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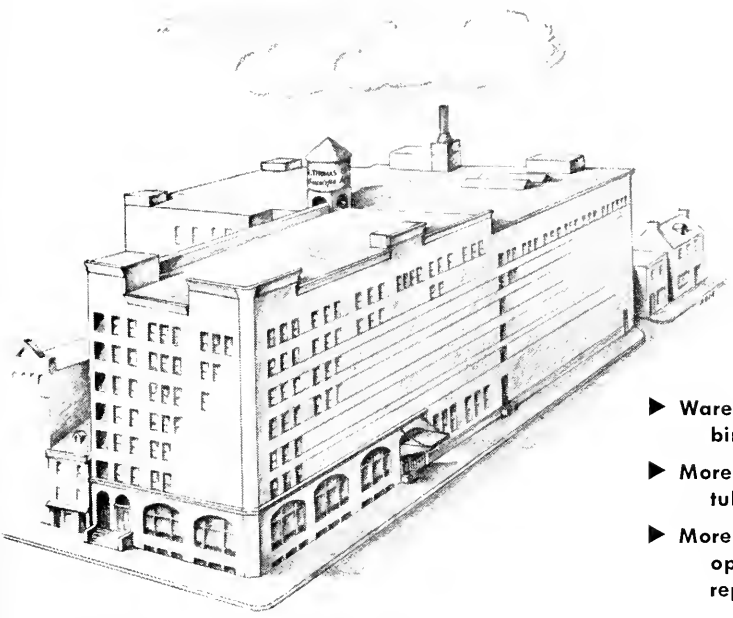

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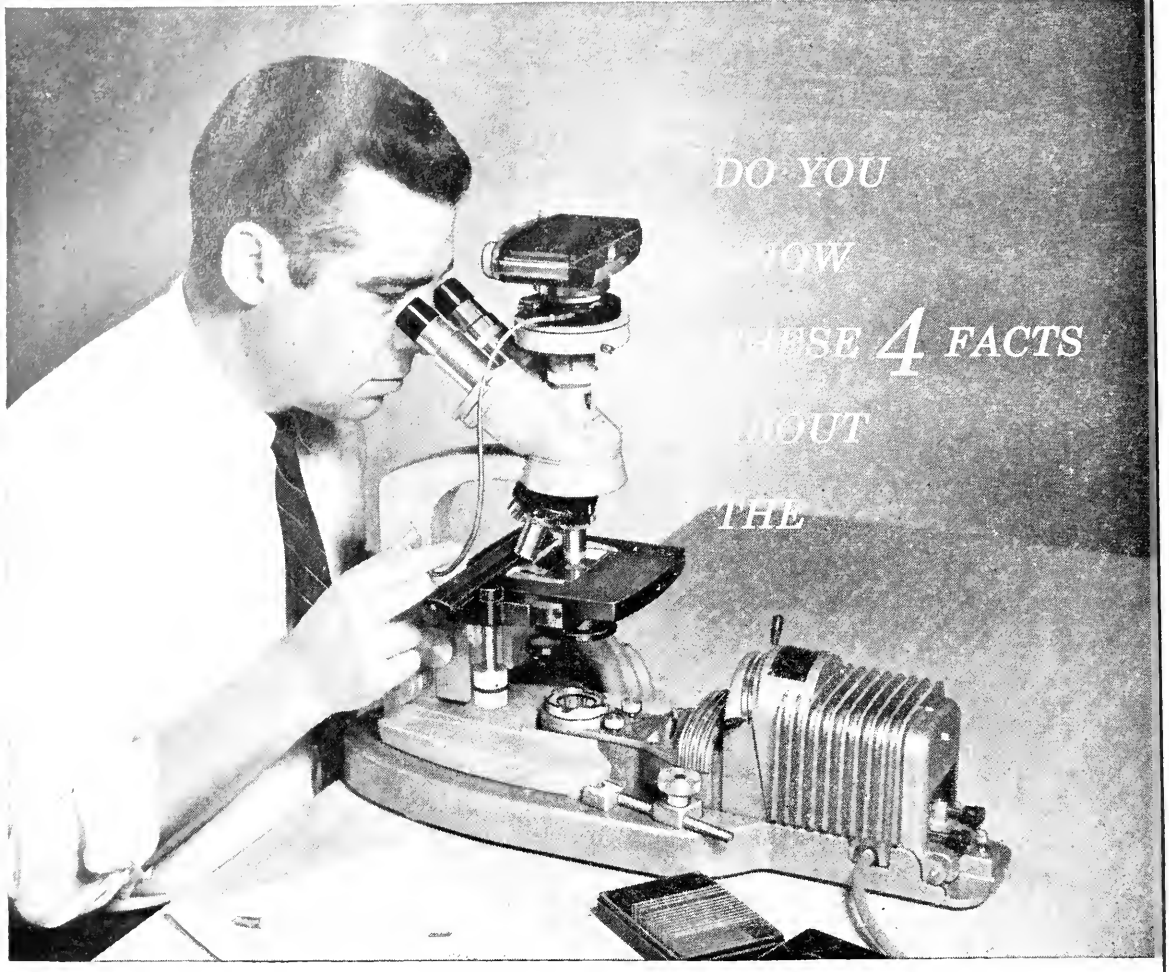

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THE
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THE DISTRIBUTION OF POLYSACCHARIDES AND BASOPHILIC SUBSTANCES DURING THE DEVELOPMENT OF THE MUSHROOM COPRINUS[1]

JOHN TYLER BONNER, ALLAN A. HOFFMAN, WILFRED T. MORIOKA AND A. DUNCAN CHIQUOINE

Department of Biology, Princeton University, N. J.

As Buller (1909 *et seq.*) has pointed out, some species of the genus *Coprinus* sow their spores once during a short interval of time and the fruiting body disappears shortly thereafter by auto-digestion. Characteristically the small buds will, all in one day, go through a period of rapid expansion and elongation, shed their spores, and deliquesce. This rapidity is no doubt related to their small size, for larger species of hymenomycetes will go through many days of continued production and shedding of spores.

The origin of the gills and their individual lamellae has been described in detail by Atkinson (1916). They are formed at an early stage by the orientation of hyphae and the final result (which is illustrated in Figure 1) has a number of distinguishable component parts: the outside is covered with the hymenium which consists of a mixture of basidia and sterile paraphyses; below this there is a subhymenial layer of small hyphae; and finally the central portion of the gill lamella, the tramal layer, which is composed of large hyphae.

Borriss (1934) has shown that the development of *Coprinus* occurs in two distinct stages, one in which cell division and the initial cell orientation takes place, and it is during this stage that the gill primordia are formed. The second stage consists of rapid cell elongation and in the latter phase of this period the spores bud from the basidia. It may be inferred, from the recent results of Madelin (1956), that this period of rapid expansion involves the transfer of material from the vegetative mycelia. Such a transfer is in keeping with the views of Buller as well as with the situation in *Agaricus campestris* (Bonner, Kane and Levey, 1956).

Using histochemical techniques, it has been possible in this study to follow the distribution of certain groups of substances in the fruiting body. It could be demonstrated that these substances accumulated at specific locations in the gills and that they were all transferred into the spores, so that by the advent of auto-digestion there were virtually no demonstrable substances left within the cells at the time of the final destruction. Not only does this indicate an efficiency, an economy in the

[1] This research was supported in part by funds of the Eugene Higgins Trust allocated to Princeton University.

fruiting process, but also it is of some interest to find that prior to sporulation the different substances are stored in different parts of the gills.

Methods

Two species of *Coprinus* were studied: *C. lagopus* Fr. and *C. curtus* Kalch. (The authors would like to thank Dr. Haig P. Papazian of Yale University for the culture of *C. lagopus*, and Dr. Alexander H. Smith of the University of Michigan for the identification of *C. curtus*.) They were grown in jars on moist, sterile horse dung and kept at room temperature (approximately 24° C.)

The entire fruiting bodies were fixed for 12 hours in Rossman's fluid (9 parts absolute alcohol saturated with picric acid plus 1 part formaldehyde) at 4° C. The selection of this particular fixative, as well as the temperature, follows from the recommendations of Deane *et al.* (1946), from their studies on the preservation and localization of glycogen in mammalian liver. The suitability of the fixation for the mushroom material is evidenced by the absence of any false localization of glycogen, "glycogen flight," due to the pathways of penetration of the fixative. Following fixation the tissues were dehydrated, cleared with methyl salicylate (14 hours), and embedded in paraffin. Sections were cut at both 5 and 10 μ and mounted on slides in the usual manner.

Polysaccharides were demonstrated by the periodic acid-Schiff reaction as described by Gomori (1952). This technique is based upon the oxidation with periodic acid of the vicinal hydroxyl groups of polysaccharides to aldehyde groups and the subsequent visualization of the aldehyde groups by reaction with the Schiff reagent. The sections were deparaffinized, run to water, and oxidized for 10 minutes at room temperature with 0.5% periodic acid. Schiff reagent was prepared according to the method of Lillie (1954) and the slides were treated with it for 20 minutes. Excess Schiff reagent was removed by three sodium metabisulphite rinses, the slides dehydrated, cleared and mounted. Control sections were treated exactly the same, although not oxidized with periodic acid. Any material which stained with the PAS technique and did not stain in the control slides is hereafter referred to as polysaccharide.

It was possible to differentiate mucopolysaccharides from glycogen by digestion of the slides with salivary amylase prior to the periodic acid oxidation. The slides were run to water as before and digested for three hours at room temperature in saliva. Following digestion the slides were run through the same PAS procedure outlined above. Any material which was PAS-positive but removable by salivary digestion is hereafter referred to as glycogen. Any material not removable with salivary digestion is referred to as mucopolysaccharides. Additional slides placed in water for three hours, in place of saliva, served as control slides. Since the pattern of localization in those control slides differed in no way from the standard PAS pattern, one can conclude that the saliva removes glycogen by enzymatic hydrolysis specific for glycogen and not by a leaching-out of materials by simple dissolution in an aqueous solution.

Regions of basophilia were demonstrated in the same material by staining with a 0.1% aqueous solution of toluidin blue. Cytoplasmic basophilia can be attributed to the presence of ribose nucleoproteins and/or acid mucopolysaccharides. Since all

basophilic material observed in this study was not PAS-positive, it is assumed that the basophilia was due to ribose nucleoproteins.

Results

A series of stages of both species of *Coprinus* was fixed and sectioned. Then duplicate slides of each fruiting body were stained by (1) the PAS method for all polysaccharides, (2) the PAS method with salivary digestion for the mucopoly-

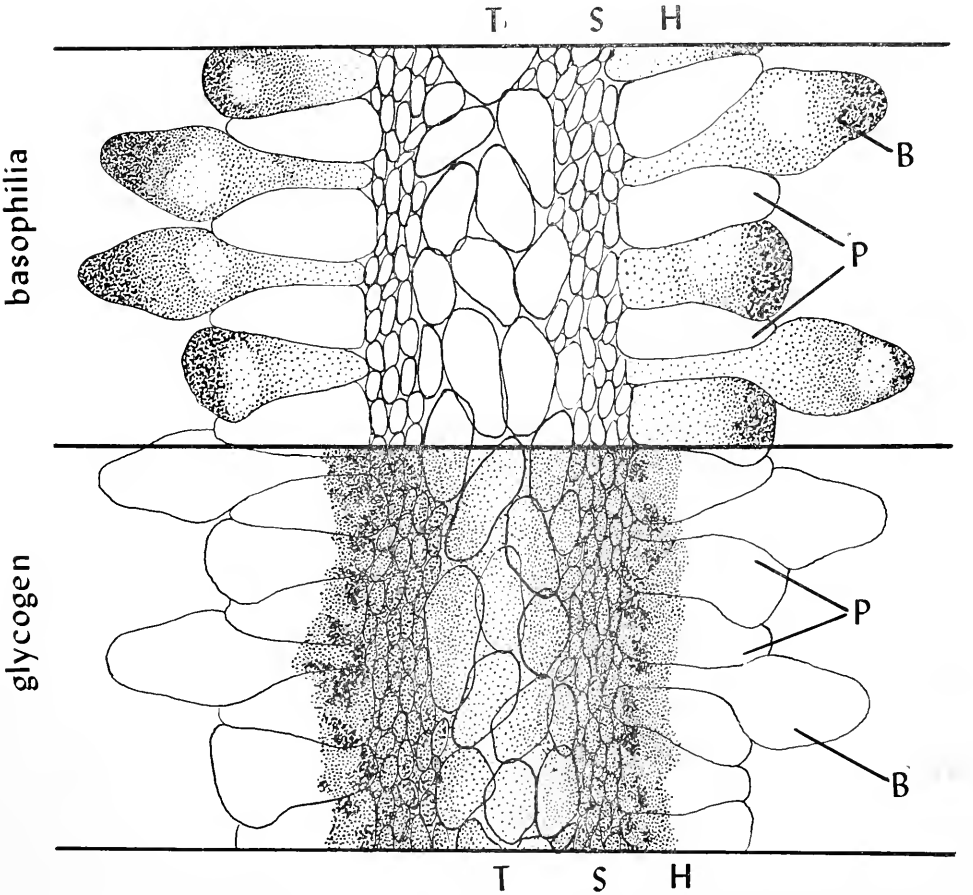

Figure 1. A section through a gill indicating the distribution of basophilic substances (above) and glycogen (below). T, tramal layer; S, sub-hymenium; H, hymenium; B, basidium; P, paraphysis. (Drawing by Miss Marcia J. Shaw.)

saccharides, and (3) with toluidin blue for the basophilic substances. Therefore, it was possible to compare a specific fruiting body at a specific stage for three substances: mucopolysaccharides, glycogen, and basophilic substances.

First of all it should be said that both species, *C. lagopus* and *C. curtus*, showed the same staining characteristics, and therefore the description given below applies

to both. The general staining properties of the cap and stipe could be seen more advantageously in the smaller *C. curtus*, while *C. lagopus* was especially suitable for examination of the gills.

An intense staining with Schiff reagent was observed in the globular cells of the cap in *C. curtus* which was not dependent upon periodic acid oxidation. There was also a faint staining of all cell walls in non-oxidized control sections, and character-

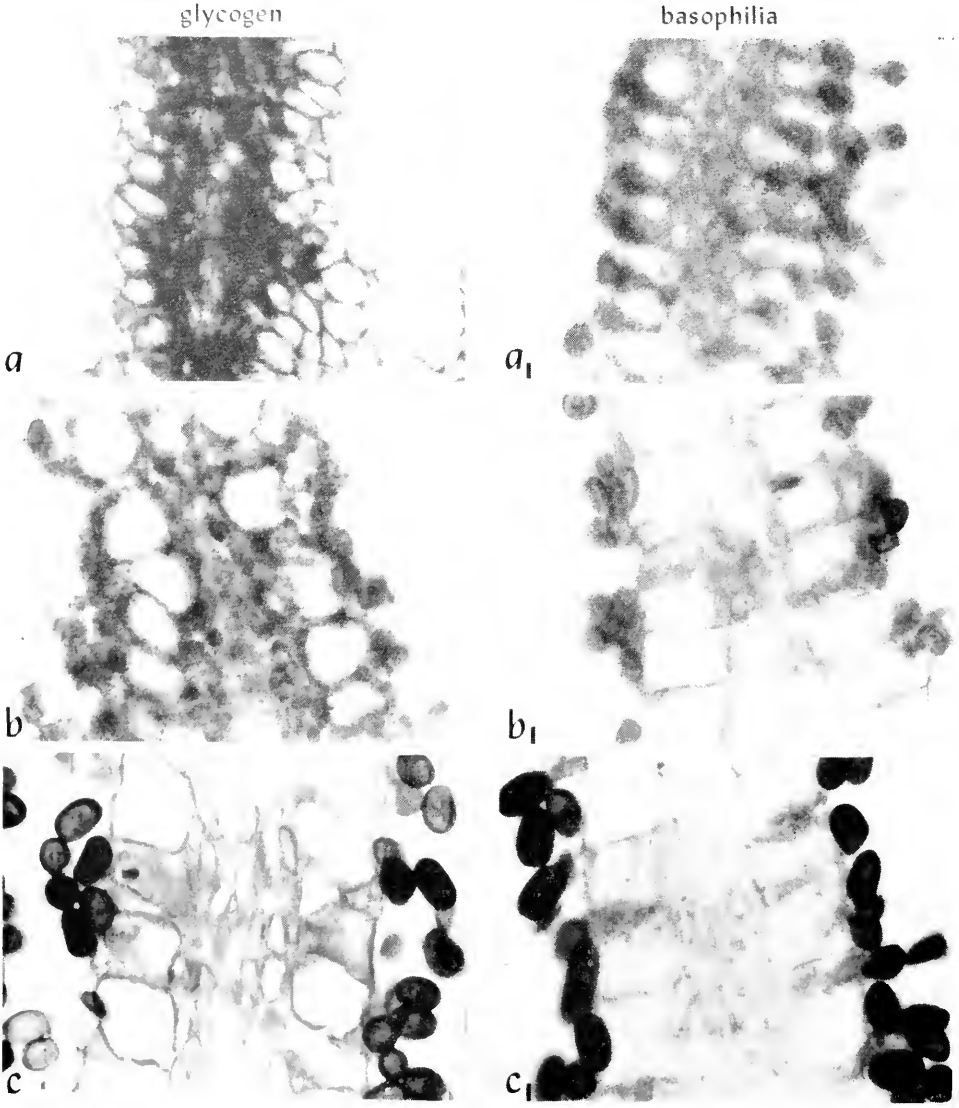

FIGURE 2. Photograph of sections through the gills showing the distribution of glycogen (left column) and basophilic substances (right column) at different stages of development; a, b, c represent the three stages of before, during, and after spore formation. Each pair (*e.g.*, a and a_1) are from the same mushroom.

istically the stain was more intense at the basal end of the mushroom. The basis for these two staining reactions is unknown. Following periodic acid oxidation the cell walls stained intensely, indicating the presence of mucopolysaccharides, presumably chitin. The cell walls were the only regions where mucopolysaccharides were observed in a significant concentration.

