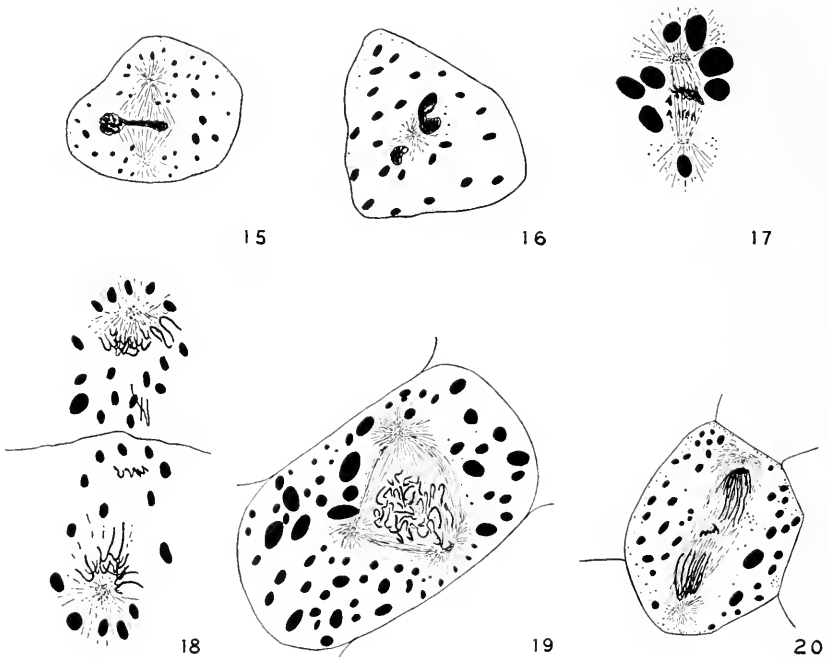


PLATE II

Explanation of Figures

Figures 15 to 20 drawn at a magnification of 1200 and reduced to ca. 600 in reproduction.

Figures 15 to 18, different stages in elimination of chromatin.

Fig. 15. Metaphase figure in a cell of the egg in Text Fig. 6. The chromosomes have degenerated into a pycnotic mass on the center of the spindle.

Fig. 16. Metaphase figure, polar view, in a cell of the egg in Text Fig. 6. The chromatin is completely removed from the spindle.

Fig. 17. Metaphase figure in a cell of the vegetal region of the egg in Text Fig. 6. Six chromosomes lagging on the spindle.

Fig. 18. Telophase mitosis in the egg AA.e. Several chromosomes lagging near the new cell membranes.

Fig. 19. Triastral figure in a cell of the egg in Text Fig. 8. Thirty-three chromosomes present.

Fig. 20. Anaphase figure in a cell of the egg in Text Fig. 8. Several chromosomes lagging on the spindle.

velopment during the early blastula stage. The majority of these eggs were incompletely segmented and contained abnormal nuclei in the cells and in the undivided areas, indicating the early irregular division of more than one sperm nucleus. The few completely segmented blastulae, although fairly normal in their cleavage, were, nevertheless, very irregular in their nuclear conditions. In these cases, the division of only one sperm nucleus probably initiated the almost normal cleavage, but even this early mitosis must have been extremely irregular.

The majority of eggs which had ceased development during the late blastula stage were incompletely segmented in certain areas of the egg. Only a few were normally formed blastulae. Chromosome counts in these eggs showed conclusively that irregular distribution of the male chromosomes had occurred earlier, and, indeed, was still going on in many cells at the time the eggs were preserved. It was of interest to note in the case of the normal androgenetic blastulae preserved while still developing, that one of these eggs possessed irregular chromosome numbers in the majority of mitoses analyzed. In the other egg, it was observed that irregular mitoses were just beginning. Cytological conditions such as these in normally developing androgenetic blastulae would be of importance in experiments involving the transplantation of haploid cells.

The nuclear conditions of the gastrulae were abnormal. About 80 per cent of the eggs ceasing development in this stage were not haploid. All of these gastrulae were abortive. Since it has been shown previously (Kaylor, 1937) that all androgenetic embryos which develop beyond the gastrula are haploid, it is apparent that the early gastrula is as far as an androgenetic egg can develop unless it possesses at least the haploid number of chromosomes in all of its cells.

These observations are in exact agreement with Fankhauser's (1934, *b*) conclusions from his excellent analysis of chromosome numbers and chromosome individuality in andro-merogonic *Triton* eggs. A complete discussion of the indispensability of a balanced set of chromosomes in early development is found in Fankhauser's papers.

These experiments on androgenesis have recently been extended to eggs of the Japanese newt, *Triturus pyrrhogaster* (Kaylor, 1940). In this species, a smaller percentage of the operated eggs die during blastula or gastrula stages. The more normal development of these eggs as compared with that in *Triturus viridescens* must be connected, then, with a more normal behavior of the sperm nuclei in early cleavage.

Summary

1. Androgenetic eggs of *Triturus viridescens* most frequently cease development in the following stages: *a.* Irregular beginning cleavage; *b.* Early blastula; *c.* Late blastula; *d.* Gastrula.

2. The causes of arrested development were investigated cytologically in eggs fixed in each of these stages.

3. Eggs of the first group were of two types, i.e., abortive cleavage, and early irregular cleavage in which a few cells were formed near the animal pole. In seven eggs of the first type, it was found that the sperm nuclei had degenerated either before or during early mitosis and cleavage furrows had disappeared. In five eggs of the second type, either all sperm nuclei had degenerated during early mitosis or one sperm nucleus divided more or less normally while "accessory" sperm nuclei either degenerated or divided irregularly in the unsegmented part of the egg.

4. In nineteen early blastulae, thirteen were incompletely segmented and six, although irregularly segmented, were fairly normal blastulae. Associated with these abnormalities in the thirteen eggs were the independent division of sperm nuclei in the yolk region without segmentation of the cytoplasm, and the presence of abnormal mitoses in the majority of cells. In the six almost normal mid-blastulae, the greater number of cells contained abnormal nuclei. Chromosome counts varied from 9 to 22 + in twelve of the nineteen eggs in which analyses could be made.

5. In twenty-three late blastulae sectioned, the same abnormalities as found in the earlier blastulae were observed. The majority of eggs were incompletely segmented and all of the eggs contained abnormal mitotic figures in some of the cells. Chromosome counts were made in fourteen eggs. None of these blastulae were completely haploid.