Exclusive of the gills, there was no general difference in the distribution of glycogen and the basophilic substances; they both appeared to be present in small quantities all over the mushroom at early stages, although the growth zone just below the cap always showed a higher concentration. The intensity of staining reactions was inversely proportional to the cell length, that is, the short cells of the growth zone at early stages were darkly stained, but after complete expansion these cells were depleted of stainable material as were the cells of the rest of the mushroom.

In the gills, prior to spore formation, there is a dark layer showing a concentration of basophilic substances, as well as a similar layer of glycogen. The interesting point is that, as shown in Figures 1 and 2, the positions of these layers do not correspond. The basophilic layer lies at the tips of the basidia, that is, the end at which the future spores will be formed. Furthermore, basophilia is observed only in the basidia and not in the paraphyses. The glycogen lies primarily in the subhymenium, although it extends to the base of the hymenium (in both the basidia and paraphyses) on one side and into the tramal layer on the other.

This staining picture is present at the earliest stages that gills are discernible, and in subsequent stages there is no obvious increase in the intensity of the staining. The only significant change is the increase in the size and the extent of the gills with age.

It was possible to show that once the spores are formed (Fig. 2, c) both the stainable layers disappear, showing a complete absence of glycogen and basophilic substances. Therefore, it is presumed that these materials enter into the spores, but the spores themselves become darkly pigmented with maturity, which masks any staining reaction they might show.

In order to verify the hypothesis that the spores contain these substances, a new series of mushrooms of intermediate size was fixed to find ones in the middle of the process of spore formation. A particular set of sections of *C. lagopus* was fortunately exactly at this stage (Fig. 2, b). The glycogen is now present in and around the hymenium, as well as concentrated in the young spores. The basophilic substances, which were at the basidial tips to begin with, appear to enter directly into the spores during their formation.

Discussion

The presence of glycogen about the base of the basidia, and its outward movement during spore formation, suggest that it might supply energy for the process of spore formation. However, a considerable portion of the glycogen enters directly into the spore and becomes part of its reserves for future germination and growth.

The basophilic substances are at the end of the basidium that forms the spores and therefore the material is transferred directly upon spore formation. Presumably the nuclear material is associated with this basophilic zone and both are transferred together.

It is of interest to note that the staining appears equally intense in the gills in

both small young mushrooms and large fully developed ones. The only difference is the extent of the gill material and from this we might presume that the gill is laid down with all its food material for sporulation right in the beginning, and during the period of growth the sole change is that the total amount of gill material is extended. From the work of Madelin (1956) it is likely that materials are constantly being drawn up from the vegetative hyphae into the mushroom during its expansion, and this material must be led directly to the newly forming gill.

The observation that essentially all stainable glycogen and basophilic substances are gone after sporulation implies an economy, and one would expect this phenomenon to be correlated with the fact that in small species of *Coprinus* there is but one short period of spore formation and discharge. Mr. Anthony J. Schmidt of this laboratory has made some preliminary PAS and toluidin blue preparations of *Agaricus campestris* and here one finds large concentrations of glycogen and basophilic substances in the cap with channels of conduction to the hymenium. Therefore in a large species which forms spores over a long period there is a large store of substances that can be continuously poured into the spores.

Summary

Two species of *Coprinus* (*C. lagopus* and *C. curtus*) were examined, using histochemical techniques, and it was found that prior to sporulation there were two distinct zones in the gills, one containing glycogen and one containing basophilic substances. The glycogen zone is at the base of the hymenium, extending into the central tramal layer. The basophilic zone is at the outer tips of the basidia. Upon sporulation both these groups of substances entered the spores, leaving no demonstrable material within the cells of the gills.

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RELATION BETWEEN POSITION OF BURROWS AND TIDAL RHYTHM OF UCA [1]

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The literature concerning tidal and semilunar rhythms of color change has been reviewed by Fingerman (1957). These rhythms were first observed in the fiddler crab *Uca pugnax* collected in the region of Woods Hole, Massachusetts, where the tides are semidiurnal (Brown, Fingerman, Sandeen and Webb, 1953). The crabs darkened by day and lightened by night in accordance with their 24-hour rhythm of color change. Superimposed upon the latter was a tidal rhythm that progressed across the 24-hour rhythm at the average rate of 48.8 minutes per day, as evidenced by a supplementary dispersion of pigment in the melanophores about the time of low tide. Also evident was a semilunar rhythm of 14.8-day frequency, the average interval between days on which the 24-hour and tidal rhythms repeat similar time relations to one another. The relationship between the time of supplementary dispersion of the melanin and the time of low tide was determined directly by the local tidal situation where the crabs were collected, as evidenced by the phase difference of the tidal rhythms of *Uca pugnax* collected from two localities where the times of low tide were different.

Persistent tidal and semilunar rhythms of color change have been observed in the blue crab, *Callinectes sapidus*, by Fingerman (1955). The rhythms were similar to those described above for *Uca pugnax* in spite of the fact that the *Callinectes* were collected in the vicinity of New Orleans, Louisiana, a region of diurnal tides. Evidently, the center of tidal rhythmicity in *Callinectes* operates on the basis of tides spaced 12.4 hours apart in spite of the fact that the crabs were collected in a region where 24.8 hours is the interval between two successive low tides.

Tidal rhythms of color change have been observed in two species of fiddler crabs in addition to *Uca pugnax*, *Uca pugilator* and *Uca speciosa* by Fingerman (1956). The latter two species were collected at Ocean Springs, Mississippi, where the tides are diurnal. The tidal rhythms of both species were similar in nature to the tidal rhythms of *Uca pugnax* and *Callinectes sapidus*. Both the *Uca pugilator* and the *Uca speciosa* were collected from limited portions of the beach. Analysis of the tidal rhythm of both species revealed that the *Uca speciosa* behaved as if low tide occurred for them 7.5 hours earlier in the day than low tide for the *Uca pugilator*.

Inspection of the beach at Ocean Springs revealed that the burrows of the *Uca speciosa* were closer to the high tide mark than were the burrows of the *Uca pugilator*. The phase difference of the tidal rhythms appeared to be due to the fact that when the water began to recede after high tide, a local low tide occurred earlier for the *Uca speciosa* than for the *Uca pugilator*. The *Uca speciosa* would be free, therefore, to leave their burrows and feed earlier than the *Uca pugilator* living

[1] This investigation was supported by Grant No. B-838 from the National Institutes of Health.

closer to the actual low tide mark. Measurements of the beach where both species were collected revealed that the water actually began to uncover the burrows of the *Uca pugilator* 4.9 hours after the burrows of the *Uca speciosa* began to be uncovered.

The present study was undertaken to investigate further the nature of the tidal rhythm of color change of the fiddler crab *Uca pugilator* and to test the hypothesis presented above that the phases of the tidal rhythm are set by the time of low tide at a limited portion of beach. The hypothesis was tested by comparing the phases of the tidal rhythm of color change of specimens of the same species collected from distinct sets of burrows different distances from the high tide mark, because the possibility existed that the phase difference between the *Uca speciosa* and *Uca pugilator* was a species difference and not due to the tides as hypothesized above.

Materials and Methods

Adult male and female specimens of the fiddler crab *Uca pugilator* were collected on the beach near the Gulf Coast Research Laboratory, Ocean Springs, Mississippi, for use in the observations reported below. The specific collection site consisted of two discrete sets of burrows. Crabs could be collected from each set of burrows with assurance that no mingling of the two groups occurred normally. Figure 1 is a diagrammatic representation of the section of the beach where the animals were collected. Included in this figure are the distances of the burrows from each other and from the high and low tide marks. A sub-surface drainage pipe, not shown on the diagram, emptied its contents between the two sets of burrows. The water flowing from this pipe at times of low tide was another factor that kept the crabs of the two sets of burrows isolated from one another.

The animals were placed into stainless steel aquaria and transported to an air-conditioned laboratory in New Orleans. From the evening of the day of collection until the end of the period of observation, the animals were maintained in darkness except for the few minutes required to make observations of the chromatophores and to change the water in the aquaria on days observations were performed. The animals were kept in inclined stainless steel aquaria containing sufficient sea water to cover approximately one-half of the bottom of the aquaria. The crabs were, therefore, free to move into and out of the water.

Chromatophores were staged in the manner described by Fingerman (1956). The average index was determined for the melanophores on the anterior aspect of a walking leg with the aid of a stereoscopic dissecting microscope and lamp. The chromatophore indices of Hogben and Slome (1931) were used in staging the chromatophores. The most concentrated state of the pigment is referred to as stage 1, the most dispersed stage 5, and the intermediate conditions stages 2, 3, and 4.

Results and Discussion

The measurements of the portion of the beach at Ocean Springs, where the animals were collected (Fig. 1), were analyzed to determine the phase difference that would be expected between the tidal rhythms of the animals from the two sets of burrows if the hypothesis of Fingerman (1956) is correct. The distance from the high tide mark to the low tide mark was 215 feet (Fig. 1). Twelve and four-tenths hours is the average interval required for the water to recede from the high

tide mark to the low tide mark. One set of burrows was 28 feet closer to the high tide mark than was the second set. If the water recedes 215 feet in 12.4 hours, then the water will recede 28 feet in 1.6 hours assuming, of course, that the slope is uniform. The set of burrows closer to the high tide mark would, therefore, begin to be uncovered by the receding water 1.6 hours earlier each day than would the set of burrows closer to the low tide mark. On the basis of the average tidal progression of 48.8 minutes per day, 1.6 hours in a tidal cycle are equivalent to two days. Therefore, a phase difference of two days was anticipated between the specimens from the two sets of burrows when they were examined in the laboratory.

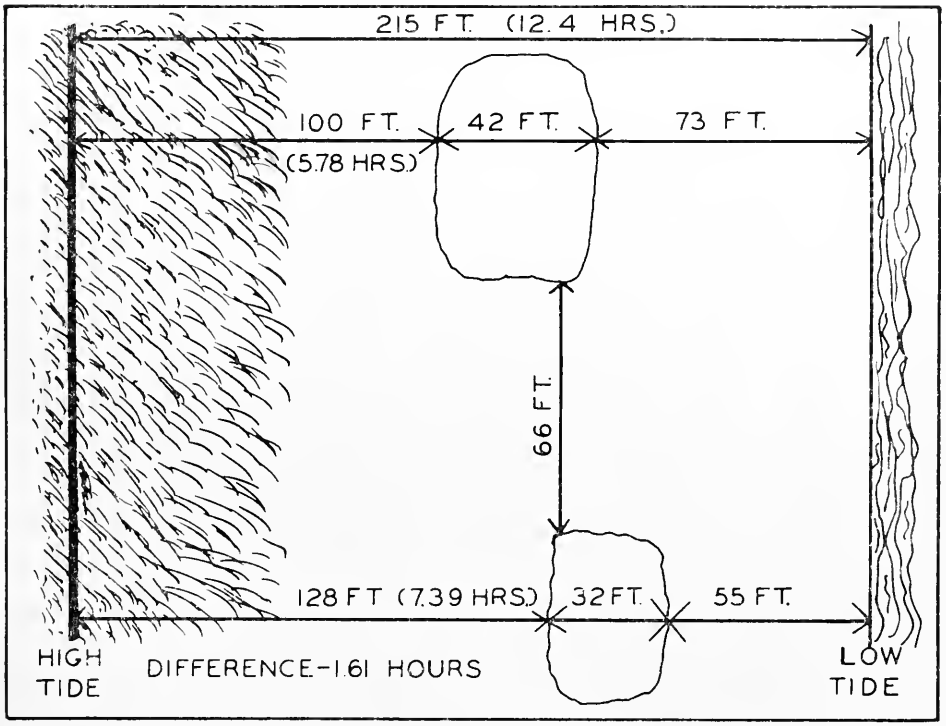

FIGURE 1. Diagrammatic representation of the positions of the burrows on the beach at Ocean Springs, Mississippi, where the specimens of *Uca pugilator* were collected.

Figure 2 presents the times of high and low tide at Ocean Springs on days chromatophores were observed in the laboratory. The diurnal nature of the tides is obvious. However, the tides did not follow the usual pattern of advancing 48.8 minutes daily across the 24-hour day. High tides occurred generally between midnight and 2 P.M. and low tides from 2 P.M. to midnight. The data for Figure 2 were selected from the tide tables published by the Coast and Geodetic Survey, United States Department of Commerce.

On July 3, 1956, several hundred crabs were collected from each set of burrows for the first series of observations that started at 8 A.M. on July 4. On days when observations were performed, 50 specimens from each group were observed hourly

from 8 A.M. through 7 P.M. and the average chromatophore stage was calculated for each group. The crabs from the burrows closer to the high tide mark will be referred to as Series A and the crabs from lower on the beach as Series B. In Series A a sufficient number of crabs survived throughout the period of observation so that 50 specimens were always available. Many of the Series B crabs died suddenly between August 6 and 8 so that this group had to be discarded. The average hourly chromatophore stages for Series A and B on each day observations were performed are presented in Tables I and II, respectively. Observations were usually made every second day, but from July 12 through 17 observations were daily.

Some of the data were taken from Tables I and II in order to prepare Figure 3. Use of this figure aids in discussion of the phase difference of the tidal rhythms

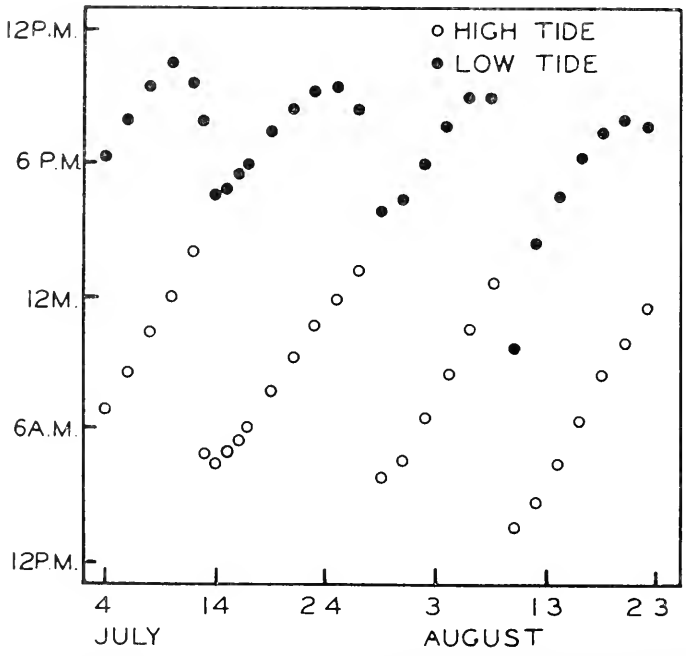

FIGURE 2. Times of high and low tide at Ocean Springs on days observations of the crabs were made in the laboratory.

of the two sets of animals. In Figure 3 the daily patterns of Series A and B crabs were arranged according to the expected two-day phase difference. According to the calculations low tide at any given hour of the day, *e.g.,* noon, would occur two days earlier for Series B crabs than for Series A crabs. Therefore, the curve of Series A for any given day was placed beside the curve of Series B that had been obtained two days previously. As is evident from inspection of Figure 3, a two-day difference in the tidal rhythms was present as shown by the similarity of the curves of Series A with the curves of Series B that had been obtained two days earlier.

The shapes of the daily curves obtained during the first three weeks of observa-

tion did not turn out as predicted on the basis of the observations of chromatophores of *Uca pugilator* performed during the summer of 1955 (Fingerman, 1956). The maxima of the curves did not progress across the day but were restricted to the left side of the curves, decidedly different from the results obtained with the same species collected in 1955 in the same area (Fingerman, 1956). The specimens of *Uca pugilator* observed during the summer of 1955 exhibited the typical pattern of tidal rhythm of color change just as was found in *Uca pugnax* by Brown, Fingerman, Sandeen and Webb (1953) and in *Callinectes sapidus* by Fingerman (1955).

TABLE I

The average melanophore index for each of the 12 daily observations of the Uca pugilator animals of Series A

		8 A.M.	9	10	11	12 M.	1 P.M.	2	3	4	5	6	7
July	4	3.7	3.4	3.6	3.3	3.6	3.8	3.8	3.4	3.2	3.6	3.3	2.9
	6	3.9	3.8	3.6	3.7	3.3	3.5	3.3	3.1	3.3	3.2	2.8	2.9
	8	3.8	4.2	4.2	3.7	3.6	3.7	3.5	3.7	4.0	3.8	3.4	3.4
	10	4.0	3.9	3.9	4.2	4.1	4.1	3.7	3.5	3.3	3.8	3.9	3.6
	12	3.3	3.2	3.9	3.9	3.6	4.0	3.8	3.2	3.5	3.4	3.2	3.1
	13	3.5	3.8	3.5	3.6	3.6	3.6	3.4	3.3	3.6	3.6	3.9	3.4
	14	3.6	3.6	3.7	3.6	3.6	3.5	3.5	3.6	3.7	3.4	3.4	3.6
	15	3.9	3.7	3.1	3.1	3.4	3.3	3.2	3.4	3.3	3.5	3.3	3.5
	16	3.8	4.0	3.6	3.4	3.5	3.2	3.0	3.1	3.1	3.4	3.1	3.2
	17	3.6	3.9	3.9	3.8	3.8	3.5	3.5	3.0	3.4	3.2	3.2	3.3
	19	3.6	3.2	3.6	3.3	3.3	3.4	3.5	3.2	3.0	3.1	2.9	2.5
	21	4.2	3.8	4.0	3.6	3.5	3.7	3.9	3.7	3.7	3.6	3.6	3.1
	23	3.9	3.8	4.1	3.2	3.4	3.0	3.3	3.2	3.3	3.2	3.0	3.6
	25	4.2	3.9	3.7	3.4	3.0	3.2	3.9	3.4	3.0	3.1	3.3	2.9
	27	3.6	3.7	3.0	2.8	3.0	3.1	3.2	3.3	3.3	3.2	3.3	2.9
	29	3.8	3.2	3.4	2.9	2.8	3.0	3.3	3.2	3.5	3.3	3.2	2.8
	31	3.6	3.9	3.2	3.0	3.1	3.3	3.0	3.0	3.0	3.3	2.9	2.8
August	2	3.6	3.4	3.5	3.2	3.1	2.9	3.2	3.0	3.1	3.2	2.9	2.7
	4	3.5	3.3	3.0	2.8	2.9	3.0	3.2	3.3	3.1	3.5	3.2	2.9
	6	2.6	3.2	3.6	2.9	3.3	3.2	3.3	3.3	3.4	3.1	2.9	3.1
	8	3.1	2.8	3.1	3.0	2.9	3.3	3.0	3.4	3.2	3.2	3.5	3.3
	10	3.0	2.9	2.7	2.6	2.8	3.1	3.0	2.8	3.2	3.3	3.5	3.0
	12	3.4	3.4	2.8	3.0	2.9	2.7	3.4	3.2	3.2	3.0	3.0	2.9
	14	3.0	3.3	3.5	2.8	3.1	2.8	2.9	3.0	2.7	2.9	2.7	2.4
	16	3.1	3.0	3.0	2.8	3.2	3.2	3.2	3.5	3.5	3.0	3.4	3.0
	18	3.3	3.1	2.7	3.2	2.8	3.1	2.8	3.1	3.0	3.3	3.0	3.1
	20	3.2	3.2	3.1	3.2	3.0	2.9	3.1	3.0	3.2	3.4	3.5	3.1
	22	3.0	3.0	2.7	2.8	3.0	2.8	2.9	2.9	3.0	3.1	3.2	3.2

The tidal maximum of pigment dispersion of the crabs observed in 1955 progressed across the 24-hour day at the usual tidal rate of 48.8 minutes per day, with the result that maximum pigment dispersion occurred in the right half of approximately 50 per cent of the curves depicting the daily pattern of pigment dispersion. However, the maximum did not progress into the afternoon during the first three weeks of observation in 1956. More will be said of this phenomenon below.

In order to reaffirm these observations specimens of *Uca* were collected on July 18, 1956, from the same sets of burrows as were the animals of Series A and B and

were treated in the same fashion as the animals collected previously. Crabs collected on July 18 from burrows nearer the high tide mark will be referred to as Series C and crabs from burrows lower on the beach as Series D. The average chromatophore indices determined for Series C and D are presented in Tables III and IV, respectively. Fifty specimens were available from Series C throughout the observations. However, with Series D 50 were available only from July 19 through August 8. The number then gradually diminished from 49 on August 10 to 26 on August 22, the last day of observation. A two-day phase difference in the tidal rhythms of Series C and D crabs was evident during the first week of observation. Maxima were restricted to the left half of the curves during this period as were the maxima of the curves of Series A and B. About July 26, however, unexpected results that will be described below appeared.

TABLE II

The average melanophore index for each of the 12 daily observations of the Uca pugilator specimens of Series B

		8 A.M.	9	10	11	12 M.	1 P.M.	2	3	4	5	6	7
July	4	3.8	3.8	3.6	3.4	3.3	3.5	3.5	3.3	3.3	3.1	2.5	2.6
	6	3.7	3.8	4.0	3.6	3.2	3.5	2.9	3.2	3.4	3.3	3.3	2.6
	8	3.6	3.8	4.1	3.9	3.9	3.6	3.7	3.8	3.2	3.6	3.6	3.2
	10	3.3	3.7	3.4	3.8	4.1	4.0	3.8	3.7	3.2	2.8	3.5	3.1
	12	3.4	3.4	3.8	3.2	3.5	3.3	3.3	3.6	3.5	3.1	3.3	3.2
	13	3.2	4.0	3.5	3.3	3.4	3.5	3.3	3.8	3.3	2.8	3.2	3.2
	14	4.0	3.7	3.7	3.3	3.2	3.5	3.0	3.3	3.3	3.3	3.2	3.1
	15	3.9	4.0	3.9	3.7	3.8	3.2	3.4	3.0	3.5	3.3	3.3	3.2
	16	3.4	3.6	3.6	3.4	4.0	3.7	3.2	3.6	3.1	3.4	3.0	3.4
	17	3.9	4.0	3.7	3.9	3.5	3.3	3.5	3.2	3.3	2.9	3.1	2.7
	19	3.4	3.9	3.7	3.5	3.4	3.3	3.3	2.9	3.2	3.1	2.8	2.4
	21	3.5	3.7	3.8	3.1	3.3	3.0	3.1	2.8	2.7	2.5	2.7	2.5
	23	3.4	3.5	3.5	3.0	2.7	2.9	2.8	3.1	2.8	2.8	2.7	2.3
	25	3.9	3.6	3.3	3.1	3.2	3.1	3.0	2.9	2.9	2.9	2.7	2.4
	27	3.2	3.2	2.7	2.8	2.5	2.6	2.7	2.6	2.5	2.5	2.6	2.3
	29	3.1	3.1	2.5	2.4	2.5	2.6	2.3	2.6	2.6	2.4	2.5	2.1
	31	3.7	2.9	2.8	2.7	2.6	2.6	2.4	2.5	2.6	2.7	2.4	2.2
August	2	3.0	3.0	2.6	2.7	2.7	2.5	2.4	2.4	2.6	2.2	2.6	2.1
	4	3.0	2.9	2.9	2.4	2.5	2.4	2.4	2.5	2.4	2.3	2.4	2.3
	6	3.2	3.0	2.9	3.0	2.8	2.8	3.1	2.8	2.9	2.6	2.3	2.2

To determine the precise rate at which the tidal rhythm progressed across the 24-hour rhythm, and to demonstrate clearly the phase difference between the tidal rhythms of the crabs from the two sets of burrows, Figures 4 and 5 were prepared as follows. The data of Series A and B were used for Figure 4 and Series C and D for Figure 5. The 12 periods of observation were divided into four periods of three hours each, 8–10 A.M., 11 A.M.–1 P.M., 2–4 P.M., and 5–7 P.M. The 12 hourly averages were summed and 12 subtracted from the total because if there had been no daily excursion of the pigment the sum would have been 12. Likewise, the average chromatophore indices for each three-hour period were summed and three subtracted from the total. The percentage of the total that each three-hour period occupied was then calculated.

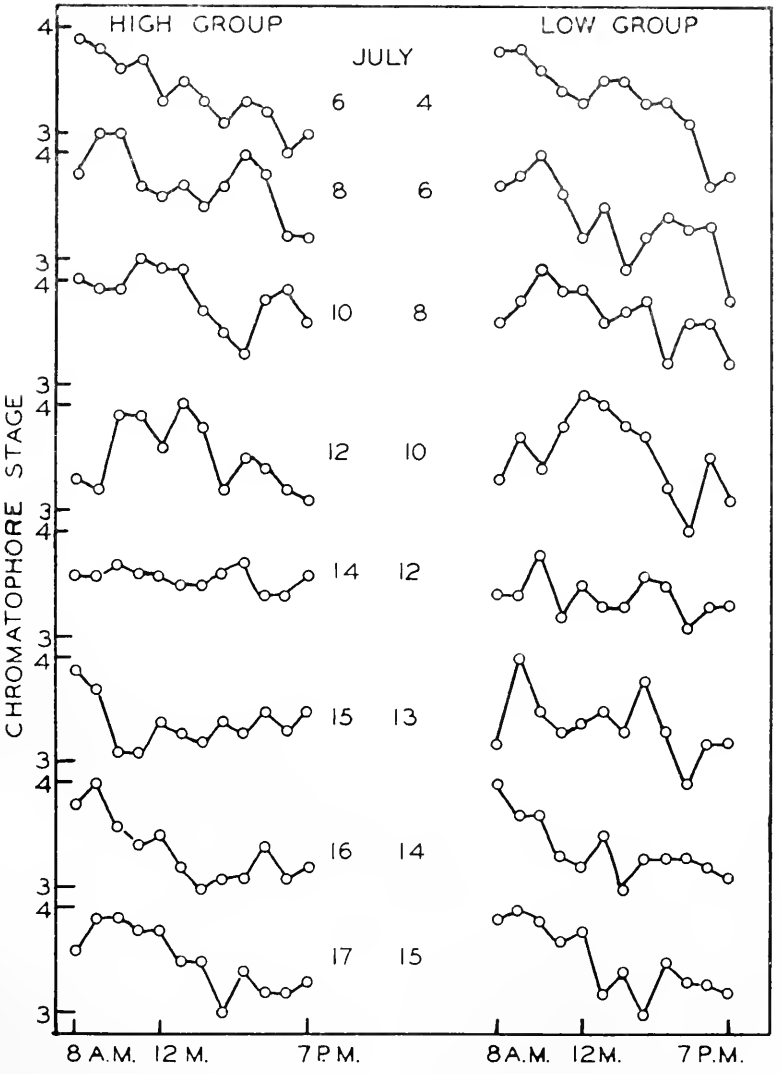

FIGURE 3. Daily pattern of melanophores of *Uca pugilator* from Series A (high group) and B (low group) on selected days.

Analysis of the data in the manner described above revealed a two-day difference, relative to the time of day, in the phases of the tidal rhythms of specimens of Series A and B and Series C and D through July 26 as indicated by the horizontal bars in Figures 4 and 5. For any given hour of the day the groups closer to the low tide mark exhibited corresponding maxima and minima two days earlier than the crabs from burrows closer to the high tide mark.

Analysis in the same manner of the data obtained with *Uca pugilator* during the summer of 1955 revealed regularly recurring 14.8-day cycles (Fingerman, 1956).

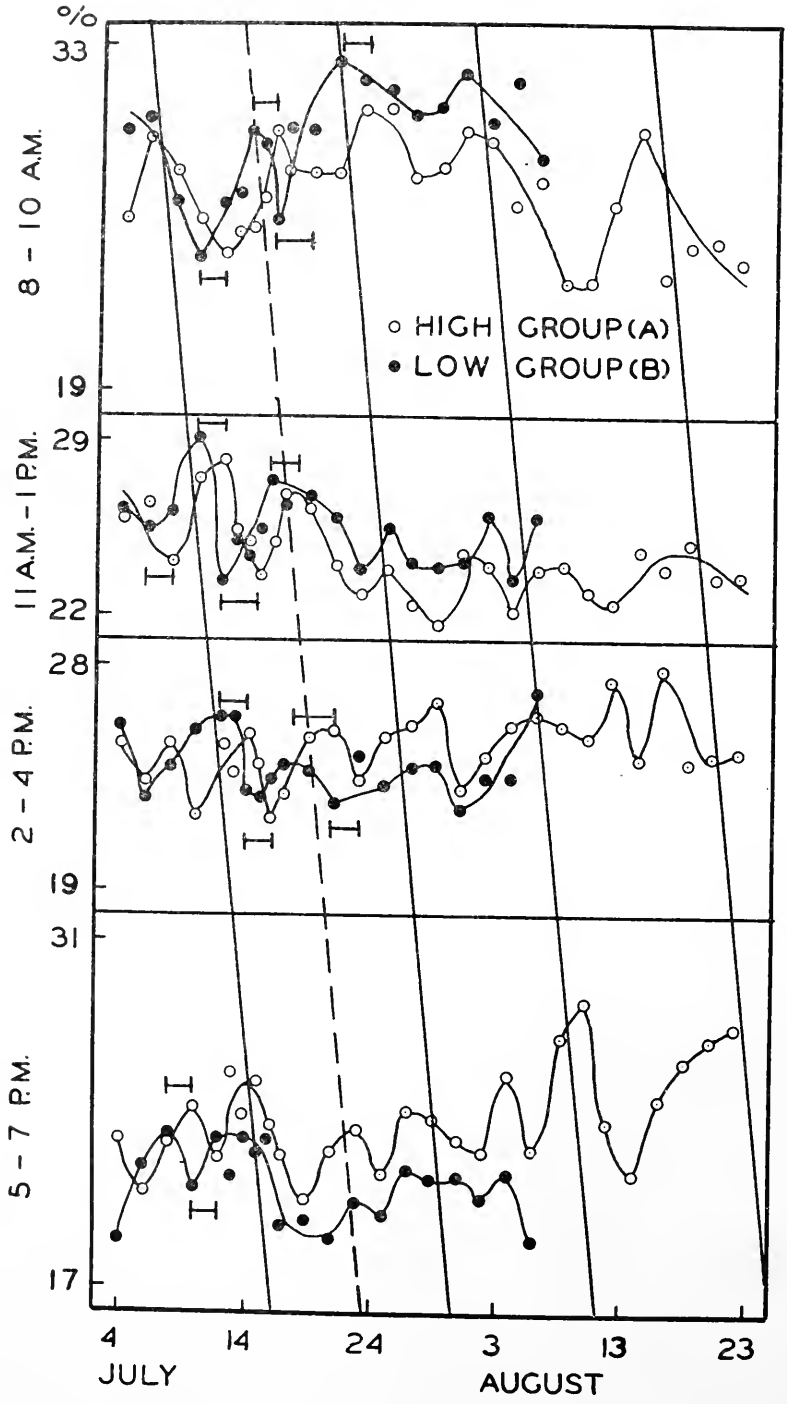
%
33
19
8 - 10 A.M.
HIGH GROUP (A)
LOW GROUP (B)
29
22
11 A.M. - 1 P.M.
28
19
2 - 4 P.M.
31
17
5 - 7 P.M.
4
14
24
3
13
23
JULY
AUGUST

FIGURE 4.

However, because of the absence of afternoon maxima through August 26 in the data collected in 1956, the pattern of 14.8-day cycles was different. Furthermore, the phase difference evident through July 26, 1956, disappeared and the four groups of crabs became rhythmically similar.

Inspection of Figure 4 reveals that the first 14.8-day cycle (shown by the first two solid diagonal lines) consisted of two 7.4-day rhythms as shown by the broken diagonal line. The diagonals were drawn midway between the two-day difference of Series A and B and C and D. The 7.4-day rhythms were due to the absence of late afternoon peaks. Instead of the tidal rhythm progressing across noon and into the afternoon the peak shifted back to early morning, thus producing a 7.4-day rhythm rather than the typical 14.8-day rhythm. Only two 7.4-day cycles were evident in Series A and B and were followed by a cycle of different frequency. A 7.4-day cycle was not evident in Series C and D because when they were collected Series A and B crabs were completing their second and last 7.4-day cycle. Furthermore, the two-day phase difference between the tidal rhythms of the crabs of Series C and D was not evident for longer than a week because of the unexpected loss of the phase difference that occurred in the four series of crabs about July 26.

TABLE III

The average melanophore index for each of the 12 daily observations of the Uca pugilator animals of Series C

		8 A.M.	9	10	11	12 M.	1 P.M.	2	3	4	5	6	7
July	19	3.7	4.0	3.5	3.8	4.0	4.0	3.5	3.9	3.4	3.4	3.4	2.6
	21	4.0	3.9	3.7	3.6	4.0	3.6	3.9	3.8	3.4	3.5	3.8	3.1
	23	3.4	4.3	4.2	3.6	4.0	3.8	4.1	3.7	3.7	3.3	3.8	3.6
	25	3.7	4.0	4.1	3.6	4.2	4.2	3.8	3.7	4.3	4.0	3.6	3.4
	27	3.7	4.1	3.7	4.1	3.7	3.4	4.0	3.8	3.6	4.1	3.8	3.7
	29	4.1	4.1	3.8	3.8	3.8	3.2	3.5	3.8	3.5	3.9	3.6	3.6
	31	4.1	4.0	4.0	4.0	3.2	3.1	3.3	3.0	3.2	2.9	3.2	2.5
August	2	4.0	3.4	4.1	3.4	4.0	3.2	3.1	2.8	3.0	3.1	3.3	3.2
	4	3.3	3.0	3.3	3.6	3.2	2.7	3.0	3.2	3.1	2.7	2.6	2.7
	6	2.8	3.1	3.4	3.6	3.5	3.4	3.4	3.5	3.7	3.3	3.1	2.6
	8	2.6	3.0	3.1	3.0	3.2	2.9	3.1	3.4	3.5	3.2	3.6	2.8
	10	2.9	2.9	2.7	2.6	3.1	2.5	2.5	2.6	2.5	3.3	3.0	3.5
	12	3.2	3.0	3.7	2.9	2.8	2.8	2.9	3.2	2.8	3.0	2.8	2.8
	14	2.9	2.7	3.4	2.7	2.6	2.7	2.4	2.4	2.1	2.5	2.8	2.7
	16	2.9	3.2	3.2	2.9	2.8	2.9	2.5	2.9	3.0	3.1	3.0	2.4
	18	3.0	3.0	3.4	3.1	2.8	2.7	2.7	3.1	3.0	2.9	3.1	3.1
	20	2.8	3.1	3.1	3.0	3.2	3.1	3.2	3.2	3.4	3.3	3.0	2.9
	22	3.0	2.9	3.0	3.0	3.2	3.3	3.3	3.4	3.6	3.4	3.3	3.5

When the phase difference disappeared a rhythm appeared with a frequency that had not been observed previously. Instead of a typical 14.8-day cycle, an 11.5-day rhythm appeared (Figs. 4 and 5). As evident from inspection of Tables I, III, and IV, maxima occurred in the afternoon on August 8. From the latter date until the

FIGURE 4. Relationship between percentage of daily melanin dispersion of *Uca pugilator* collected July 3 that occurred at each of four periods during the day and the day of the month. Circles represent fiddler crabs from the burrows closer to the high tide mark (Series A). Dots represent fiddler crabs from the burrows closer to the low tide mark (Series B).