6. Nine gastrulae examined were abortive. No abnormal mitotic figures were found in these eggs, but in seven gastrulae the chromosome numbers varied above and below the haploid number, indicating that abnormal mitoses had occurred during earlier cleavage stages. Two gastrulae were haploid and it is assumed that these are examples of the abnormalities which many haploid embryos exhibit when differentiation of parts or of structures first takes place.

7. These observations confirm and extend those of Fankhauser and of Fankhauser and Moore. In order to develop beyond the gastrula stage, an androgenetic egg must be at least completely haploid.

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REVERSAL OF SEX PRODUCTION IN MICROMALTHUS

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Introduction

There is only one known example of paedogenesis in the Coleoptera and there are relatively few cases in the whole insect class. Hence any information which relates to the nature of paedogenesis in the beetle *Micromalthus debilis* has a general biological importance. One important question relative to paedogenesis in the beetle *Micromalthus* is: what is the mechanism which determines the strict separation of male production from female production in two types of larval mothers? This paper shows that the mechanism has an environmental rather than a genetic basis.

Many groups of animals produce unisexual broods. Thus some, aphids, Hymenoptera, Diptera, certain rotifers and Isopoda and some nematodes etc., produce broods of one sex and in some cases a partial explanation of the mechanism of this unisexual propagation is known. In many cases the broods are consistently female and involve a more or less constant process of diploid parthenogenesis, but in a few cases unisexual male progenies also occur. In the genus *Sciara,* Metz (1931) has disclosed a genetic basis which determines the sex of brood and a sex-linked gene is responsible. The thelytokous wasp, *Nemeritis canescens,* studied cytologically by Speicher (1937) is a perfect example of constant female production; no males were found in some fifty generations! The mechanism here also appears to have a genetic basis. It controls sex of progeny by determining a constant type of maturation. In the paedogenetic fly, *Miastor metraloas,* unisexual broods are apparently almost inviolably the rule and although Gabritschevsky (1928) ascribes this to a genetic mechanism, the contrary conclusion is indicated by Ulrich's (1936) work on *Oligarces.* Ulrich has shown that in the paedogenetic fly, *Oligarces paradoxus,* many broods show unisexual propagation although some broods contain both sexes. Hereditary differences among the larvae of *Oligarces* are not the determiners of the sex of the brood. It is the environment that is of primary importance.

It is the physiological state of the presumptive paedogenetic mother and the environment which primarily determine the sex of the brood in *Micromalthus,* just as in *Oligarces.* It is the purpose of this paper to describe a reversal of sex of brood which can be made to occur experimentally in the larval male-producer of *Micromalthus.* All the members of the first brood are male and all of the second brood are female.

Review of the Reproductive Types

In a previous paper (1938) I have described extensively the life history of *Micromalthus* and have outlined the reproductive anatomy of the various types. It is necessary for the purpose of discussion to review the reproductive types and more especially to describe the male producer with considerable care.

There are in the American variety of *Micromalthus debilis* five sexually mature reproductive types: (1) an adult female, (2) a female-producing paedogenetic larva, (3) a male-producing paedogenetic larva, (4) an adult male, and (5) a paedogenetic female larva with a mixed brood. This last is a modified male-producing larva and is the subject of research reported here. It is essential to note that the modified male-producer (amphoterotokous female) is simply a later developmental stage of the male producer. They rarely occur in nature but can be produced in large numbers experimentally. It is of incidental interest to refer here to the apparent absence of male-producers in the South African variety of *Micromalthus* which has recently been reported by Pringle (1938).

Normal History of the Male Producer

The male-producer (arrhenotokous female) is the only source of the adult male. She arises viviparously from a female-producing paedogenetic mother (thelytokous paedogenetic female) as one member of a large brood, sometimes twenty or more. In the first instar all these viviparous larvae possess legs which are lost at an early moult. They are all identical in appearance, indeed, it is impossible to distinguish the male-producer from other types until shortly before the last moult when inspection by dissection shows an ovary of a very special character in the male-producer. This early ovary is often recognizably distinct when only about 80 microns in length when a few egg cells (from one to five in each ovary) first begin to grow (Speicher, 1937). These continue to grow until they are of relatively large size and have become the shape of a hen's egg. The eggs of the thelytokous paedogenetic female are elongate so that the sex of the embryo resulting from either type

egg is predictable long before maturation. This adds another animal to the list showing sexual dimegaly of the ova (Wilson, 1925). When they are mature, the eggs of the male-producer begin development by haploid parthenogenesis in contrast to the diploid parthenogenetic development of the viviparous young (Scott, 1936). The one male that is successful in emerging from the mother is shed as a very young embryo in late June or early July. It is most peculiar, however, that although several embryos may be present in the ovary, only one is born. This new-born male remains for some four or five days adherent to the outside of the mother as is shown in Fig. 2 of Plate I. By that time he has developed sufficiently to insert his head into her genital aperture, which is shown at the arrow in Plate I, Fig. 1. Within a few days more the male has devoured his mother completely. These canabalistic males pupate and soon emerge as male adults.

This astonishing form of reproduction raises several perplexing questions. (1) Why is but one embryo shed by the male-producer when others equally advanced in development are present? (2) Is any one of the embryos more likely to be born than any other; viz., (*a*) does the position of the male in the mother have any bearing on successful emergence? Or (*b*) does the age of the embryo affect his ability to emerge? (3) Can any one of the other embryos be shed if the one that has been born is not allowed to feed upon the mother? (4) What becomes of the male-producer if her son is prevented from eating her?

The Birth Process

Why is only one male embryo shed by the male-producer? I can give no answer to the question but can only indicate some additional facts. Only seven male-producers in a group of three hundred and fifty-seven have given birth to two embryos. Fifty-eight male-producers have shed their male embryos in isolation. The females had previously been placed each in a shallow depression made in black wax and kept in a moist chamber. It is apparent from this that removal from their gallery in the wood does not affect their ability to give birth to the male embryo. Four of the fifty-eight individuals which shed in isolation,

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PLATE I

Fig. 1. Feulgen preparation of male producer with one egg visible. The genital aperture is shown at the arrow.

Fig. 2. Male producer and successful male offspring.

Fig. 3. Ovary of the male producer with three embryos, all in the same developmental stage.

Fig. 4. Ovary of the reversing male producer with a newly-developed female embryo, and an exceptionally well-developed male embryo still within its follicle.

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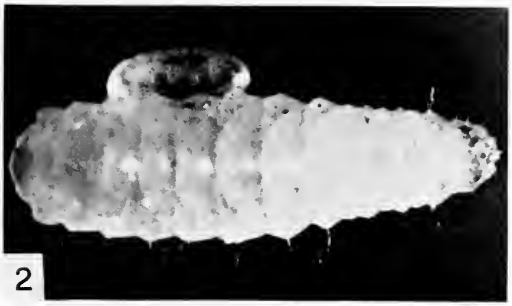

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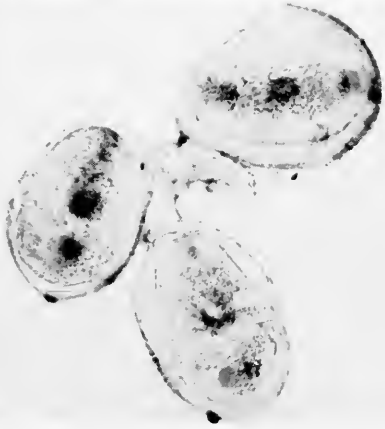

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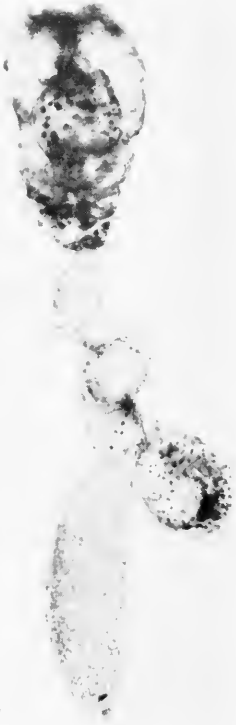

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PLATE I

shed two eggs, thus it is possible that removal from the wood favors the birth of a second embryo.

Two other factors might conceivably affect the birth process, that is,—(1) position in the mother and (2) stage of development of embryo within the mother. I have previously shown that there is no favored position in the ovary from which an embryo is shed. (Scott, 1938, Fig. 13.) The successful male embryo may have occupied any position within the ovary. Indeed, the successful embryo may sometimes occupy such a position within the mother that it must experience some mechanical difficulty at birth, since other embryos appear to block its exit.

I do not think that the most mature male embryo is necessarily the most likely to emerge, for frequently the difference in age of the embryos is negligible, as is shown in Plate I, Fig. 3, and moreover, an embryo may occasionally develop into a rather well-developed larva while still within the follicle of the ovary, as illustrated in Plate I, Fig. 4.

No factual explanation of the mechanism governing this uniparity is available. However, a very plausible hypothesis can be formulated from the point of view of natural selection. The male has no other source of food during his larval life than his mother's body and since a second male would compete for this food supply, a process may have been developed which prevents this competition. This process very probably involves the active coöperation of the mother in that some internal physiological mechanism prevents a second male from being born. This mechanism is quite conceivably a failure of the muscular contractions which normally expel the egg. The continued presence of the born male on the mother is not necessary to prevent birth of the remaining embryos. The birth of one in some way sets the mother against further activity of the ovary and ducts. Does a hormone govern the contractions involved in the ovulation-birth process?

A New Brood in Male Producers

The fate of the mother after the emerged male has been removed is quite unexpected and is, I believe, a quite unprecedented observation. In practically every surviving case after approximately four weeks time a new, small brood is born. The members of the new broods have not yet been reared, nor have chromosome counts been possible, but the offspring are judged to be females with considerable certainty for the following reasons: (1) the shape of the egg is in every way similar to that of the female-producing female, (2) the development is in every way identical with that of the ordinarily produced females, and is vastly

different from the development of the male embryo, (3) the appearance of the newly-hatched embryos is identical with that of the more normally produced female larvae. That is, these second brood embryos possess well-developed legs and well-differentiated jaws, whereas new-born male

TABLE I

	Dead Dissected 7/24	Alive Dissected 7/25	Total	Percentage
Females showing female embryos on dissection	1	43	44	21.8
Females showing no female embryos	47	2	49	24.3
Females in which ovaries were not located	13	4	17	8.4
Died before examination			91	45.2
Total number involved in experiment—201. Mortality—75.7%				

embryos are rarely beyond the germ band stage. The second brood larvae will, therefore, subsequently be referred to as females.

It should be stressed that this process is not an occasional one but is quite normal for those larval mothers that survive long enough. Thus in the summer of 1938, 93 females from which the male had been removed gave rise to a new female brood. Of this number, 21 mothers shed their brood and the rest showed female embryos on dissection. Since mortality records were not kept in 1938, the experiment was repeated in 1939.

On July 1, 1939, 201 females, each with the shed male removed, were isolated in black wax depressions in Syracuse dishes, 20 to each

TABLE II

New brood born before July 24	4
Found with female brood on dissection 7/24	10
Dead when dissected, ovaries disintegrated	8
Dead, no new brood developing	2
Lost	1
Total	25

dish and kept in a moist chamber. The mortality was severe, therefore the larvae were dissected before they could have given birth to their new brood. Table I summarizes the results.

In another experiment 25 male-producers were removed from the wood and the adherent male was removed from each one. They were likewise placed in a moist chamber at 35° C. ± 1°. The results are shown in Table II.

In this group 56 per cent developed a new brood of female embryos.

It is impossible from these data to decide whether or not every male-producer can, under favorable conditions, give rise to a new female brood but it is certainly indicated by the fact that out of the 63 animals that were still alive at the end of their respective experiments, only 6 did not show indications of a new brood. It is reasonable to expect that the larvae that died during the course of the experiment would also have given rise to a female brood had they survived.

The mortality, high in both experiments, is less severe at higher temperatures. The difficulty is largely due to the susceptibility of the larvae to mold. Perhaps a sterile technique would obviate the trouble.

Apparently no structural feature of the male producer prevents viviparity of the new female brood since a considerable number have been kept long enough to allow normal birth. The birth process is in every respect similar to that which takes place in the normal female-producing, paedogenetic female.