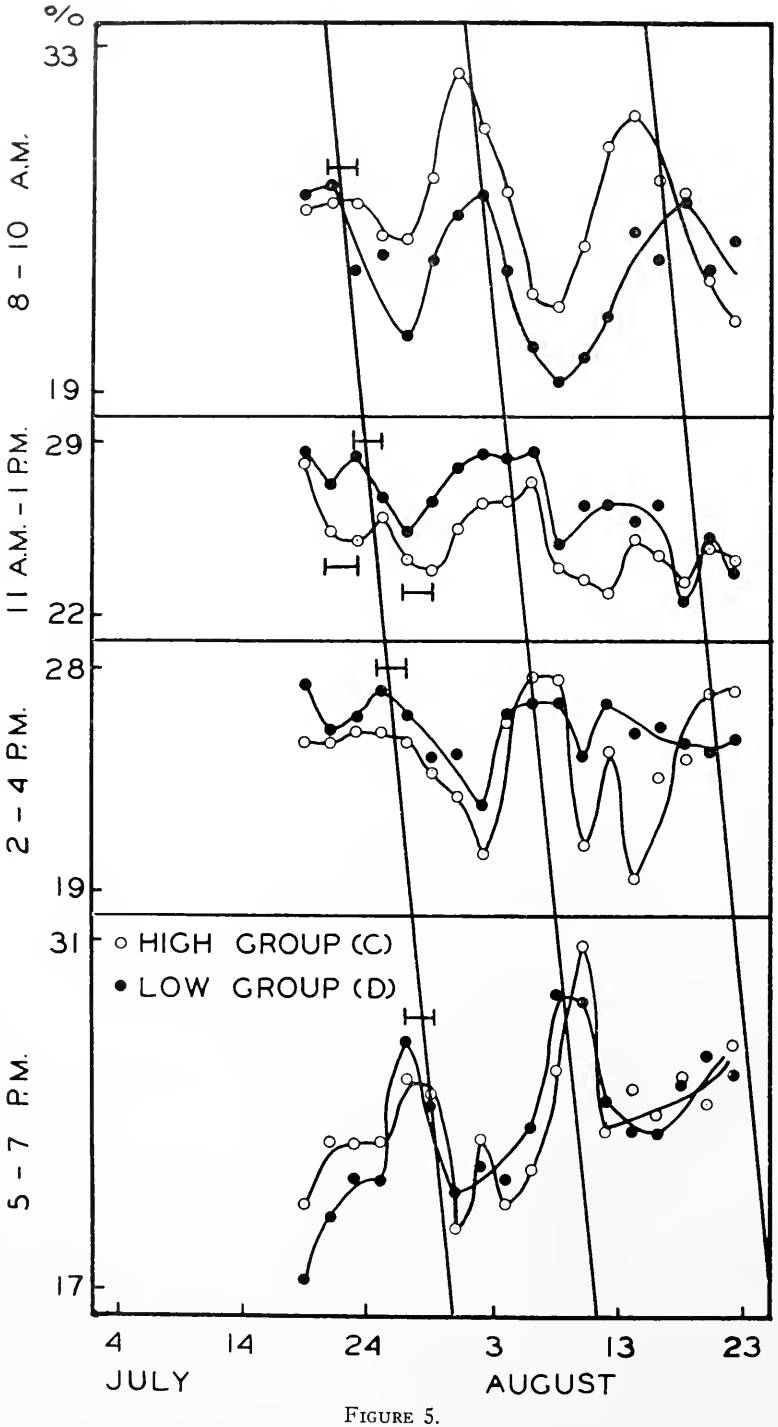
%
33
19
8 - 10 A.M.
29
22
11 A.M. - 1 P.M.
28
19
2 - 4 P.M.
31
17
5 - 7 P.M.
○ HIGH GROUP (C)
● LOW GROUP (D)
4
14
24
3
13
23
JULY
AUGUST

FIGURE 5.

observations of Series A, C, and D ended on August 22, the daily patterns exhibited a typical tidal progression of the peak across the day as observed with *Uca pugilator* during the summer of 1955. A typical 14.8-day cycle followed the 11.5-day cycle concomitant with the gradual progression of the tidal maximum across the day.

The relationship between the times of high and low tide (Fig. 2) and the maximum of the chromatophore readings determined each day of observation could not be determined with accuracy, since at the start of the observations the maxima of the chromatophore readings did not progress across the day but were confined to the morning hours. On some days the maximum would occur later than the time of

TABLE IV

The average melanophore index for each of the 12 daily observations of the Uca pugilator crabs of Series D

		8 A.M.	9	10	11	12 M.	1 P.M.	2	3	4	5	6	7
July	19	3.1	3.0	3.7	3.4	3.5	3.3	3.5	3.4	3.0	3.0	2.6	1.8
	21	3.2	3.7	3.6	3.8	3.2	3.5	3.4	3.3	3.3	3.3	2.8	2.4
	23	2.3	3.0	3.7	3.7	3.3	3.1	3.2	3.2	3.1	3.1	2.7	2.6
	25	2.7	3.5	3.8	3.8	3.3	3.5	3.6	3.4	3.7	3.1	3.1	2.9
	27	2.1	3.2	2.9	3.1	3.1	3.0	3.2	3.0	3.2	3.2	3.3	3.1
	29	2.2	3.4	3.5	3.3	3.3	3.1	3.0	3.1	3.0	3.1	3.2	2.9
	31	2.9	3.5	3.6	3.8	3.4	3.3	3.2	3.1	3.3	2.8	3.1	2.7
August	2	2.5	3.3	3.4	3.2	3.3	3.1	3.0	2.7	2.5	2.7	2.6	2.8
	4	2.4	2.8	3.1	3.2	3.1	3.0	3.1	2.8	2.9	2.8	2.8	2.2
	6	2.0	2.7	3.3	3.4	3.2	3.3	3.4	3.1	2.9	3.3	2.9	2.5
	8	1.6	3.0	2.8	2.8	3.0	2.8	3.0	3.1	2.9	3.1	3.2	3.2
	10	2.0	2.0	3.1	3.1	3.2	3.2	2.9	3.0	3.1	3.4	3.5	3.1
	12	2.4	3.0	3.5	3.3	3.4	3.4	3.3	3.4	3.4	3.3	3.2	3.1
	14	2.7	3.1	3.4	3.1	3.1	3.1	3.3	2.9	3.0	3.1	2.9	2.7
	16	2.9	2.9	2.6	3.1	2.9	2.9	2.8	2.9	3.0	2.7	2.9	2.6
	18	3.1	2.7	3.1	2.9	2.5	2.6	2.7	2.8	3.0	2.9	2.8	2.9
	20	2.8	2.8	3.0	2.9	2.8	3.2	2.9	2.9	3.0	3.1	3.1	3.0
	22	2.9	2.9	2.8	2.9	2.6	2.8	2.9	2.9	2.8	3.0	2.9	2.8

the tide and on other days earlier since the tides occurred at all hours of the 24-hour day but the maxima were restricted to the morning hours.

The relationship between the maximum of pigment dispersion and the time of tide from day to day must have been different at the end of the period of observation than at the beginning because of the 11.5-day cycle that intervened between the two 14.8-day cycles shown in Figure 4. The tides progressed at the typical semilunar rate of 14.8 days throughout the period of observation so that the phase relationship must have changed by 3.3 days, equivalent to 2.7 hours in a tidal cycle, in addition to the complication imposed by the appearance of typical cycles and maxima in the afternoon.

FIGURE 5. Relationship between percentage of daily melanin dispersion of specimens of *Uca pugilator* collected July 18 that occurred at each of four periods during the day and the day of the month. Circles represent fiddler crabs from the burrows closer to the high tide mark (Series C). Dots represent fiddler crabs from the burrows closer to the low tide mark (Series D).

The disappearance of the phase difference between the groups of crabs was in all probability due to some exogenous factor and not to removal of the crabs from direct contact with tides. If the latter explanation were correct, then crabs collected July 3 (Series A and B) would have lost their phase difference and taken on an 11.5-day cycle two weeks prior to the animals of Series C and D collected July 18. Since the crabs were not exposed in the laboratory to any overt stimuli capable of evoking the 7.4- and 11.5-day cycles observed during the summer of 1956, some exogenous factor might be considered as the mediating agent although natural rhythms have neither 7.4- nor 11.5-day cycles. Brown, Bennett and Ralph (1955) obtained evidence for a reversible effect of cosmic ray showers upon the responses of the chromatophore system of the fiddler crab *Uca pugnax*. The response of crabs exposed to increased intensity of cosmic ray showers, as compared with controls, was decreased pigment dispersion during the start of the day phase of the endogenous 24-hour cycle and increased dispersion during most of the remaining hours of the day.

Brown and Stephens (1951), in an investigation of the influence of length of photoperiod upon the amplitude of the daily pigmentary excursion of *Uca pugnax,* found that the longer the daily photoperiod the greater was the amplitude of the daily pigmentary excursion after the crabs were placed in constant darkness. Crabs with their burrows close to the low tide mark experience a shorter daily photoperiod than do animals whose burrows are close to the high tide mark. When the water covers the burrows the crabs are in darkness and emerge when the burrows are uncovered by the receding water. Burrows close to the low tide mark are uncovered later than burrows near the high tide mark and are covered earlier each day. Therefore, crabs living in burrows close to the low tide mark experience a shorter photoperiod each day than do crabs in burrows near the high tide mark.

The averages of the daily totals obtained for the first 33 days the crabs of Series A and B were in darkness were 40.96 and 37.59, respectively; averages for the first 34 days the crabs of Series C and D were in darkness were 39.65 and 36.62, respectively. The values for the crabs closer to the low tide mark (Series B and D) were less than the values for the crabs from the burrows close to the high tide mark (Series A and C).

An influence of change in day-length is also evident from inspection of the averages of the amplitude values. The time from sunrise to sunset in New Orleans was nine minutes less on July 18, 1956, than on July 3, 1956. The amplitude values for groups of crabs from the same set of burrows likewise decreased. Analysis of the daily amplitudes obtained with *Uca pugilator* during the summer of 1955 (Fingerman, 1956) also reveal an effect of change in photoperiod upon the amplitude of the daily pigmentary dispersion. On June 15, 1955, the time from sunrise to sunset was seven minutes more than on June 1, 1955. The average amplitude for *Uca pugilator* from the same set of burrows for the first 34 days in the laboratory was 37.75 for those collected on June 1, 1955, and 39.22 for those collected June 15, 1955. In this instance the day-length had increased between the first and second collection and the amplitude was consequently greater.

General Discussion

The results presented above support the conclusions of Fingerman (1956) concerning the tidal rhythms of color change of the fiddler crab *Uca pugilator,* as

well as provide new information about the nature of the rhythm. The time the burrows are uncovered at a particular level of the beach, and not the time of actual low tide on the beach, appears to be the primary determinant of the phases of the tidal rhythm relative to the 24-hour rhythm of color change.

Chromatophore rhythms with frequencies other than 12.4 and 24.0 hours and 14.8 days have not been observed previously. In the present investigation 7.4- and 11.5-day rhythms were described. The latter two, however, did not persist for more than one or two cycles. Evidently, the center or centers of rhythmicity of the fiddler crab can be set at one of several frequencies, some persistent and others not. Brown, Webb and Bennett (1955) have shown that *Uca pugnax* has the endogenous ability to mark off periods of solar and lunar day lengths in the absence of all possible rhythmic external signals. The persistent frequencies because of their correlation with frequencies of environmental events are probably of adaptive significance. On the other hand, no cosmic phenomenon has a 7.4- or 11.5-day frequency nor do fiddler crabs carry on activities correlated with these frequencies. The non-persistent rhythms of 7.4 and 11.5 days may have been imposed upon the crabs by exogenous factors and when these factors were no longer able to express themselves the crabs returned to the usual rhythm of 14.8-day frequency.

Summary and Conclusions

1. The tidal and semilunar rhythms of color change of the fiddler crab *Uca pugilator* have been subjected to further analysis.

2. The phases of these rhythms appear to be set according to the time the burrows of the crabs begin to be uncovered by the receding water following a high tide and not the time of actual low tide on the beach.

3. The amplitude of the daily pigmentary dispersion is also influenced by the time when the burrows are covered and uncovered. Crabs in burrows close to the high tide mark experience a longer daily photoperiod and consequently exhibit a greater daily amplitude of pigment dispersion.

4. A two-day phase difference, equivalent to 1.6 hours in a tidal cycle, was found between two groups of crabs collected from two discrete sets of burrows different distances from the high tide mark. The tidal maximum of pigment dispersion at any given hour of the day occurred two days earlier in the crabs collected closer to the low tide mark.

5. Measurements of the beach where the crabs were collected revealed that the receding water begins to uncover the burrows close to the high tide mark 1.6 hours earlier than the burrows closer to the low tide mark begin to be uncovered, the same difference observed in the laboratory.

6. The daily patterns of pigment dispersion were different during the first two weeks of observation from the patterns observed in previous investigations. Maxima of pigment dispersion did not occur in the late afternoon during the first three weeks of observation but were restricted to the morning hours. The absence of peaks in the afternoon resulted in cycles with a 7.4-day frequency.

7. An 11.5-day cycle also appeared and was followed by a typical 14.8-day cycle accompanied by maxima of the daily curves in the late afternoon.

8. The results are discussed in terms of possible exogenous causes of the 7.4- and 11.5-day cycles.

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EVIDENCE FROM SEA URCHIN–SAND DOLLAR HYBRID EMBRYOS FOR A NUCLEAR CONTROL OF ALKALINE PHOSPHATASE ACTIVITY

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Several investigators (Hultin, 1948a, 1948b; Bohus Jensen, 1953; Tyler and Metz, 1955) have shown that hybridization in echinoids can be facilitated by treatment of the eggs with trypsin. A recent review by Moore (1949) has covered the problem of inheritance in hybrid plutei of echinoids. It seemed of interest to determine the activity of two enzymes (acid and alkaline phosphatase) in the developing embryos of two separate species and of the hybrids. In the hybrids it was hoped that it would be possible to assess the relative role of the nuclei and the cytoplasm in directing the synthesis and activity of these two enzymes.

Alkaline phosphatase activity rises rapidly from gastrulation on to the pluteus stage (Mazia *et al.*, 1948; Gustafson and Hasselberg, 1950), while acid phosphatase maintains a constant level of activity during sea urchin development according to Gustafson and Hasselberg (1951). However, the latter authors have noted a slight but definite rise in acid phosphatase activity in the sea urchin *Paracentrotus lividus*.

Paternal antigens arising by the late blastula stage in sea urchin hybrids have been demonstrated serologically by Harding, Harding and Perlmann (1954), but little information exists dealing with quantitative chemical differences in hybrids. Brachet (1954) has expressed the belief that the nucleus exerts a very important control over enzymes of the microsomes, as evidenced from work upon nucleated and enucleated halves of amoebae.

Utilizing the Gomori–Takamatsu cytochemical technique Krugelis (1947b) has noted that nuclear alkaline phosphatase activity increases from gastrulation to the pluteus stage while that of the cytoplasm declines. In the unfertilized egg the weak staining reaction for the enzyme is primarily located in the cytoplasm (Krugelis, 1947a) and this is borne out by Mazia *et al.* (1948) who utilized biochemical methods and found equal activity in nucleated and enucleated halves of unfertilized eggs separated by the Harvey method. The Gomori-Takamatsu technique has been criticized by Novikoff (1951) and Johansen and Linderstrom-Lang (1952); these authors believe that diffusion obscures the accurate localization of the calcium phosphate precipitate and that nuclei may adsorb the precipitate. Novikoff *et al.* (1950) isolated rat liver nuclei and found less alkaline phosphatase activity in that fraction as compared to the cytoplasm, and Stern *et al.* (1952), utilizing a non-aqueous medium for isolating nuclei (Behren's technique), obtained similar results for nuclei isolated from the thymus gland. However, Dounce (1943) found a higher con-

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centration of alkaline phosphatase in the isolated nuclei from rat liver as compared to the whole tissue. Danielli (1953) expresses the belief that the Gomori-Takamatsu cytochemical technique can successfully localize alkaline phosphatase in the cell and this would mean that a number of workers who cite a nuclear localization of alkaline phosphatase (Danielli, 1946; Krugelis, 1947b; Brachet and Jeener, 1948; and Bradfield, 1950) are correct. The question of localization of this enzyme is an open one and it was hoped the method of hybridization might help to clarify it.

It was originally planned to hybridize reciprocally *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus* (see Moore, 1943, for a discussion of maternal and paternal inheritance in this cross), but it was found that there was essentially no difference in acid and alkaline phosphatase activity between the two species. Also it is known that when *S. franciscanus* eggs are fertilized by *S. purpuratus* sperm, development is blocked at the late blastula stage (Moore, 1943). Hybridization of *S. purpuratus* and *Dendraster excentricus* is advantageous in that these genera are more distantly related; they differ markedly in their speed of development, and the cross can be made reciprocally.

Materials and Methods

Eggs and sperm of *Dendraster* and *S. purpuratus* were obtained by injection of 0.5 *M* KCl (Tyler, 1949) into the body cavity. The eggs of *Dendraster* could be fertilized by the usual dilute suspension of *S. purpuratus* sperm used in the homologous crosses. In order successfully to fertilize *S. purpuratus* eggs with *Dendraster* sperm (Tyler and Metz, 1955), the eggs were placed in a 0.05% trypsin (crystalline-lyophilized preparation from Worthington Biochemical Sales Co.) solution for ten minutes and the eggs were then washed several times with sea water to remove the trypsin. They were then fertilized with an amount of 1% sperm (1 drop of dry sperm/5 cc. sea water) which was in forty-fold excess of the volume of sea water containing the eggs. After the eggs were left in this concentrated sperm solution for 30–40 minutes (with frequent agitation of the solution), the eggs were then washed four or five times to remove the excess sperm and cultured in a slowly rotating four-liter flask which floated in a tank of running sea water. The temperature of this running sea water was usually about 10° C. With this procedure about 40% of the eggs were successfully fertilized and hatched swimming blastulae could be separated from the unfertilized eggs.