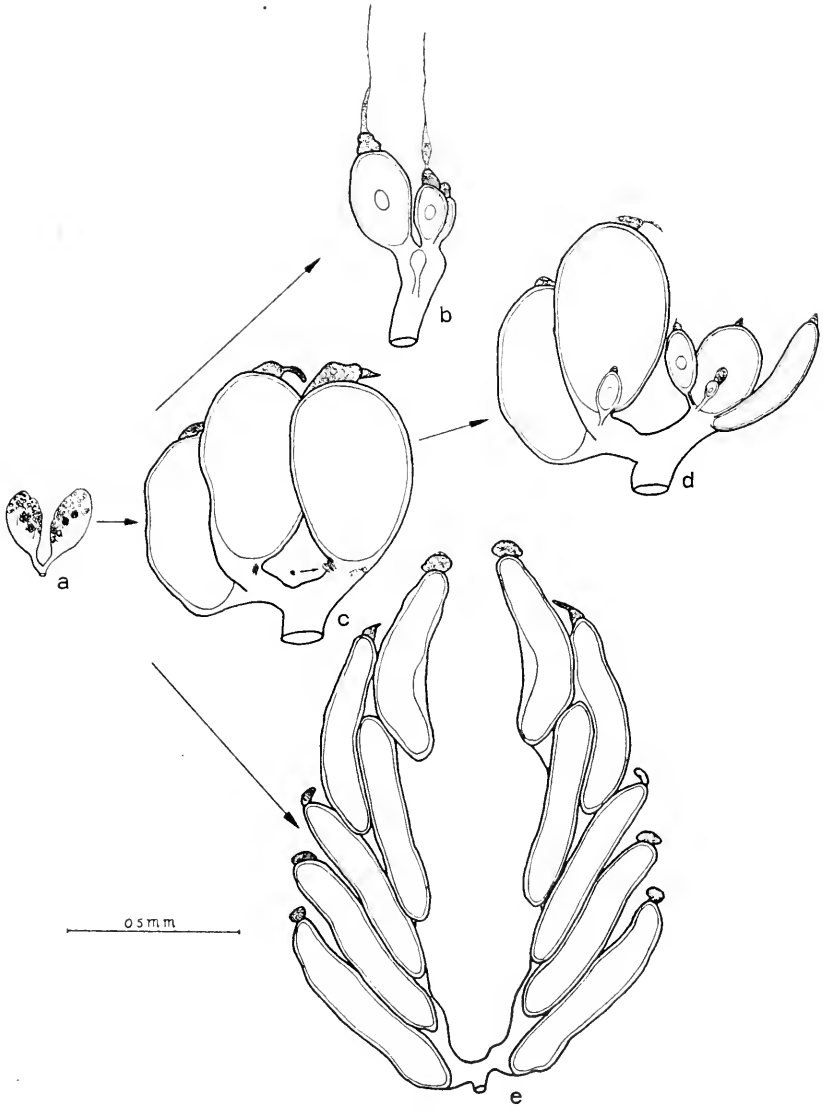
The size of the second brood of the reversing male-producer is intermediate between the size of normal female broods and the size of male broods. Normal female broods are frequently more than ten while male broods are rarely as many as four. An examination of the ovaries of forty reversing male-producers showed that the average number of new eggs formed was 4.2.

Study of the ovaries of this same group of forty reversing male-producers showed that a few females *failed to shed even one male, yet they developed a new brood of female larvae notwithstanding.*

The production of a new brood is not, therefore, absolutely dependent upon the experimental removal of the emerged male from his mother. Male-producers whose emerged male embryo dies will evidently give birth to a new all-female brood in the natural course of events. Indeed, in August, 1937, I found eighteen individuals with a new brood developing, obviously the result of this natural event. Dissection of these eighteen larval mothers showed an empty follicle from which a male had emerged and apparently died.

It seems altogether possible that a third brood might be produced by the original male-producer if it lived long enough. However, a single individual that lived thirty days after the production of the second brood showed no sign of new eggs when dissected.

It will be of some interest to test similarly the further reproductive capacity of the thelytokous paedogenetic female after the birth of her first brood.



TEXT FIG. 1. A diagrammatic representation of the developmental possibilities of the basic ovary as it occurs in the several reproductive forms: (*a*) basic ovary (schematic) showing several undeveloped ovarioles in each ovary; (*b*) the ovary of the adult female with three ovarioles developing; (*c*) the ovary of the male producer (three ovarioles and eggs greatly enlarged, four others have remained small, see asterisk); (*d*) the ovary of the reversing male producer experimentally produced (the rudimentary ovarioles have enlarged and are developing female-producing eggs; (*e*) the ovary of the female producer with numerous ovarioles developing.

Histology of the Reversing Ovary

Studies of the ovaries of the male-producer indicate that the cells which give rise to the new crop of female-producing eggs are already present on the oviducts of the male-producer before the male embryo is born; indeed, they were probably present at the time of the first differentiation of the male-producing ovary. In the mature ovary of the male-producer these cells are located in little clusters around the ventral and lateral surfaces of the oviducts at the point of junction of oviduct and follicle. (Text Fig. 1*c* at the asterisk and Plate II, Fig. 5*b*). Structurally these groups of cells are undeveloped ovarioles. They doubtless represent ovarioles which did not enlarge during the first period of development of the male-producing eggs. The detailed structure of these ovarioles is shown in Plate II, Figs. 5 and 6. In both figures some differentiation can be seen within the ovariole and although no single egg cell can be identified with certainty, still, terminal cells of the germarium, nurse cells, and duct cells can be seen in Plate II, Figs. 6*a*, 6*b*, and 6*c* respectively.

In *Micromalthus* the ovaries of the four reproductive types are fundamentally similar. The general plan of the ovary in each of the female reproductive types is meroistic and acrotrophic, since the nutritive cells are all located at the apex of the ovariole. The variations in structure which the ovaries of the several reproductive types present may all be considered as modifications of a basic, undifferentiated ovary illustrated in Text Fig. 1*a*. This basic ovary possesses multiple ovarioles at the ends of a forked oviduct; it is the development or non-development of these potential ovarioles that determines the nature of the mature ovary. If the ovary develops within an adult female, then three or four of the ovarioles will enlarge with their contained eggs, as indicated in Text Fig. 1*b*. When, however, the basic ovary develops within a female-producing paedogenetic mother, a number of eggs, each in a different

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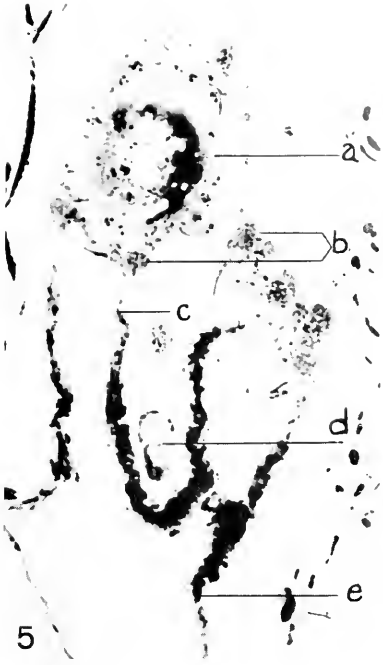
PLATE II

Fig. 5. Frontal section of ovary of male producer: (*a*) Follicle; (*b*) undeveloped ovarioles (the follicle on the right side appears in another section); (*c*) oviduct; (*d*) last ganglion; (*e*) vagina.

Fig. 6. Oil immersion photograph of undeveloped ovarioles of the male-producing ovary. No enlargement has as yet taken place: (*a*) germarium; (*b*) potential nurse cells; (*c*) potential duct segment; (*d*) oviduct.

Fig. 7. Total Feulgen preparation of a reversing male-producing ovary: (*a*) unshed male egg; (*b*) female-producing egg; (*c*) oviduct; (*d*) new segment of oviduct; (*e*) empty follicle (out of focus); (*f*) vagina or terminal duct.

Fig. 8. Total Feulgen preparation of the ovary of a male producer with a new brood of female embryos: (*a*) female embryo; (*b*) empty follicle; (*c*) retained degenerating male embryo.



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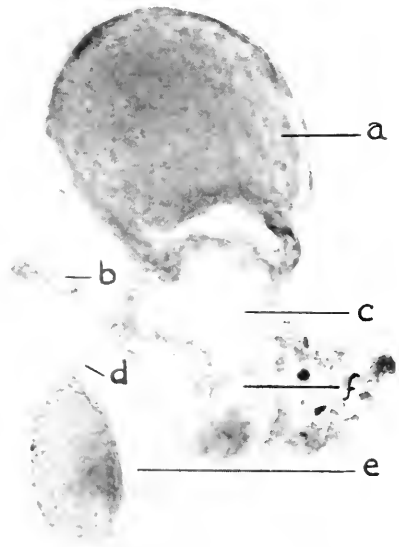

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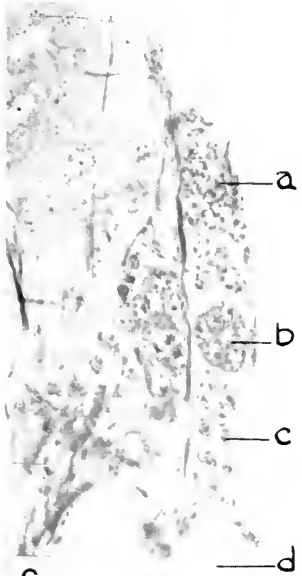

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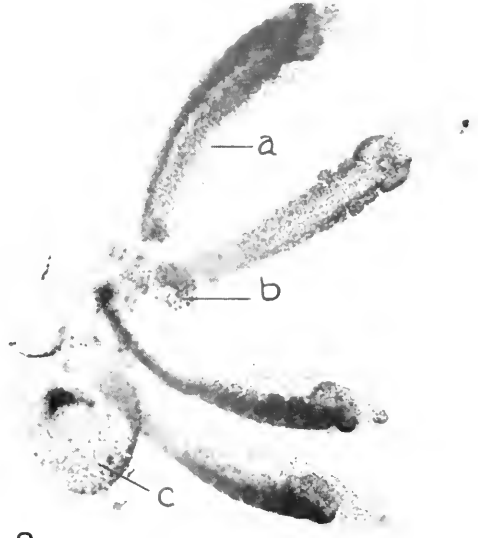

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PLATE II

ovariole, enlarge to determine the characteristic ovary of that type of larva. This is illustrated in Text Fig. 1*c*. When the basic ovarioles develop within a male-producer, again only a few of the ovarioles enlarge. In this case, too, only a few eggs develop, one in each ovariole, so that the fully developed ovary of the male-producer, shown in Text Fig. 1*c*, still possesses a number of undeveloped ovarioles at the base of the enlarged follicles. It is these undeveloped ovarioles that enlarge to give rise to the second all-female brood under the conditions noted above and illustrated in Text Fig. 1*d*.

The development of the new eggs involves the production of other new parts of the reproductive system, for although the old oviducts and vagina are utilized by the larvae of the second brood on emergence, it will be apparent from Plate II, Figs. 6*c* and 7*d*, that a new segment of oviduct is added during the development of the new crop of eggs. The potentialities of the ovariole tissue are such, therefore, that it gives rise to the following reproductive structures: (*a*) oviduct, (*b*) follicle cells, (*c*) eggs, one per ovariole, (*d*) nurse cells and (*e*) germarium. The new oviduct segment is at first relatively long, but it is inconspicuous in late development, as Plate II, Fig. 8 shows. Perhaps it is incorporated into the follicle as the egg grows.

It should be added that the development of the new eggs is not particularly related to the stage of development of the retained males, for the latter may be in any stage of development from a post-maturational stage to a well-developed larva. Frequently, indeed, the retained males undergo an abnormal type of development which also has no apparent effect on the new brood.

Summary

1. The paedogenetic, arrhenotkous female in the beetle, *Micromalthus debilis* (Leconte), gives birth to but one male embryo, although unshed males also develop.

2. Factors which determine this uniparity are still uncertain, but neither greater age nor more favorable position in the mother are determining factors.

3. When the single successful male offspring is not allowed to devour his mother, a new crop of eggs develops in the ovary.

4. These new eggs are all of the elongate female type. They develop into a larva identical in appearance with the first stage larva of the thelytokous paedogenetic female.

5. Histologically the new eggs originate from undeveloped ovarioles which failed to develop during the first period of growth of the male-producing eggs.

6. Sex of brood in *Micromalthus* is obviously determined by environment, intrinsic or extrinsic, and not by the hereditary constitution of the mother.