On the first, second, third, and fourth days after fertilization embryos were collected by centrifugation and were washed several times with the appropriate buffer. These washes were carried out rapidly so as to prevent any loss of the enzyme. For the acid phosphatase assays this was a 0.1 *M* sodium acetate-acetic acid buffer of pH 5.32; for the alkaline phosphatase assays a 0.1 *M* sodium veronal-HCl buffer (0.0015 *M* $MgCl_2$) of pH 9.0 was utilized. For a given assay the embryos were suspended in 4 cc. of the acid or alkaline buffer and disintegrated in an all-glass homogenizer which was kept immersed in an ice bath during homogenization. Then a two-cc. aliquot of the brei was added to two cc. of the substrate (0.1 *M* sodium β glycerophosphate); for the acid phosphatase assays the pH of the substrate solution was adjusted to 6.0. One of the mixtures of brei plus substrate was immediately inactivated by the addition of 0.8 cc. of 60% trichloracetic acid while the other was allowed to incubate at 25° C. for a two-hour period at which time a simi-

lar amount of trichloracetic acid was added. The control and experimental samples were then centrifuged at approximately 10,000 G and the trichloracetic acid supernates were collected. Phosphorous assays were made upon these samples utilizing the Fiske-Subbarow technique (1925) and the control values subtracted from the experimental ones. The trichloracetic acid precipitates were each suspended in one cc. of water and total nitrogen determinations (Umbreit *et al.,* 1948) were made upon aliquots from these samples. Acid or alkaline phosphatase activity is stated as the ratio of total micrograms of phosphate released in a two-hour period divided by the total micrograms of trichloracetic acid-insoluble nitrogen. Acid-insoluble nitrogen is a good standard for these measurements since it remains fairly constant during early development.

Results

Preliminary assays of acid phosphatase in developing embryos of *Strongylocentrotus purpuratus* and *S. franciscanus* showed the activity of this enzyme to be essentially similar in the two species, and preliminary determinations of acid phosphatase in *Dendraster* gave values within this same range. Since acid phosphatase could not be used quantitatively to distinguish *S. purpuratus* from *Dendraster,* this enzyme was not assayed in the hybrids nor were further determinations made in the homologous species. The series of determinations for any given batch of eggs seemed to indicate a slight increase in activity of acid phosphatase during development, but the average values only substantiated an increased activity from the blastula to the gastrula stage.

The activity of alkaline phosphatase was quite different in *Dendraster* and *S. purpuratus,* rising quite sharply for the sand dollar embryos and much more slowly for the sea urchin embryos (Fig. 1). The points in Figure 1 represent two series of experiments, all of which were conducted under similar conditions and gave excellent reproducibility. It was of interest to see if the activity of this enzyme in the hybrids was characteristic of the maternal or the paternal species, or if it might be intermediate between the two. If the latter alternative held true, this would be a quantative indication of a nuclear control of alkaline phosphatase activity, whereas a maternal rate of activity would indicate a cytoplasmic independence of this enzyme. It was not expected that a paternal activity rate would be found.

Hybridization of *Dendraster* eggs by *S. purpuratus* sperm results in normal development up through gastrulation but in the experiments reported here the embryos then became abnormal and gave rise to so-called spherical plutei. These "plutei" did not swim actively and died within a few days. It is believed that the low alkaline phosphatase activities which were found here are a reflection of the poor condition of the embryos. Even if these embryos had developed normally, this would not have been the best cross to ascertain nuclear or cytoplasmic dependence of the enzyme since the maternal species (*Dendraster*) normally has a high alkaline phosphatase activity by the pluteus stage. An intermediate value in this case would be a decrease from the normal activity of the maternal species and might be ascribed to lowered vitality of the hybrids. However, in the *S. purpuratus* ♀ × *Dendraster* ♂ hybrids an intermediate value would be an increase from the normal activity of the maternal species and could clearly be ascribed to the effect of the paternal nuclear material during development. It was fortunate that these hybrids

developed perfectly normally. Some were kept as long as 12 days after fertilization and they were still perfectly healthy plutei at that time.

The *S. purpuratus* ♀ × *Dendraster* ♂ hybrids were strictly maternal in terms of rate of development both before gastrulation and up to the pluteus stage; *Den-*

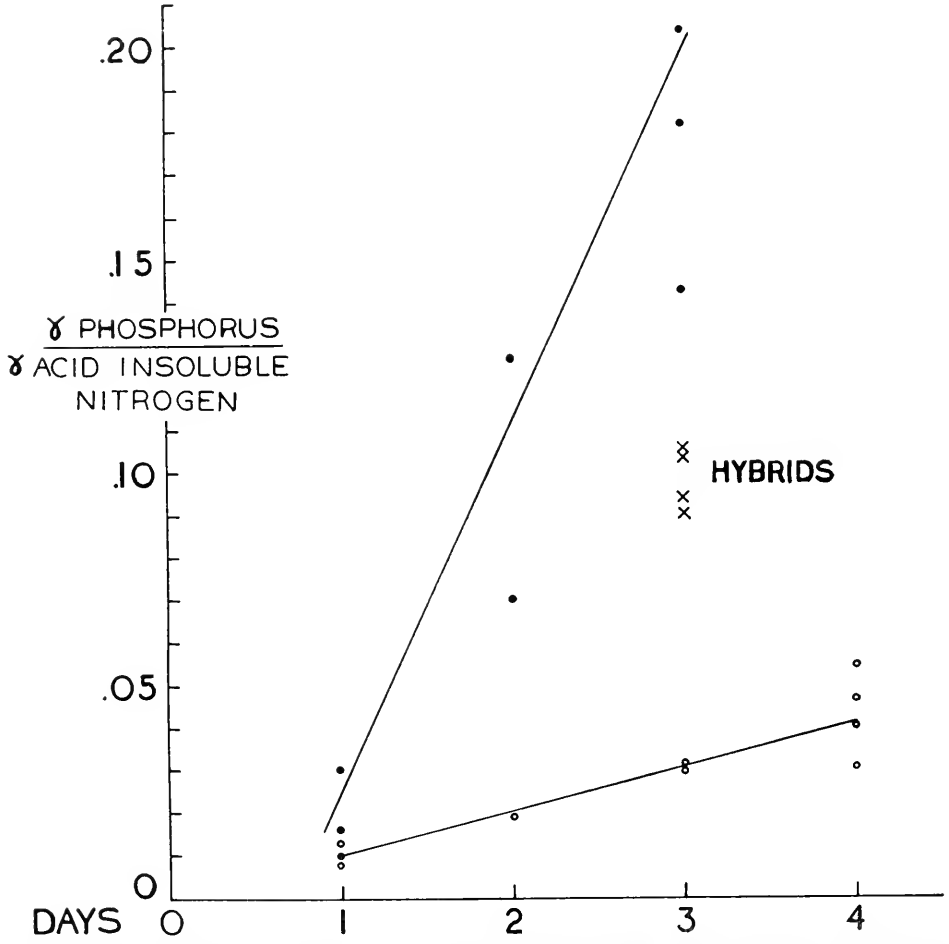

FIGURE 1. Alkaline phosphatase activity of *Strongylocentrotus purpuratus, Dendraster excentricus* and *Strongylocentrotus purpuratus* ♀ × *Dendraster excentricus* ♂ hybrids. Expressed as the ratio of micrograms phosphorus released in a two-hour period per microgram of acid-insoluble nitrogen. Solid dots indicate determinations upon *Dendraster*, open circles are for *S. purpuratus* and × indicates determinations upon the hybrids.

draster reaches the pluteus stage three days after fertilization whereas *S. purpuratus* takes four days. The day after fertilization (first day) the hybrids were early blastulae, the next day (second) they were hatched swimming blastulae or early gastrulae, the third day they had reached the prism stage and on the fourth day after fertilization they were plutei. In terms of morphological appearance the hybrid

plutei look very much like those of *S. purpuratus*. They lack the extended oral and anal arms of the *Dendraster* plutei and the skeletal rods are located as they are in *S. purpuratus* plutei.

Alkaline phosphatase determinations in the *S. purpuratus* ♀ × *Dendraster* ♂ hybrids were routinely made on the third day after fertilization when the hybrids had reached the prism stage. Assays were made at this time (third day) since no estimations were made upon *Dendraster* plutei after this period, and also by this third day there was a marked disparity between the alkaline phosphatase activity of the two homologous species which is not true at the earlier stages when alkaline phosphatase activity of the two species is more similar. The enzyme estimations revealed values intermediate to those of either homologous species (Fig. 1) at a similar time after fertilization. These hybrid prism activities were not only greater than those of *S. purpuratus* prisms but also distinctly higher than the values obtained for four-day *S. purpuratus* plutei. This indicates that alkaline phosphatase activity is not merely reaching an appropriate level for a given stage of development, as is the case with so many metabolic activities. These data are interpreted as an elevation of alkaline phosphatase activity due to the presence of *Dendraster* nuclear material in the developing embryos. It is not known if this nuclear stimulation of alkaline phosphatase indicates a nuclear localization of the enzyme, but it certainly seems to demonstrate a quantitative nuclear control for this enzyme with the intermediate value resulting from the action of both maternal and paternal nuclear elements.

Several attempts to induce higher acid and alkaline phosphatase activities in *S. purpuratus* embryos were carried out by adding sodium β glycerophosphate (final concentration 0.1 M) to several of the cultures at the mesenchyme blastula stage. The blastulae were allowed to develop for two days in the sea water containing the substrate and then acid and alkaline phosphatase assays were made at the pluteus stage. However, in two sets of experiments involving such cultures and their controls (where no substrate was added) no increase in activity was noted. Usually the activity of the controls was slightly higher than that of the cultures to which glycerophosphate had been added.

In order to determine if inadequate homogenization might account for the low alkaline phosphatase values, *S. purpuratus* embryos were frozen and thawed several times before homogenization so as to facilitate rupture of the cells. This procedure had no effect upon alkaline phosphatase activity since similar values were obtained for fresh material, once frozen, and twice frozen samples. Microscopic examinations of the homogenates were made routinely in all experiments. In all cases no large clumps of cells were observed, but undoubtedly not all cells were broken by the homogenization. This does not affect the interpretation of the results since the embryos of *S. purpuratus, Dendraster* and the hybrids were disintegrated in the same manner, and an equal degree of homogenization apparently was obtained in each case. It might also be expected that the hybrids would yield homogenates much like those of *S. purpuratus* since the only *Dendraster* component of the hybrids is nuclear and hence they are probably of equal fragility.

The author wishes to express his gratitude to the staff of the Friday Harbor Laboratories for their many kindnesses during the summer.

Summary

1. Alkaline phosphatase estimations upon sea urchin embryos (*S. purpuratus*) showed a slow rate of increasing activity up to the pluteus stage while sand dollar embryos (*Dendraster excentricus*) showed a very rapid increase up to this same stage.

2. Hybridization between *Strongylocentrotus purpuratus* ♀ and *Dendraster excentricus* ♂ resulted in alkaline phosphatase activity at the prism stage which was intermediate between that of the two homologous species and higher than that of the maternal species. This is interpreted as indicating a nuclear control of alkaline phosphatase activity.

3. Addition of substrate (0.1 *M* sodium β glycerophosphate) to cultures of developing *S. purpuratus* embryos did not affect an increase in acid or alkaline phosphatase activity.

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OBSERVATIONS ON OSMOREGULATION IN THE ARCTIC CHAR (SALVELINUS ALPINUS L.)[1]

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The different groups of euryhaline fishes have developed somewhat different mechanisms for maintaining the relative constancy of the concentration of their "milieu interieur" in the face of large changes in the external osmotic pressure. Following such an external change, many change their internal concentrations only transitorily, in the same direction as the external variation. A return to essentially the original conditions usually follows shortly (in adult female eels (*Anguilla*): Boucher-Firly, 1935; Duval, 1925; in sticklebacks (*Gasterosteus*); Gueylard, 1924; Koch and Heuts, 1943; in killifish (*Fundulus*): Burden, 1956). Anadromous salmonid fishes, however, have long been known to regulate their blood concentrations on two distinct levels (probably the end-points of an acclimation curve). In almost all such salmonids studied so far, the transition from salt to fresh water (or the reverse) is accompanied by a fall (or rise) in total blood concentration of about 25% (the Atlantic salmon, *Salmo salar,* and the Chinook salmon, *Oncorhynchus tshawytscha*, however, change by only 12% (Fontaine and Koch, 1950; Greene, 1926)). Plasma freezing point depressions vary from species to species, being 0.67–0.90° C. in salt water, 0.55–0.72° C. in fresh water (Benditt *et al.,* 1941; Fontaine, 1943, 1948; Fontaine, Callamand and Vibert, 1950; Fontaine and Koch, 1950; Greene, 1904; Kubo, 1953).

The Arctic char (*Salvelinus alpinus* L.) is an anadromous salmonid fish common in fresh and coastal salt waters throughout most of the Arctic. As in other sea-going char, its migrations from fresh to salt water and back again are somewhat different from those of other salmonids in that its winter periods in fresh water lakes and rivers are long compared to its summer periods in the ocean, rather than vice versa (Andrews and Lear, in press; Backus, 1952; Sprules, 1953). Osmoregulation in this form has not been studied, probably due to its inaccessibility. The present paper describes some observations made on the osmoregulatory abilities of adult Arctic char on their return (spawning) migration to fresh water after a summer in the ocean.

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The material is of a preliminary nature in many ways, but serves to show that the Arctic char is similar to other salmonid fishes in regulating its blood concentration on two levels. Agreement between experimental results and data obtained from fish living in fresh and salt waters provides a basis for further experimental study of osmotic phenomena during the migrations of these fish. Some data on regulation of muscle concentrations are also presented. Differences in results obtained from char in northern Labrador and Hudson Bay indicate the possibility of physiologically different populations in this species.

Materials and Methods

Regulation of total plasma concentration, chloride and potassium, and of total muscle solids, chloride, and potassium was studied in mature adult char of both sexes by means of observations on fish living in salt or fresh water, and by time series of observations immediately following direct transfers of fish from salt to fresh water. Conditions of temperature, stage in life cycle, etc., were kept fairly constant in these experiments. Char were taken by means of gill nets from Hebron Fjord, Labrador, and Hudson Bay near Churchill, Manitoba, during late July and early August of 1954 and 1955, respectively.

Seven char were used to establish the normal salt water ranges for plasma freezing point and chloride concentration in fish in Hebron Fjord (water of 28.6 ‰ salinity, 9° C. temperature). Five Churchill char were used similarly, observations on these consisting of plasma chloride and potassium concentrations, and total muscle solids, chloride, and potassium concentrations (Hudson Bay water was of approximately 26 ‰ salinity, 10–12° C. temperature).

Fresh water ranges for plasma freezing point and chloride concentration were determined at Hebron in several small land-locked char from a small lake, in some pre-sea-run parr from a river, and in an adult char that had returned to fresh water by itself. No fresh water char were obtainable at Churchill.

The osmotic stresses these fish undergo during the course of their migrations from the sea were approximated by transferring four Hebron fish and seven Churchill fish directly from salt to fresh water. Temperatures at Hebron were: salt water, 9°, fresh water, 5–10°; at Churchill: salt water, 10–12°, fresh water 14–16°. Changes in plasma freezing point with time were followed in three of the Hebron fish via serial blood samples taken at intervals up to 77 hours following transfer. The fourth fish was sampled initially and after 77½ hours. Two of the Hebron char were then returned to the Fjord and sampled again following death (after some fifteen hours in salt water).

Only two of the Churchill char survived for more than one hour after transfer from salt to fresh water—these for two and six hours, respectively. Blood and muscle concentrations were determined in these, after the periods mentioned, as in the salt water fish. The approximately 5° C. thermal shock to which these fish were subjected, combined with a lack of running fresh water, hence a need for aeration by hand dipping, probably explains in great part the lowered survival as compared with the Hebron fish. The speed with which death followed transfer makes it seem unlikely that this is the complete explanation, however.

Blood samples were taken via heart puncture from all fish. The samples were heparinized, centrifuged, and the plasma pipetted off. Plasma freezing points

were determined at Hebron using the method of Pounder and Masson (1934) modified for field use (Scholander *et al.*, in press). Plasma chloride determinations were made using the method of Schnohr (1934). Plasma potassium concentration was determined on the Churchill samples following dilution with Pyrex-distilled water on a Baird Associates internal standard flame photometer. Probably due to coagulation of the proteins in these samples, which may have interfered with activation of the ions by the flame, only three of these analyses gave what appear to be reasonable values.

Four samples of tissue from the dorsal muscle mass, averaging about 100 mg. wet weight, were also taken from each of the Churchill fish. Total solids were determined by weighing these before and after complete drying in an oven at 105° C.

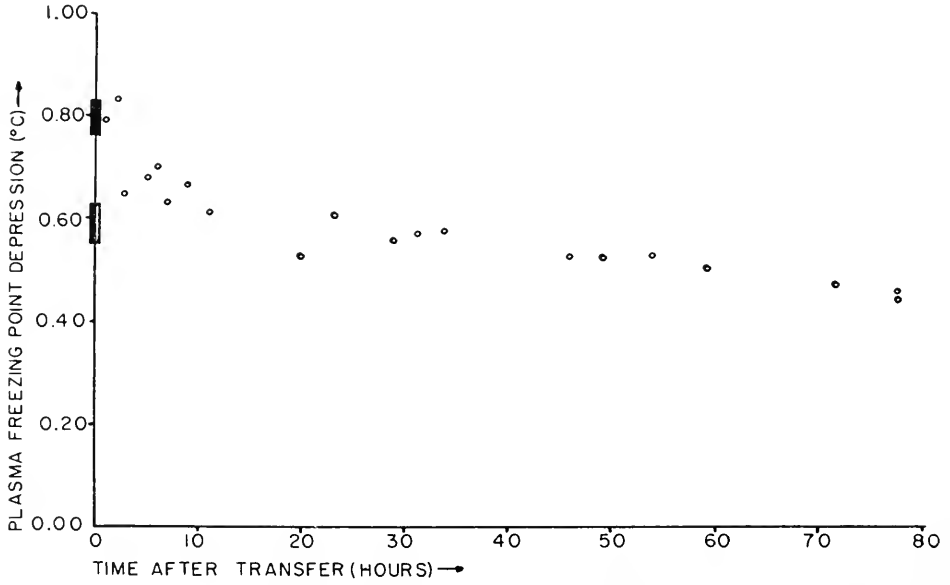

FIGURE 1. Changes of plasma freezing point depression with time following direct transfer from salt to fresh water of Arctic char (*Salvelinus alpinus* L.) from Hebron Fjord, Labrador. Ranges for fish naturally acclimated to salt water (black bar) and fresh water (black and white bar) indicated on ordinate. Temperature 5–10° C.