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THE TIME–TEMPERATURE RELATION OF DIFFERENT STAGES OF DEVELOPMENT

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The phenomenon of development appears as a series of processes which are visibly unlike. Many investigators have demonstrated that some of these events can also be separated on the grounds that their rates possess different temperature coefficients. For example, the rate of growth of the gill filaments in the frog is more depressed by a low temperature than is the rate of body growth (Atlas, 1935; Doms, 1915). Again, the rate of embryo formation in *Salmo* has a higher temperature coefficient than the rate of growth in wet weight (Gray, 1928). In view of this and other evidence it has always seemed curious that development could yield approximately the same differentiated product over a wide range of temperatures. As a possible solution to the problem, Tyler (1936*a*) has shown that in some marine invertebrates the temperature coefficients of various cleavages are not only the same but are also identical with those for later stages of differentiation. Yet his results are not comparable with those on the frog obtained by Hertwig (1898) and Krogh (1914) whose data show, although the authors do not point it out, that the temperature relation of cleavage is different from that of later development. The experiments on the egg of the frog to be reported here were designed to discover the temperature relations of some of the more clear-cut events of development which could be accurately measured.

Rana pipiens from Vermont were caused to ovulate at 15° by pituitary injection. Batches of about 25 eggs were shed into finger bowls and fertilized artificially. The sperm suspension was replaced after 5 minutes by 200 cc. of 10 per cent Ringer's solution at the temperature at which the eggs were to be kept and the bowls were then distributed to constant temperature environments. When the jelly swelled, the egg mass in each container was cut into bunches containing about 5 eggs apiece. The 10 per cent Ringer's was replaced daily by solution at the same temperature. Cold rooms, water baths and incubators were used to maintain constant temperatures. Generally the temperatures were constant within 0.1° C. (except at 10° and 8.5° where ice-boxes were used; the maximum observed variations here were 1.0° and 0.5° re-

spectively). The temperature in the finger bowls seldom changed more than 0.5° during observation on the stage of a binocular microscope and observation never lasted as much as 5 minutes. Mortality during early development was less than 5 per cent and development was normal at temperatures between 25° and 8°. Above and below these temperatures mortality increased and abnormalities became frequent, so that at 29.6° usually less than 50 per cent of the eggs hatched and abnormalities were very common.

The times to various cleavages, gastrulation and gill circulation were measured from fertilization for embryos remaining constantly at one of the several temperatures. Cleavage was considered begun when the first slight furrowing was seen on the surface of the egg; gastrulation when the dark line of pigment associated with the initial dorsal lip invagination appeared; and gill circulation when the initial blood corpuscles could be seen circulating in the anterior gill. When the critical time approached, repeated observations were made until about 50 per cent of a batch of eggs had reached the initiation point, at which time it was considered that the stage was entered. All of the embryos in a group of 25 entered a stage well within 10 per cent of the total time necessary to reach that stage. The maximum deviation of any batch of eggs from the average time was about 10 per cent.

In order to portray the relation between the times to different stages at different temperatures a semi-logarithmic plot was chosen (see figures). The logarithm of time was placed along the ordinates and the abscissae represent either temperature, in which case the curves are for different developmental intervals, or stages, in which case the curves are for different temperatures. The choice of one of these abscissae was made so as to employ the largest number of points per curve. In either event it is possible to compare what types of function of time the stages are at different temperatures. Spacing of temperatures along the abscissa was obtained by plotting the data for one stage as a straight line. This arbitrary abscissa was then used as a base for the times to other stages. Stages were spaced along the abscissa in a similar fashion. This method is preferred to the comparison of temperature coefficients inasmuch as: (1) it does not entail a selection of points but involves all of the data; (2) it avoids attributing one of the several controversial numerical constants to the temperature relation; and (3) the linear arrangement of points obtained by a distortion of one axis permits immediate visual comparison of the time-temperature relation.

Figure 1 compares the temperature relation of the different stages of development in *Rana pipiens*. The curve for time between gastrulation and gill circulation (Stages 10–20) has the greatest slope. The curve

for time between fourth cleavage and gastrulation has a lesser slope which is, however, greater than the slope of the curves for all the cleavages. These differences are real. If the curves actually were parallel to that for gastrulation to gill circulation, a time error of 25 per cent would have to be assumed at both ends of the gastrulation curve, an error of about 45 per cent at both ends of the curve for first cleavage and an error of about 45 per cent at both ends of the curve for second, third and fourth cleavage. Such errors are highly improbable because the

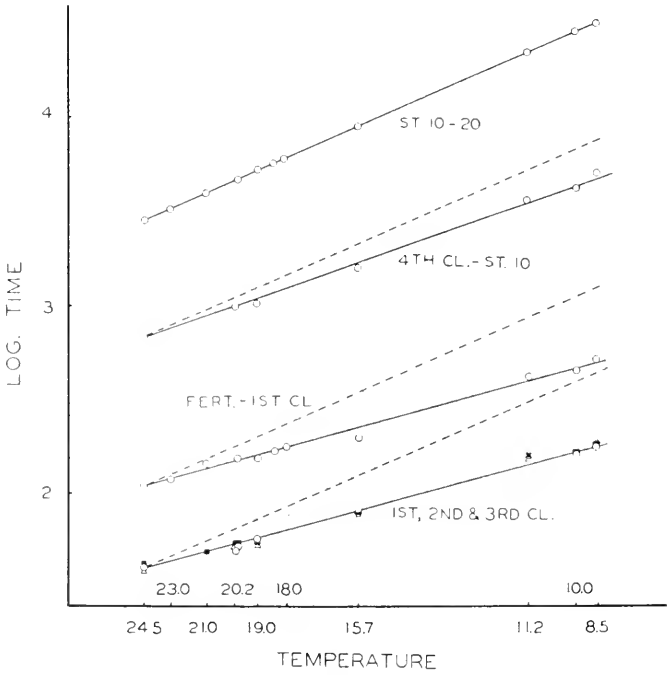

FIG. 1. The relation, at different temperatures, of developmental intervals to time in *Rana pipiens*. Ordinate, logarithm of time in minutes between the specified stages; abscissa, temperature in °C. The data for development between stages 10 and 20 are plotted as a straight line by the arbitrary distortion of the temperature axis. The latter is used as a base for the data for other intervals. The upper complete curve describes development between gastrulation (stage 10 of Pollister and Moore, 1937) and gill circulation (stage 20); the next, between fourth cleavage and gastrulation; the next, between fertilization and first cleavage. In the lowest curve, the circles describe development between first and second cleavage, the squares, between second and third, and the triangles, between third and fourth. Twenty points are single determinations; the remaining thirty-four points are the averages of from two to ten determinations. One hundred and seventy-two determinations were made in all. The broken lines were drawn through the points at 24.5° parallel to the curve for stages 10 to 20. These broken lines emphasize the real nature of the slope differences among the curves for different intervals.

maximum deviation of a point from any one of the curves is only 10 per cent. The deviations of points for the cleavages from the parallel curves drawn through them are at random and are about the size of the expected experimental error (10 per cent). Hence, in *Rana pipiens* the temperature relations of cleavages are alike, but they are different from those of later development.[1] Differences in temperature relation are apparent even in rate-temperature plots (Ryan, 1941) where, in addition to a difference in μ values, the curve for later development shows a "break" at about 18° C., while the curves for cleavage "break" around 14° C.

The data of Krogh (1914) for *Rana butyrhina* when placed on the semi-logarithmic plot (Fig. 2) completely confirm this difference between cleavage and later development. Even though the times to later stages are from fertilization and must include some time during cleavage when the temperature relation is like that of the upper curve, a significant difference in slope is visible. If all the curves actually were parallel, an error of about 25 per cent must be postulated at both ends of the cleavage curve. Such errors are extremely improbable with Krogh's method. His precision in measuring cleavages should be better than that for later stages and yet no such errors are visible in his later stage data (for example, the maximum deviation of a point from the straight lines in Fig. 2 is equivalent to an error in time of only 5 per cent). Despite this difference between cleavage and later development, the parallelism of curves among different stages of later development in Fig. 2 shows that the temperature relations of the latter are alike.[2] Apparently in contradiction to this, Bělehrádek (1926) has calculated for Krogh's data a series of b values increasing from 1.76 to 2.52 between medullary groove closure and 7.8 mm. tadpole formation. However, from the same data and for the same stages b values of 1.6 and 1.7 (and Q_{10}'s decreasing from 4.2 to 3.5) can be calculated according to the points selected for comparison.

[1] Atlas' (1935) Fig. 8 indicates the same temperature characteristic over the low temperature range for the rates of different stages of development in *Rana pipiens*, but the column he uses to include all the points obscures the difference between the temperature relations of the different stages. At higher temperatures the temperature relations of cleavages, of gastrulation, and of later development show the same sort of differences as are visible in Fig. 1 of this paper. Neither the data for *Rana pipiens* described in this paper nor Atlas' data (Fig. 2) for the same animal show the adaptation in the rate of later cleavages found by Hoadley and Brill (1937) in *Arbacia* and *Chaetopterus*, although this may be because the temperatures used were not close enough to the maximum.

[2] Times from fertilization or first cleavage, instead of the length of developmental intervals, are used in this and all subsequent figures in order not to exaggerate errors in timing the events of later development which are difficult to measure.

Confirmation of the similarity in the temperature relations of events in later development can be found in semi-logarithmic plots of the data of Moore (1939) for *Rana pipiens, R. sylvatica, R. clamitans* and *R. palustris,* and of Knight (1938) for *Triton alpestris.* The curves for

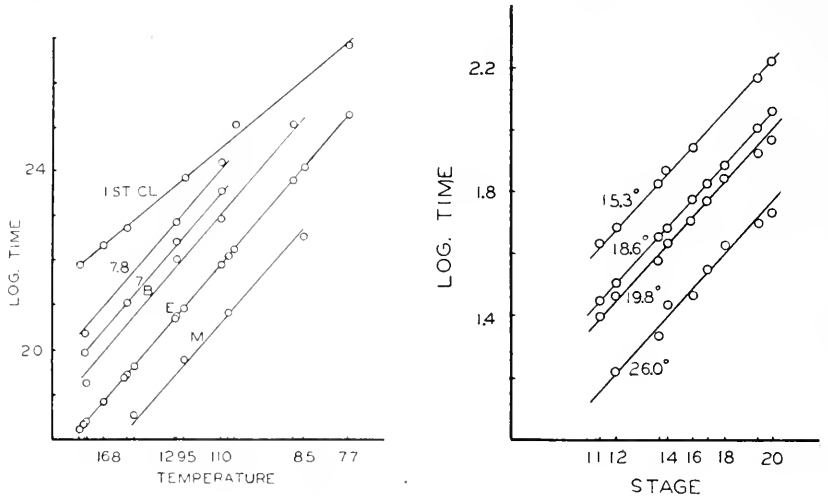

Fig. 2 (left). Krogh's (1914) data for *Rana butyrhina* showing the relation at different temperatures of stage to time from fertilization.[3] Ordinate represents the logarithm of time from fertilization (for cleavages in minutes, for later stages in hours); abscissa, temperature in °C. The symbols over the curves represent first cleavage, 7.8 and 7 mm. length, branched gills, external gills, and medullary groove closure. For the sake of ready comparison, the data for the formation of external gills are plotted as a straight line by the arbitrary distortion of the temperature axis. The latter are used as a base for the data for other stages.

Fig. 3 (right). Moore's (1939) data for *Rana pipiens* showing the relation, at different temperatures, of stages between gastrulation and gill circulation to time from first cleavage. Ordinate represents time from first cleavage in hours; abscissa, stage of development (Pollister and Moore, 1937). For the sake of ready comparison the data for 18.6° are plotted as a straight line by the arbitrary distortion of the stage axis. The latter are used as a base for the data from other temperatures.

later development are all parallel. For example, in Fig. 3 Moore's data for *Rana pipiens* are presented. These supplement the data in

[3] It should be pointed out that the similarity in temperature relation among these stages does not *necessarily* imply that each step in the formation of a given stage has the same temperature relation. There may be differences of short duration which might be in opposite directions and cancel one another. At any rate, if there are such differences, they are not additive, for the overall sort of examination made does not reveal them. The stages in between gastrulation and stage 20 are not clear-cut enough to obtain easily sufficiently accurate determinations to solve this problem.

Fig. 1 inasmuch as they show that many different events between yolk plug and gill circulation have the same temperature relation in *Rana pipiens*. Indeed, the significance of the difference between the temperature relation of the period from fourth cleavage to gastrulation and that of the interval between gastrulation and gill circulation (Fig. 1) is dubious. The process of gastrulation might have the same temperature relation as other stages of later development, but when the measured time also includes cleavages (between fourth cleavage and gastrulation), the observed overall temperature relation would be intermediate. Assuming that the curve for fourth cleavage to gastrulation is just such a composite, a calculation shows that the cleavage temperature relation would prevail into early blastula stages. However, it is very difficult to measure blastula stages accurately enough to break this period into its components and settle the problem.