Following digestion with concentrated nitric acid and 30% hydrogen peroxide, duplicate chloride and potassium analyses were carried out. Methods were as above.

Precision of the freezing point determinations is ± 0.05° C. Plasma chloride analyses agreed within 1%, as did total muscle solids. Muscle chloride and potassium duplicates agreed within 5%.

RESULTS AND DISCUSSION

Figure 1 shows the behavior of total blood concentration in control and experimental char from Hebron Fjord. The total plasma concentration in Hebron char living in fresh water is about 25% lower than the salt water concentration. The char is thus like most other anadromous salmonids in this regard. Beginning

two hours after transfer from salt to fresh water, the experimental fish also decreased their plasma concentrations by about 25% over a period of approximately twenty hours. They then became fairly stable. It therefore seems that sudden transfer experiments duplicate at least some of the physiological events occurring during the migrations of these fish. This last might have been expected, since individual salmonids frequently make the transition from salt to fresh water and back again as rapidly as they can swim through the estuaries involved (Benditt *et al* 1941; Greene, 1910; Killick, 1955).

The continuing decrease in plasma concentration below the normal fresh water level which occurred in the experimental fish after about 35 hours in fresh water could have been the result of several influences. First, the fish were not fed; second, they may well have lost salt via their urine as a result of having been handled

TABLE I

Blood concentrations in arctic char

Source of fish	Plasma Δ_F (° C.)	Plasma $[Cl^-]$ (meq./l.)	Equivalent Cl^- Δ_F (° C.)*	Plasma $[K^+]$ (meq./l.)
Hebron Fjord, salt water	0.80	148	0.28	
	0.80	191	0.36	
	0.81	191	0.36	
	0.82	213	0.40	
	0.82	214	0.40	
	0.83			
	0.76			
Hebron Fjord, fresh water (land-locked)	0.59	144	0.27	
	0.61	144	0.27	
	0.63	161	0.30	
Churchill, salt water		177	0.33	5
		153	0.28	8
		156	0.29	1
		130	0.24	1
		132	0.25	2
Churchill				
2-hr. transfer		134	0.25	1
6-hr. transfer		124	0.23	8

* Equivalent $Cl^-\Delta_F$ calculated from: $\Delta_F = 1.86\ [Cl^-]$

(laboratory diuresis of Grafflin, 1931, 1935, and Forster, 1953); and third, their skin permeability, hence rate of water uptake in a hypotonic medium, may well have been increased as a result of loss of slime during handling.

The two Hebron char transferred back to the Fjord after the experiment, after fifteen hours in salt water, had plasma freezing points of $-0.75°$ C. This is essentially the original salt water value.

Table I summarizes the data on blood concentrations obtained from both Hebron and Churchill control fish and Churchill transfers. With the exception of the first salt water char from Hebron, the calculated equivalent Cl freezing point is essentially a constant fraction of the total freezing point (45–50%). More exact regulation of the concentrations of other plasma components is thus indicated. Similar behavior of chloride and total concentrations has been noted in brook and brown trout (unpublished data of the writer and van Dam). Fontaine, Callamand and

Vibert (1950), however, found a decrease of only 4% in plasma chloride in Atlantic salmon (*Salmo salar*) when total concentration dropped 13%.

The Churchill material generally supports the Hebron results. Plasma chloride concentration in the Churchill fish in fresh water for six hours is approximately 18% lower than the mean plasma chloride concentration for Churchill char in salt water. Plasma freezing point behaves similarly in the Hebron char. Note, however, that plasma chloride concentrations in the Churchill fish are generally much lower than in the Hebron fish. The mean difference of 20% seems too large to be the result of acclimation to differing salinities (the salinities differing only by 10%.). The marked differences between Hebron and Churchill char with respect to survival following transfer were noted earlier. Even allowing for the poor conditions encountered at Churchill it seems likely that real physiological differences exist between these populations. Marked differences in growth characteristics differentiating these two groups (Andrews and Lear, in press; Backus, 1952; Sprules, 1953; unpublished data of the author) also make this seem likely (though differences in food supply might well account for this last).

TABLE II

Muscle concentrations in Churchill arctic char

Source of fish	Total muscle solids (gm./kg. wet weight)	Muscle [Cl^-] (meq./kg. wet weight)	Muscle [K^+] (meq./kg. wet weight)
Salt water	276	22	123
	224	20	130
	266	8	120
	226	8	120
	260	6	125
2-hr. transfer	242	4	127
6-hr. transfer	244	3	150

Table II indicates that the one Churchill fish surviving transfer for six hours regulated total muscle concentration very well. Muscle potassium, however, seemingly increased markedly. The concentrations of the same muscle components in brook and brown trout under similar conditions behave very differently, however (data of the author and van Dam). In these other forms there is a close parallelism between changes in muscle concentrations and changes in the blood. Further work on the char is obviously needed.

In closing it should be noted that the blood and muscle potassium concentrations reported by Jones (1956) for fresh water brown trout agree very well with the figures given in Tables I and II for the Churchill salt water char (low plasma potassiums excepted).

SUMMARY

1. Adult Arctic char (*Salvelinus alpinus*), taken in summer from Hebron Fjord, Labrador, and Hudson Bay near Churchill, Manitoba, were transferred directly from salt to fresh water under fairly constant conditions.

2. Decreases in blood freezing point and chloride concentration of the order of 25% were found, the char thus being like most other anadromous salmonids in this

respect. The possibility of much better regulation of muscle concentrations is indicated.

3. Data are presented on plasma freezing point, chloride, and potassium, muscle solids, chloride, and potassium.

4. Physiological differences between populations of char are indicated.

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A COMPARATIVE STUDY OF THE GILL AREA OF CRABS

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In a comparative study of the gill area of marine fishes (Gray, 1954), it was shown that a definite correlation exists between the size of the gill area, the degree of activity, and the habits of the fishes concerned. It was found that sluggish bottom-dwelling species have proportionately much less gill surface than do fast swimming pelagic fishes. In this paper an attempt is made to find out if similar correlations exist in crabs from different habitats. Several correlations pertinent to the present discussion have already been pointed out by others. Ayers (1938) indicated that intertidal and land crabs consumed oxygen at a higher rate than did the strictly aquatic species. Pearse (1929a, 1929b, 1950) in his study of the emigration of animals from the sea has reported that there is a lessening of gill volume as crabs emigrate toward land. Pearse determined only the gill volume, not the gill area. More recently Vernberg (1956) has shown in a series of crabs that oxygen consumption of the whole animal and of gill tissue is highest in terrestrial species and decreases progressively as the habitat approaches ocean depths.

This paper presents the results of a study of the gill areas of sixteen species of brachyuran crabs from six taxonomic families, and representing both pelagic and benthic species, and those living below the low tide level, those of the intertidal zone, and those that live out of water most of the time.

Grateful acknowledgment is made to the Duke University Research Council for partial support of this research and to Miss Darlene Connor and Miss Barbara Galloway for the technical assistance.

Materials and Methods

The method used in the determination of gill area in crabs was similar to that employed in the determination of gill area in fishes (Gray, 1954). With crabs, however, since the gill platelets are so much larger, the procedure is somewhat less tedious. Each crab was weighed after first removing surplus water from the body and gill chambers with paper toweling. The gills were then removed from one side and each placed in a separate Petri dish. The total number of platelets for each gill was counted under a dissecting microscope or computed after measuring the length of each gill with vernier calipers and determining the average number of platelets per millimeter of length. After preliminary trials to observe the range of sizes, what appeared to be average size platelets from each gill were removed and mounted in sea water on slides. Using a dissecting microscope, camera lucida drawings were made of the selected platelets and the area of these determined by means of a planimeter. Knowing the magnification used and the total number of platelets, the total gill area could be readily calculated. A weak point in the pro-

cedure is that it calls for judgment on the part of the observer in selecting average size platelets for the camera lucida drawings. However, the method seems no less accurate and is far less cumbersome than the mathematical methods employed by Riess (1881), Putter (1909), and Price (1931) in estimating the gill areas of fishes.

Gill areas were obtained for the following species, listed more or less in order from the most land-adapted to the strictly aquatic: the ghost crab, *Ocypode albicans;* the wharf crab, *Sesarma cinerea; Sesarma reticulata;* the fiddler crabs, *Uca minax, Uca pugnax,* and *Uca pugilator; Panopeus herbstii;* the stone crab, *Menippe mercenaria;* the spider crabs, *Libinia dubia* and *L. emarginata; Hepatus epheliticus;* and five portunids, the blue crab *Callinectes sapidus, Areneus cribarus, Ovalipes ocellatus ocellatus, Portunus gibesii,* and *Portunus spinimanus.*

The spider crab, *Libinia emarginata,* was obtained at the Marine Biological Laboratory, Woods Hole, Massachusetts and collected from the north side of Cape Cod. All other species were collected in the vicinity of the Duke University Marine Laboratory, Beaufort, North Carolina.

Habits and Habitats of the Crabs

Of the crabs studied, the ghost crab, *Ocypode albicans,* is by far the most land-adapted. It may make its burrows in the dunes at considerable distances from the high tide mark. It spends very little time in the ocean and, in fact, cannot survive prolonged submergence. It can, however, withstand desiccation to only a very limited degree and in hot weather comes out mainly at night and feeds near the water's edge. The wharf crab, *Sesarma cinerea,* also spends most of its time on land, hiding out under the drift in the daytime and coming out to feed at the water's edge at night. Both *Ocypode* and *S. cinerea* are very active and move at a rapid rate when disturbed.

The fiddler crabs usually make their burrows near the high tide mark where they will be submerged for at least a portion of each day. The sand fiddler, *Uca pugilator,* is found in large numbers along the sandy protected beaches of the estuaries near the ocean. It appears from its burrows with the receding tide, migrates to the water's edge, and may be exposed on the moist sand of the beach for several hours. Of the fiddlers, *Uca minax* is farthest removed from the ocean, making its burrows in mud banks of the marshes and ditches, often quite far from the sea and where tidal effect is less than on the open beaches and where the salinity may be greatly reduced. It does not travel as far from its burrows as *U. pugilator* and is exposed for shorter periods of time. *Uca pugnax* occupies a somewhat intermediate habitat between that of *U. minax* and *U. pugilator* and tends to occupy the salt marshes. *Sesarma reticulata,* more robust and less active than *S. cinerea,* lives in mud banks with *Uca minax.*

Panopeus herbstii may be easily captured at low water in the lower intertidal zone of exposed oyster reefs, in crevices and under stones of rock jetties. The stone crab, *Menippe mercenaria,* can be found in crevices of rock jetties at and below the low tide level. *Panopeus* and *Menippe* are not as active as the fiddler crabs, but are much more active than *Libinia.*

Libinia dubia and *L. emarginata* are slow moving bottom species living well below the low tide level. These are the most sluggish of the crabs studied. In

marked contrast, another bottom-dwelling species, *Hepatus epheliticus,* is very active indeed and can dig rapidly in the bottom sand where it is prone to hide.

The portunid crabs are all active swimmers, quick in movement, and capable of rapid burrowing in the sandy bottom. The blue crab, *Callinectes sapidus,* invades both the open sea and the estuaries. *Areneus cribarus* frequents the area of surf along the outer beaches. *Ovalipes ocellatus, Portunus spinimanus,* and *P. gibesii* are small crabs commonly taken in shrimp trawls in 20 to 60 feet of water, but are also occasionally found in the estuaries.

Results and Discussion

Table I presents the gill areas and number of gill platelets per gram of body weight for the sixteen species of crabs, arranged according to families. In addition

Table I

Gill area and gill platelet number in crabs

Species	No. of determinations	Body weight grams			Platelet number			Gill area mm.²/gm.			Body vol.* Gill vol. ratio	Oxygen** μl./gm./min.
		Min.	Max.	Aver.	Max.	Min.	Aver.	Max.	Min.	Aver.		
Ocypodidae:												
Ocypode albicans	31	11.2	77.3	45.8	93	13	31	446	197	325	67.4	2.35
Uca minax	33	3.8	11.8	6.9	238	74	131	904	282	513	40.0	1.28
♂	21	4.1	11.8	7.9	199	74	115	701	282	482	45.9	
♀	12	3.8	6.7	5.1	238	117	159	904	402	567	34.1	
Uca pugnax ♂	5	1.0	2.5	2.1	739	306	487	889	658	770	57.1	
Uca pugilator ♂	7	1.6	3.0	2.3	431	241	321	817	455	624	60.3	2.03
Grapsidae:												
Sesarma cinerea	13	0.9	2.0	1.5	1178	567	830	874	480	638	63.3	2.21
Sesarma reticulata	8	7.1	11.0	8.9	208	138	183	749	493	579		
Xanthidae:												
Panopeus herbstii	38	3.3	43.0	19.2	430	27	135	1561	543	874	35.8	0.93
Menippe mercenaria	55	14.3	646.1	162.7	205	10	56	1532	386	887	33.7	0.51
Inchidae:												
Libinia dubia	12	14.8	392.0	147.2	215	10	48	1355	441	748	27.6	0.42
Libinia emarginata	26	32.3	640.8	194.9	80	6	31	1007	377	566		
♂	15	82.3	640.8	299.2	35	6	16	577	377	481		
♀	11	32.3	83.5	52.7	80	35	51	1007	535	682		
Callipidae:												
Hepatus epheliticus	6	12.1	91.8	44.3	198	28	84	1486	729	1099		
Portunidae:												
Portunus spinimanus	9	8.5	60.5	29.8	355	82	170	1107	816	901		
Portunus gibesii	5	6.2	15.3	10.3	462	212	321	1214	831	1003		
Ovalipes o. ocellatus	6	15.1	19.5	17.7	225	181	192	1512	1079	1288		
Areneus cribarus	3	70.0	220.2	122.0	65	25	53	1582	957	1301		
Callinectes sapidus	38	12.8	309.8	142.5	336	14	62	2038	734	1367	22.7	1.14

* From Pearse (1929a).
** From Vernberg (1956).

to the averages it is necessary to show the ranges of platelet number and gill area for, as will be shown, these vary inversely as the weight changes. Included also are the body volume-gill volume ratios of Pearse (1929a) for some of the same species, and, as an indication of metabolic activity, data on oxygen consumption from Vernberg (1956). Species averages of gill area within each family are relatively close, but family averages in some cases differ widely.

Pearse (1929a, 1929b) has shown that there is a tendency toward reduction in the number and volume of the gills as crabs emigrate from ocean over the beaches to land. There are sixteen gills in the wholly aquatic portunid crabs, compared to only

twelve in the Ocypodidae. Pearse also found that the ratio of body volume to gill volume varied from 22.7 in aquatic *Callinectes* to 64.7 in land-living *Ocypode*, with the more transitional species somewhere in between. Pearse did not determine gill area, which is more significant than gill volume. While it may apply in general it cannot be safely assumed that gill volume is necessarily in all cases an indication of gill area. The blue crab, *Callinectes*, and the spider crab, *Libinia dubia*, according to Pearse, have quite similar body volume-gill volume ratios (22.7 and 27.6, respectively) and for this reason might be expected to have similar gill areas, yet, as may be seen in Table I, the gill area of the blue crab is nearly double that of the spider crab.

Nevertheless, as is evident from Table II where the crabs, with their average gill areas, are arranged according to habitat, there is adequate support for Pearse's contention. There does appear to be a tendency toward reduction of gill area in those that spend part of their time on land compared to the strictly aquatic species. An aquatic crab with less gill area than an intertidal or land crab undoubtedly has lower metabolic activity. Obviously habitat is important in determining the needs

TABLE II

Crabs, with their average gill areas per gram, arranged by habitat

Aquatic		Low tide		Intertidal		Above tide	
Callinectes	1367	*Menippe*	887	*U. pugnax*	770	*S. cinerea*	638
Areneus	1301	*Panopeus*	874	*U. pugilator*	624	*Ocypode*	325
Ovalipes	1288			*S. reticulata*	579		
Hepatus	1099			*U. minax*	513		
P. gibesii	1003						
P. spinimanus	901						
L. dubia	748						
L. emarginata	566						

of respiratory surface. With an abundant oxygen supply crabs living part of the time in air do not need as much respiratory area as those living wholly in water, provided they can keep their gills moist. It is suggested that to prevent desiccation is the reason the ghost crab and the wharf crab are mainly nocturnal in the summer months. Both species have been known to succumb within fifteen minutes when confined on hot dry sand.

Habitat alone, however, does not account for the differences in gill area; rate of metabolism is also important. The portunids and the land crabs, *Ocypode* and *S. cinerea*, are very active and have high rates of metabolism. *Ocypode* has reduced gill area but at the same time has developed accessory respiratory structures for breathing in air. Ayers (1938; p. 526), comparing *Libinia, Menippe, Callinectes, Panopeus, Uca* and *Ocypode*, showed that in this series of crabs at Beaufort "there is an increase in O_2 consumption as the habitat approaches land and coincident with this there is an increase in activity of the crabs." While this holds true in a general way, Ayers' own figures show exceptions, for *Callinectes*, an aquatic species, has higher oxygen consumption and is far more active than the intertidal species. Probably the metabolic activity of *Callinectes* is indicative of that of the other

portunids. Also, judging by its gill area, *Hepatus*, a very active benthic species, probably has a metabolic rate equal to or above that of the intertidal species. *Libinia* is recognized as the most sluggish of the crabs studied by Pearse (1929a), by Ayers (1938), by Vernberg (1956), and in the present discussion. Its low oxygen consumption, as reported by both Ayers and Vernberg, is in keeping with its low gill area. Although the intertidal and land crabs do not need as great respiratory surface, even though their metabolism is higher, as do the wholly aquatic species, aquatic species with high metabolism, such as the portunids, need greater gill area than do aquatic species with low metabolism, like the spider crabs.

A question may be appropriately raised at this point. If it is an adaptation for an intertidal species to have reduced gill area for breathing in air, how does the crab

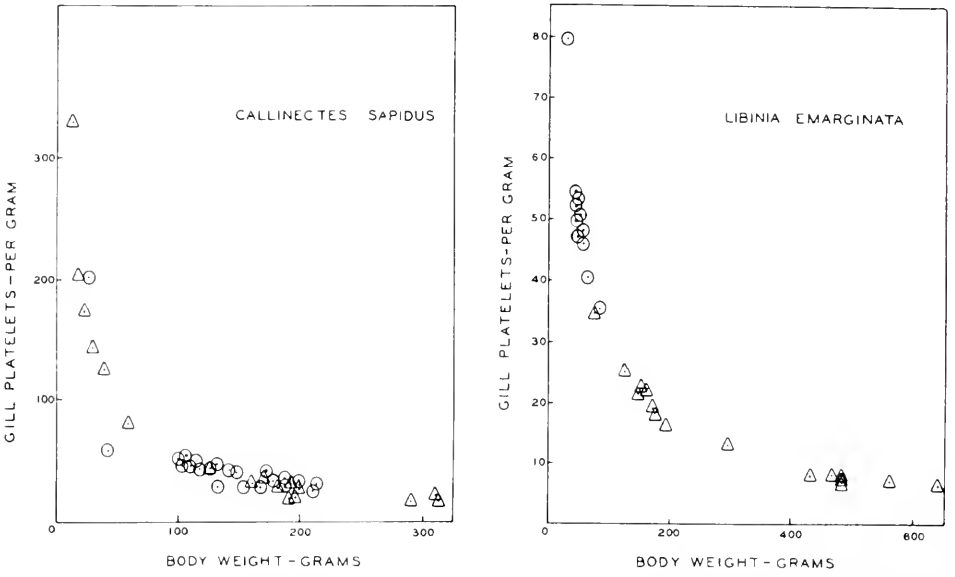

FIGURE 1. Relationship between the number of gill platelets and body weight in *Callinectes sapidus* and *Libinia emarginata*. Circles indicate females, triangles males.

survive when it is submerged? The fact that it does survive is an indication that the gill area is adequate and suggests that the crabs not only have enough gill surface for their normal needs, but enough for emergencies, too. Also, it has been demonstrated (unpublished data) that the crabs, *Callinectes* and *Panopeus* at least, go into a state of suspended animation for several hours when the O_2 tension is greatly reduced. This is long enough to carry them through a good part of a tidal cycle. In this connection, if one may be permitted to guess without supporting experimental evidence, it would be to predict that the oxygen consumption of fiddler crabs, while in their burrows with the tide covering them, is very low.

Presenting the gill areas of the various crabs as average amounts per gram of body weight suggests that the gill area per unit of measurement is relatively constant throughout life. This is not the case, however. Putter (1909) determined

the gill areas of a few crabs and fishes and maintained that the young had proportionately greater gill surface than did older animals. Krogh (1941) stated that while this was undoubtedly true Putter's work did not prove it. Figure 1, showing in *Callinectes* and *Libinia* how the number of gill *lamellae* per gram varies inversely with the weight of the crabs, and Figure 2, showing the decrease in gill area per gram of body weight with increased body growth in *Menippe* and *Libinia,* appear to substantiate the claim of Putter. Gill platelet number per gram, relatively high in very young crabs, falls off rapidly as the crabs grow older. The curves tend to level off in the older crabs. Evidently the addition of new platelets does not keep pace with the growth of the crab. Gill area per unit of weight, though more difficult to demonstrate as clearly, follows a somewhat similar pattern. Very young crabs have a greater gill surface per unit of weight than do the adults. Five small

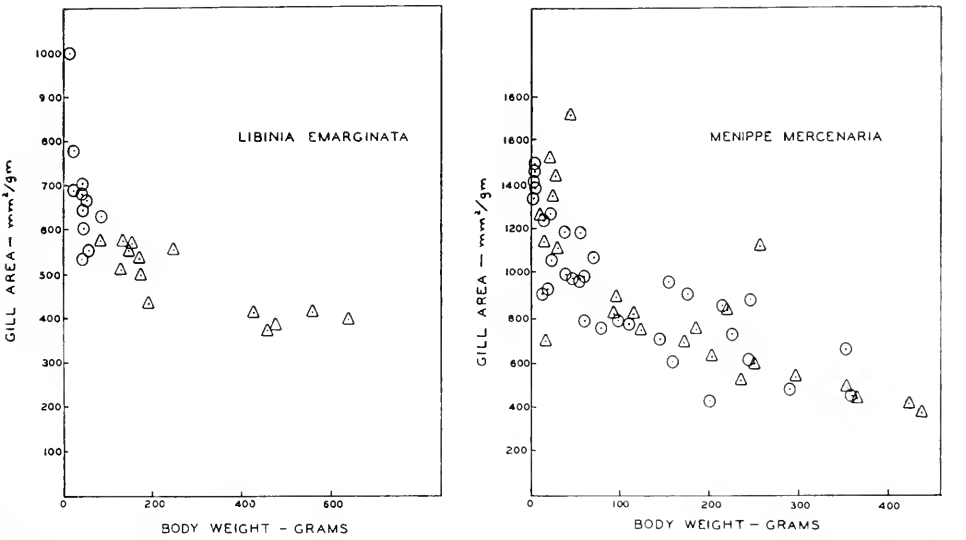

FIGURE 2. Relationship between gill area and body weight in *Libinia emarginata* and *Menippe mercenaria.* Circles indicate females, triangles males.

specimens of the genus *Menippe,* not included in Table I, weighing between 1.5 and 2.8 grams, had an average gill area of 1443 (range 1349–1496) sq. mm. per gram of body weight, whereas the gill area of individuals of the genus *Menippe* varying in weight from 10 to 600 grams averaged only about half this amount. Similarly, two crabs of the genus *Ocypode* weighing 1.0 and 2.5 grams had gill areas of 713 and 472 sq. mm. per gram of body weight, both values greater than the maximum for 31 crabs varying between 10 and 77 grams which averaged but 325 sq. mm. per gram. Though still apparent, the falling off of gill area per unit of weight is not as pronounced in older crabs as they increase in weight as it is in the young. The relative decrease in gill area is more easily demonstrated in large species that have great differences in weight between young and old than in the small species where individual variations may obscure the pattern.

It is quite possible that had Pearse (1929a) made enough determinations he

would have found that the body volume-gill volume ratio was not uniform for crabs of all sizes within the same species.

A factor deserving of comment is the percentage of inert skeleton. This differs among various species but is least in the fast-moving active land crabs, *Ocypode* and

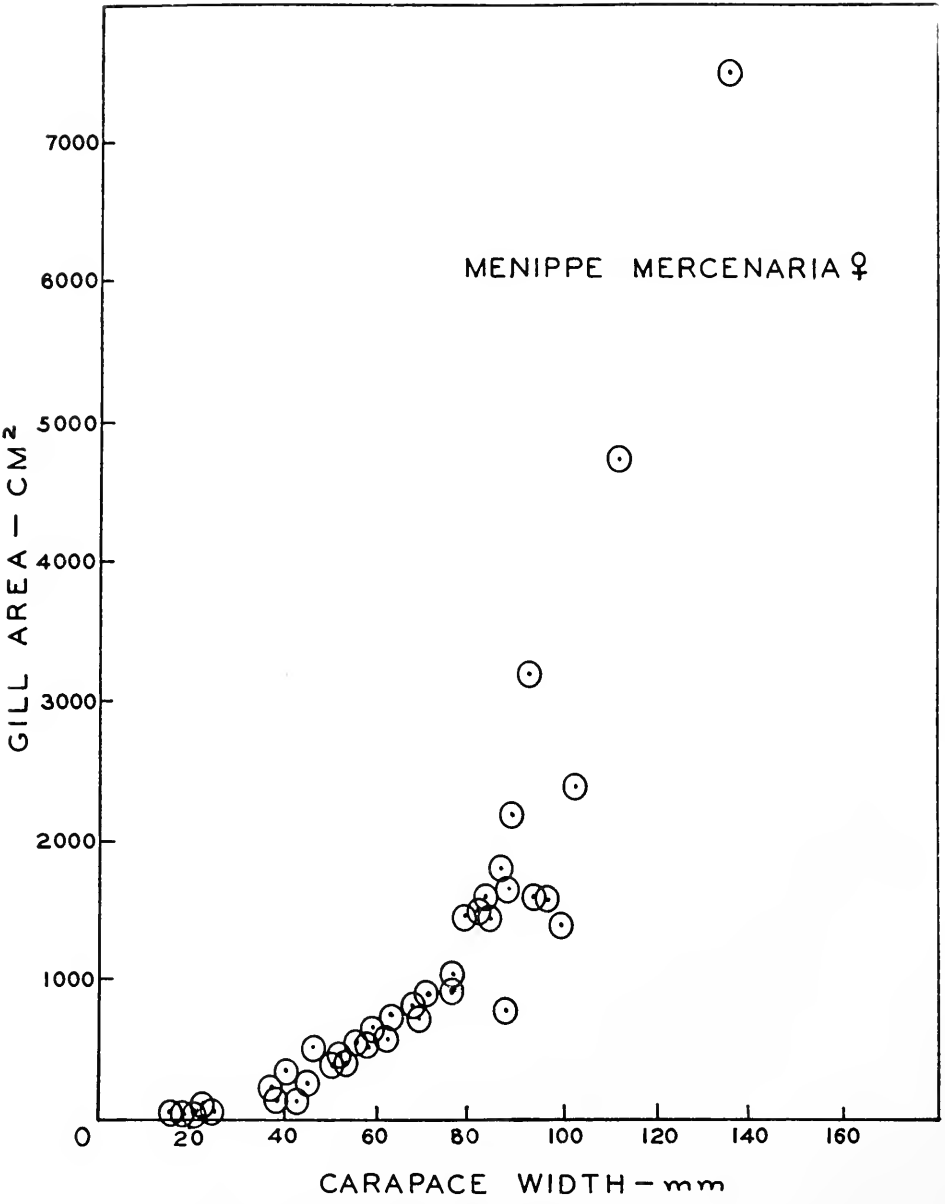

FIGURE 3. Correlation between the growth of the carapace and increase in gill area in *Menippe mercenaria.*

S. cinerea, and greatest among the heavier bodied, slower moving crabs, *Menippe* and *Panopeus.* Among the aquatic species the exoskeleton of *Callinectes* accounts for approximately 16 per cent of the total weight and that of *Libinia* about 22 per cent (unpublished data). The differences in skeletal weight, however, do not alone account for the differences in gill areas among the different species.

It may be argued that weight is not a satisfactory basis for comparing gill areas of crabs. This perhaps is true, but seems much more adequate than either body surface area or linear measurements which vary so greatly in different species. Within a species, however, linear measurements may be directly correlated with gill area. This is demonstrated in Figure 3, which shows the normal increase in total gill area of *Menippe* as the carapace increases in width.

There appears to be little or no sexual dimorphism among crabs as far as gill area or platelet number is concerned, except in those species where a major skeletal difference exists between males and females. In fiddler crabs, as illustrated by *Uca minax* (Table I), males, with greater weight because of the large chela not possessed by females, have smaller gill areas per unit of body weight than do females. In the spider crab, *Libinia emarginata,* males attain much larger size than females and may weigh several times as much. Per gram of weight the females have a greater number of gill platelets and larger gill area than do the males. It seems obvious from Figures 1 and 2 that these differences between males and females are not so much a matter of sex as of body weight. It has been found with other species, as well as with *Libinia,* that the relative number of gill platelets and the relative size of the gill surfaces decrease as the crabs grow larger and heavier.

Summary

1. A comparative study has been made of the size of the gill areas of 16 species of brachyuran crabs from six families and representing land, intertidal, and wholly aquatic habitats.

2. The size of the gill area is correlated with both habitat and metabolic activity.

3. There is a tendency toward reduction in gill area per unit of weight in going from wholly aquatic to intertidal to land species.

4. Among wholly aquatic species the active, fast moving crabs (portunids) have greater gill area than do the sluggish bottom-dwelling species (Libinia).

5. Both the gill area and the number of gill platelets per unit of weight, relatively high in very young crabs, decrease as the crabs grow older.

6. Apparent sexual dimorphism in gill area is a function of weight differences between the sexes.

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AN ANALYSIS OF RESPONSE TO OSMOTIC STRESS IN SELECTED DECAPOD CRUSTACEA

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Krogh (1939) and Prosser *et al.* (1950) have reviewed the subject of osmotic behavior in aquatic animals. Beadle (1943) has reviewed the importance of osmotic regulation in the evolutionary migration of marine animals to fresh water habitats. Pantin (1931) has discussed the origin of body fluids in animals. Robertson (1949, 1953) presents extensive information concerning ionic regulation among several groups of invertebrates. Höber *et al.* (1945) consider the physical chemistry involved in osmotic regulation. Jones (1941) showed that the crab *Pachygrapsus crassipes* regulates in dilute or concentrated sea water after 72 hours of immersion. However, osmotic regulation as a function of time for *Pachygrapsus* apparently has not been studied. This would seem to be an essential parameter in its ecologic importance, especially in the first few hours.

Salt and water pools have been suggested several times (Hukuda, 1932; Scholles, 1933; Beadle and Shaw, 1950; Gross, 1954.) The present investigation demonstrates that in the crabs studied, osmotic changes in the blood are brought about mainly by salt exchanges and not water. The presence of functional salt and water pools is considered.

Exoskeleton permeability is very unequal among decapods. Nagel (1934) found a correlation between regulating ability and permeability of the exoskeleton to applied sodium iodide in several crabs. The correlation is also established in the present study on the permeability for electrolytes and water, comparing six species of crabs and a crayfish.

The gills as seats of salt and water exchange and organs of regulation in crabs have been implicated mainly by eliminating other probable structures (Margaria, 1931; Nagel, 1934; Krogh, 1938). Webb (1940) produced histological evidence of a correlation in crabs between the ability to regulate and the complexity of the gills. Pieh (1936) demonstrated that isolated gills of regulating crabs show increased respiratory rates when exposed to osmotic stresses, thus suggesting increased work for regulation in these tissues. Koch *et al.* (1954) have produced direct evidence that *in vitro* the gills of *Eriocheir* can remove salts from a medium against a gradient. The work presented here offers direct evidence that the gill chamber of *Pachygrapsus* is a locus of electrolyte and water exchange, and that an osmotic gradient can be held in the chamber during an osmotic stress.

The energy expended for osmotic regulation has repeatedly been a subject of study by oxygen uptake determination. Schlieper (1929), Schwabe (1933) and Flemister and Flemister (1951) demonstrated that the metabolic rate increases when crabs are under osmotic stress. This they attributed to added osmotic work.

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Krogh (1939), Wikgren (1953) and Potts (1954) throw doubt on this interpretation.

The present study indicates that rates of oxygen consumption do not manifest increased osmotic work, but muscular activity.

Materials and Methods

The principal subjects for this investigation were three species of decapod Crustacea: (1) *Birgu latro* Linnaeus, the anomuran coconut crab, native of the Indo-Pacific region and an inhabitant of land, was collected on the island of Guam and maintained in the laboratory in Los Angeles. (2) *Emerita analoga* Rathbun, the common anomuran sand crab, found on sandy beaches burrowed in the sand near the level of the washing waves, was collected at Santa Monica and Corona Del Mar, California. (3) *Pachygrapsus crassipes* Randall, the brachyuran shore crab, found in high intertidal zones and in semi-terrestrial situations, was collected at Ballona Creek and Flat Rock Point, California.

The concentration of fluids was determined in two manners: (a) melting point method as described by Gross (1954), which permitted determinations on volumes as small as one $mm.^3$; (b) conductivity measurements using a 1000-cycle bridge. This allowed determinations on a two-ml. sample which was not necessarily expended but could be returned to the experimental vessel. Of course, this method measured only electrolytes. Units of resistance were converted to per cent of a standard sea water.

Oxygen consumption was measured by means of the Scholander-Wennesland respirometer as described in Wennesland (1951). All determinations were made at 16° C., a temperature to which the experimental animals were accustomed. Particular care was taken to assure that immersed animals were completely covered. No readings were made until the animal remained in the chamber for at least one hour; this was to allow them to become accustomed to the new environment.

Results

1. Osmotic Regulation as a Function of Time

Measurements of blood concentration during immersion in water were made throughout the range of water salinity, in which life could be sustained. The behavior of a species was determined partially from single readings on specimens exposed to certain stresses for given periods. However, regulation as a time function in individual specimens was followed over extended periods by two methods: (1) melting point determination on blood samples extracted periodically during immersion, and (2) periodic measurements of losses or gains of conductivity of the medium. A combination of these two methods was found best for studying individual responses over extended periods.

Emerita, a non-regulator

It was first established by melting point determinations that the body fluids of *Emerita* are isotonic to normal sea water (3.46% salt) and that this animal cannot sustain an osmotic gradient between its blood and external medium as a steady-

state condition. When three specimens were immersed in each of the following concentrations of sea water: 50, 75, 90, 110, 125, and 150% (total of 18 animals), their body fluids were isotonic to their respective external media within two hours or less after immersion. Thus *Emerita* shows no ability to regulate osmotically.