Peter (1905) computed the Q_{10}'s for Hertwig's (1898) data on *Rana fusca* and claimed that the temperature coefficients gradually increased with the age of the animal. However, when the Q_{10}'s between 15° and 24° are compared for different cleavages, there is no significant difference from the average of 1.37. The same holds for later stages where there is no significant difference from the average of 2.36. Peter's difficulty resides in the fact that he included Q_{10}'s computed for low lethal temperatures where development began but was never completed. In this range the difference between high and low temperatures becomes progressively greater with age. When Hertwig's data are put on a semilogarithmic plot (Fig. 4), it can be seen from the parallelism of curves that the times to stages of later development are the same type of function of temperature but are a different type from that for times to the first three cleavages. Hertwig could have a 25 per cent time error at both ends of each of his three cleavage curves. But since the maximum deviation of a point from the straight lines in Fig. 4*A* is only 10 per cent, the difference between cleavage and later development is probably real. The displacement of the points for gastrulation from the curves in Fig. 4*B* may be real (at 10° there is a time discrepancy of 20 per cent). This is in accordance with the fact shown in Fig. 1 that the rate of gastrulation increases to a degree intermediate between cleavage and later development with a temperature rise. Since gastrulation is compared with cleavage in Fig. 4*A*, the difference between the temperature relations of cleavage and later development is all the more convincing. In summary, it is definite that in Amphibia not only is the temperature relation different for cleavages and later stages, but there are extremely long periods during cleavage and during embryo formation over which the temperature relation remains constant.

It is not surprising that a difference should exist between the temperature relations of cleavages and morphogenesis because visibly these phenomena are unlike and probably have different causes. The amazing thing is the similarity in response of so many different stages to temperature. Second, third, and fourth cleavages are enough alike so that it is not hard to believe that they are the result of the same process. Between fertilization and first cleavage, however, there occur the completion of the second maturation division, release of the second polar

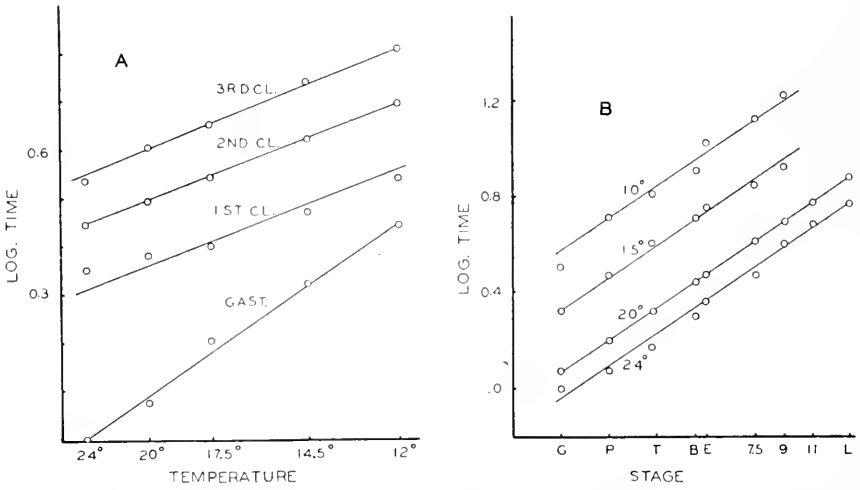

FIG. 4. Hertwig's (1898) data for *Rana fusca* showing the relation, at different temperatures, of stage to time from fertilization. *A*. Ordinate represents logarithm of time from fertilization (for cleavages in hours, for later stages in days); abscissa, temperature in °C. *B*. Ordinate, logarithm of time from fertilization in days; abscissa, stage of development from gastrulation to limb bud formation. At temperatures of 6° and below the parallel relation does not hold, but also development does not go to completion at these temperatures. The abscissae in *A* and *B*, using the data for second cleavage and for 20° respectively as the base curves, have been distorted as in Figs. 1 and 3.

body, and the fusion of pronuclei, before the process begins to resemble later cleavages. Since the total process of first cleavage is, then, different from that of later cleavages, it would be expected *a priori* that the temperature relations should differ. But they do not (Fig. 1). The coincidence may be chance or due to the independence of the cytoplasmic cleavage (which is being measured) from the nuclear phenomena (wherein lies the difference between first and later cleavages) or due to a fundamental process which controls all of the phenomena and imposes its temperature relation upon them. The latter seems more likely

inasmuch as it affords an explanation of the even more astonishing similarity in the temperature relations of stages between gastrulation and stage 20 (Fig. 3). Here such strikingly different processes as neurulation, tail bud and gill formation, onset of circulation etc. have the same relation. Either each operation independently has achieved this or all are controlled by an underlying process which imposes its temperature relation upon its various expressions. Atlas (1938) has shown that in *Rana pipiens* the temperature coefficient of the rate of oxygen consumption during development is approximately the same as that of the rate of development. In many marine eggs temperature affects the rate of early development and the rate of respiration in the same way (Tyler, 1936*b*). These correlations suggest that the "primary gear shaft" (Needham, 1933) integrating developmental processes is some part of the respiratory metabolism.

If this were so, then there should be a difference in the type of metabolism during cleavage in the frog's egg from that prevalent during morphogenesis. Brachet (1934) has, indeed, shown that in the frog the respiratory quotient changes abruptly at gastrulation from about 0.7 to 1.0. Again, in Tyler's (1936*a*) studies of marine invertebrates, there should be the same type of metabolism during cleavage as during later development because the temperature relations of both processes are the same. Accordingly, in *Urechis* where the temperature coefficients of the first four cleavages are the same (Tyler), the respiratory quotient remains unchanged over an equivalent period of time (2½ hours at 20° C.) (Horowitz, 1940). Thus there is real evidence for the belief that the coördinator of the various processes of differentiation, the factor which permits development to be reproducible over a wide range of temperatures, is the respiratory metabolism.

Summary

1. Stages in embryo formation among Amphibia between yolk plug and gill circulation have similar time-temperature relations.

2. The time-temperature relation of cleavage, although constant from first to fourth cleavages, differs from that of embryo formation.

3. It is suggested that the different time-temperature relations of cleavage and of morphogenesis represent different controlling processes; while the similarity of the time-temperature relations among cleavages and among stages of later development is the expression of a common controlling process in each case. These controlling processes are probably parts of the respiratory metabolism and they prevent temperature from disorganizing development.

I wish to thank Dr. H. B. Steinbach for criticisms of the manuscript.

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