TABLE I

Solute space calculated from the relationship between concentration changes in the medium and the blood of the animal immersed in 5 times its volume of water

Specimen number	Medium (% sea water)	Change in blood (% sea water)	Change in medium (% sea water)	$\frac{\text{Change in blood}}{\text{Change in medium}}$	Calculated solute space (water equivalent) % body weight
			Emerita		
1	50	46.3	3.7	12.5	36
2	50	45.6	4.4	10.4	44
3	60	36.5	3.5	10.4	44
4	75	23.0	2.0	11.5	40
5	75	23.1	1.9	12.2	37
6	75	22.8	2.2	10.4	44
7	125	23.2	1.8	12.9	38
8	125	23.1	1.9	12.2	37
9	150	45.3	4.7	9.6	47
10	150	46.3	3.7	12.5	36
Mean				11.5	40
			Pachygrapsus		
1	25	22.6	2.7	8.4	50
1	25	15.3	1.9	8.1	51
2	50	13.7	2.0	6.9	60
3	39	13.2	1.8	7.3	57
4	50	14.7	1.9	7.8	53
5	50	14.2	1.8	7.9	53
6	50	9.5	1.3	7.3	57
7	125	10.5	1.4	7.5	56
8	145	7.9	1.0	7.9	53
9	150	15.8	2.0	7.9	53
Mean				7.7*	54

* $S = \frac{5/7.7}{1.2} \times 100 = 54\%$.

Individual specimens which had never been removed from normal sea water, were then immersed in dilute or concentrated media of 5 times the volume of the animal. Then the electrical resistance changes in the medium were followed until the resistance was stabilized and here the body fluids could be considered to be isotonic to the medium. The change in the blood concentration is therefore equal to the difference between the final concentration of the stress medium and the concentration of normal sea water. If, then, a constant ratio between osmotic changes in the blood and medium could be established, a conductivity variation in the medium

could be interpreted in terms of a change in the blood concentration, so that at any time the osmotic pressure of the blood could be estimated by such conductivity readings. Data in Table I demonstrate that such a ratio is relatively constant over a wide range of osmotic stresses, the mean value showing a change in the body fluids equivalent to 11.5% sea water for each 1% sea water change in the medium.

The rate of approaching equilibrium with the external environment suggests a physical, diffusion phenomenon. The curves are indicated in Figure 3. However, there are individual variations which result particularly with size, the smaller animals reaching equilibrium first.

Exploratory experiments suggested that *Callianassa affinis, Upogebia* sp., *Cancer antennarius, C. gracilis,* and *Pugettia producta* behave similarly.

Regulating forms immersed in water

Pachygrapsus in normal sea water is not necessarily isotonic to the medium. (Note the variation in initial blood concentration in Figure 1.) Specimens were immersed in stress media of five times their respective volumes and salinity changes in the medium were repeatedly noted by the conductivity bridge. After a significant change in the medium was observed, a melting point determination was made on the blood.

As with *Emerita,* a relatively constant ratio between conductivity change of the external medium and osmotic pressure change in the body fluids of the animal was demonstrated (Table I). On the average, a change in the external medium equivalent to 1% sea water meant a change in the blood equivalent to 7.7% sea water.

Therefore, if the initial or final blood concentration of *Pachygrapsus* were known, conductivity measurements of the medium could be converted to represent the approximate osmotic pressure of the blood.

The crabs were able to tolerate the small volumes of medium for long periods, if moved to a fresh medium of the same conductivity at least every three hours.

Over extended periods the rate of regulation in an individual specimen of *Pachygrapsus* could be estimated by converting changes in the medium to body fluid concentrations assuming the above mean ratio of change in blood to change in medium. Occasional checks were made by melting point determinations of the blood. Subsequent deviations did not exceed 10%. Such an error could not obscure a trend. When readings were not needed at close intervals, the specimen was placed in a large volume of the desired salinity and after an appropriate period a melting point determination was made on the blood. This was done on most of the late readings (*e.g.,* 72 hours). Figure 1 illustrates osmotic regulation in *Pachygrapsus* as a function of time. It should be pointed out that a small error is introduced by using the conductivity method, for when a change occurs in the medium, the osmotic gradient is consequently reduced. In the extreme case where a change in the blood was equivalent to 40% sea water, about a 5% error was effected. However, since the conductivity method was used for brief periods, not exceeding 12 hours, osmotic changes detected were small and the consequent errors caused by reducing the osmotic gradient were usually insignificant.

As shown in Figure 1, *Pachygrapsus* can regulate osmotically in both hypotonic and hypertonic media. This confirms the work of Jones (1941). However, while Jones showed this to be true after 72 hours, the present investigation shows that

regulation is established immediately, and is sustained perfectly by some specimens in moderate stresses for a few hours. Regulation then diminishes gradually until equilibrium is reached, usually within 24 hours. However, several plateaus and steps on the osmotic behavior curves may be produced before equilibrium is finally reached. Equilibrium in the case of *Pachygrapsus* does not mean that the body fluids are isotonic to the external medium. Rather, it means that the blood of the animal has reached a steady state with respect to osmotic pressure.

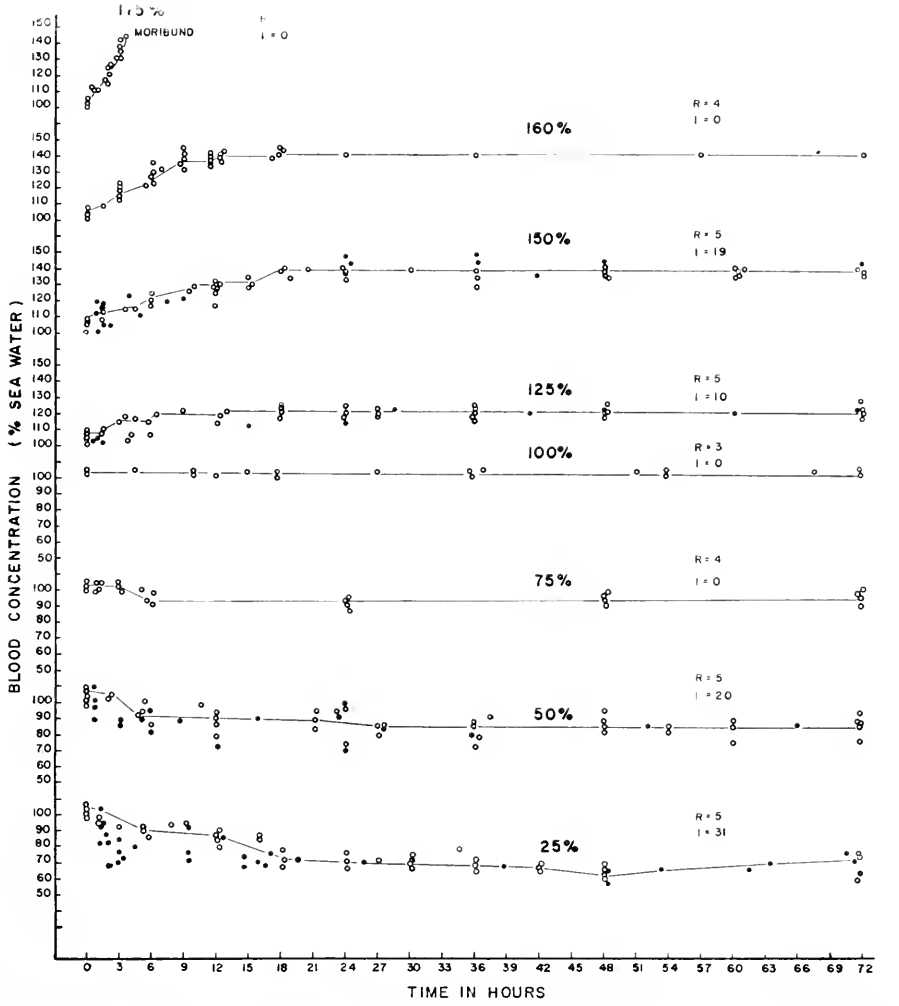

FIGURE 1. Osmotic regulation in *Pachygrapsus* as a function of time. "R" represents those specimens whose behavior was followed for extended periods by repeated determinations and is indicated by open points (○); "I" represents those specimens on which only one determination was made and is indicated by solid points (●). Solid line represents approximate median and is drawn through points representing the actual behavior of one specimen. All points are indicated unless coinciding with others. Concentration of the medium is indicated in per cent of normal sea water above the respective curve.

The plateaus and steps on the osmotic behavior curves reflect grading of the active regulatory processes or changes in accessory processes, *e.g.*, water movement over the gills. Only occasionally do fluctuations or dips occur. Such behavior is shown in Figure 1 by a crab immersed in 25% sea water.

A high degree of individual difference is demonstrated by the wide spread of points and the varying slopes among individual histories. Such variations could be caused by differences in size, age, sex, metabolic rate variances caused by physiological periodicities, *e.g.*, molting cycle, or external environment changes.

Concurring with Jones (1941), it was demonstrated that *Pachygrapsus* regulates better in hypotonic media than in hypertonic media. This phenomenon becomes evident from (a) the period of perfect regulation, (b) the slope of the regulation curve, (c) the total change in the concentration of the blood at equilibrium and (d) the extreme osmotic stresses endured by the crabs. These lines of evidence become apparent with inspection of Figure 1. However, it is worth mentioning that no animal lived in 175% sea water for as long as 6 hours. Yet several crabs actively survived 25% sea water for 72 hours. Prosser *et al.* (1955) demonstrated relatively strong tolerance to 170% sea water by *Pachygrapsus*. However, their experimental animals were gradually acclimated to lesser stresses before immersion in 170% sea water (personal communication).

There seems to be variation in the blood concentration of *Pachygrapsus* living in normal sea water. Jones (1941) reports that the body fluids of *Pachygrapsus* are hypotonic to normal sea water. Pearse (1931) found the body fluids of this crab hypertonic to normal sea water. As can be seen from Figure 1 the crabs used in the present investigation were usually hypertonic to their external medium when they were immersed in normal sea water. Such variations possibly can be explained by the fact that the osmotic pressure of the blood is a function of the molt cycle (Baumberger and Olmsted, 1928).

Token experiments suggested that *Uca* and *Hemigrapsus* regulate similarly to *Pachygrapsus*. Confirming the work of Jones (1941), *Uca* was found to regulate more strongly in hypertonic and hypotonic sea water than *Pachygrapsus*. In the salinity ranges from 50% sea water to 150% sea water, this form maintained almost perfect regulation for 36 hours.

While Jones (1941) found that *Hemigrapsus* could regulate strongly in dilute sea water, he was unable to show regulation in concentrated sea water after 72 hours immersion. The present investigation revealed that this crab can regulate up to 33% perfectly for 20 hours in 150% sea water.

Osmotic regulation in the land crab Birgus latro

The implications of osmotic regulation in the land crab *Birgus latro* have been discussed by Gross (1955). As mentioned there, these anomurans will drown when completely immersed for a day or so. It was therefore necessary to allow all the animals to rise slightly out of the water for exposure of their respiratory membranes to air. By this operation they partially released themselves from the imposed osmotic stresses. However, most of the external surface was immersed all of the time. Because of the limited number of specimens, only one specimen of *Birgus* could be studied in the representative stresses which were: 25, 50, 66 and 137% sea water, respectively (four specimens). In these cases the changes in the

osmotic pressure of the blood were followed by repeated melting point determinations on blood samples extracted at chosen times. Results are illustrated in Figure 2. These data show at least that *Birgus* is a strong regulator in both dilute and concentrated sea water. The moribund condition of the crabs after prolonged immersion in 25% and 137% sea water is not believed to be the direct result of the osmotic changes, since the two blood concentrations, specifically 70 and 119% sea water, are readily tolerated by this species (Gross, 1955). Anoxia seems to be a more satisfactory explanation for the moribund condition of these two specimens; however, high oxygen tensions failed to revive them. It is perhaps pertinent that

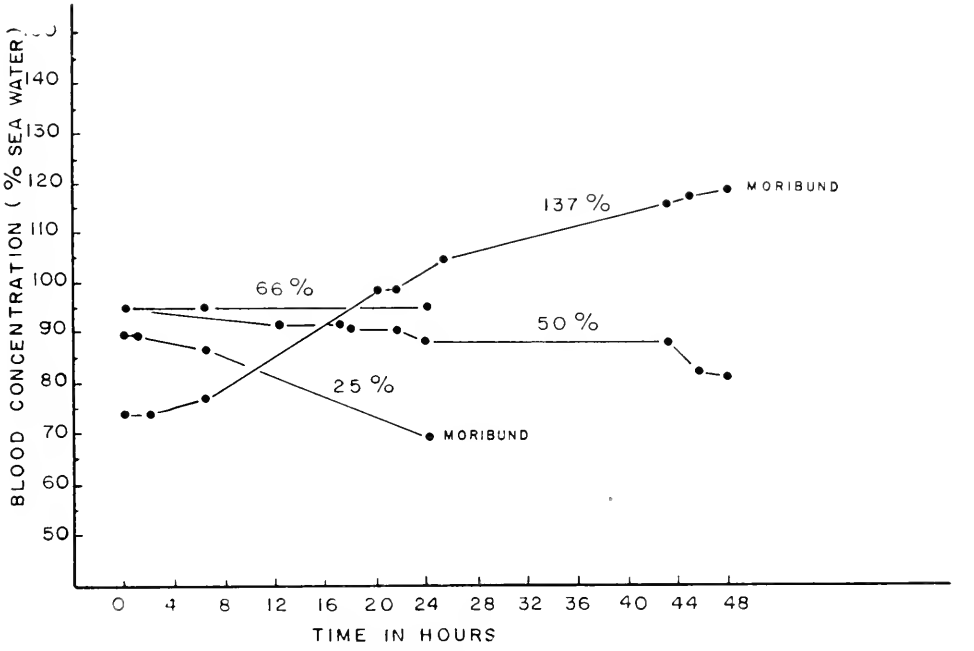

FIGURE 2. Osmotic regulation in *Birgus* as a function of time. Behavior of four specimens demonstrated by repeated melting point determinations on the blood. Concentrations of the medium are indicated in per cent of normal sea water over the respective curves.

the two animals exposed to the greatest stresses were most affected, but the fact that *Birgus* can regulate in concentrated sea water corroborates the observation that hypo-osmotic regulation is common among crabs showing some degree of terrestrial behavior (Jones, 1941; Gross, 1955).

Salt exchanges

When *Emerita* and *Pachygrapsus* are immersed in dilute or concentrated sea water, the osmotic pressure of their body fluids changes but corresponding weight changes under these conditions are small. In the case of *Emerita* a change in the body fluids equivalent to 25% sea water resulted in a weight change of less than 2% of the body weight. If pure water, this could effect a concentration change in the

blood of less than 6% on the assumption of 40% of the body weight being osmotically active water. This of course means that changes in the concentration of the body fluids are effected mostly by net changes in the solute content rather than water; 20% of the total concentration change of the blood was caused by water and 80% by solutes, in this case.

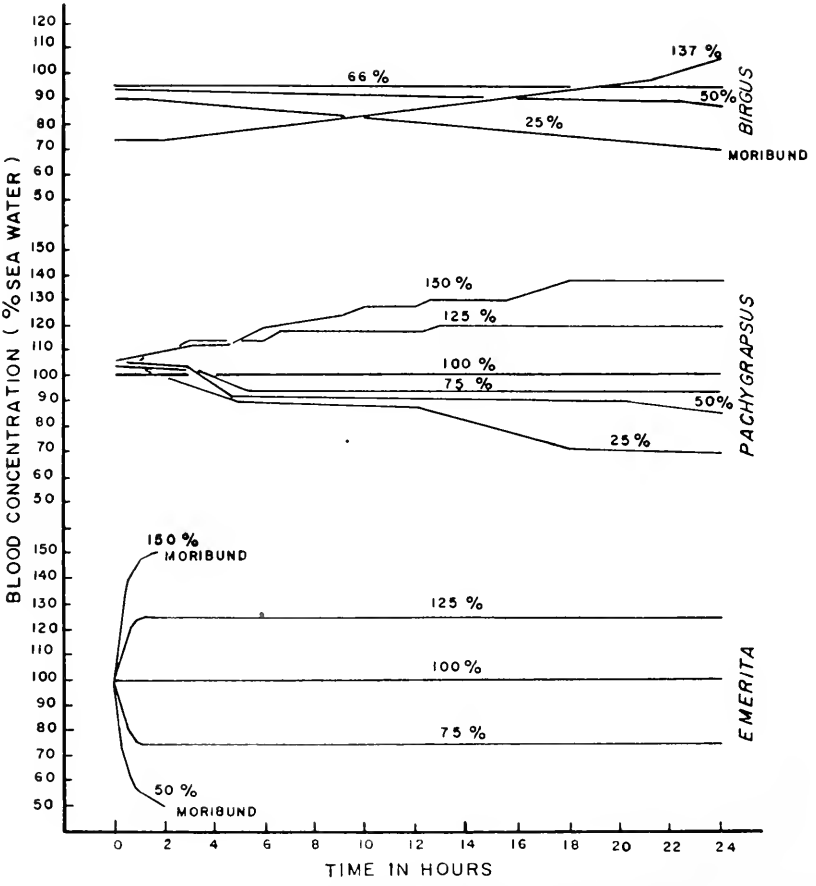

FIGURE 3. Comparative osmotic behavior of *Emerita, Pachygrapsus* and *Birgus*. Solid line indicates behavior of individual specimen which, in the case of *Emerita* and *Pachygrapsus,* represents the approximate average behavior of all individuals investigated for a given osmotic stress. All cases of *Birgus* are illustrated. Medium concentrations are indicated in per cent of normal sea water over the respective curves.

Curves for *Emerita* uncorrected for salt losses to the medium.

On the other hand, weight changes in *Pachygrapsus* during prolonged immersion in dilute and concentrated sea water deviating 75% from normal were so small they could hardly be considered significant. If water does cause changes in the blood concentration of these crabs, its effect is small. These findings are in accord with the work of Hukuda (1932).

Now the relatively persistent ratio between the concentration change in the blood and the external medium (Table I) suggests a method for estimating values for solute space which can be calculated from the equation:

$$S = \frac{v/p}{d} \times 100,$$

where S = solute space (equivalent in water) in per cent body weight,

$$v = \frac{\text{volume medium}}{\text{volume of specimen}},$$

$$p = \frac{\text{change in body fluids}}{\text{change in external medium}},$$

d = specific gravity of specimen.

Mean solute space values thus calculated were 54% body weight for *Pachygrapsus* and 40% body weight for *Emerita* (Table I). There was no evidence of a trend; that is, the values for solute space did not vary with different magnitudes of change in the osmotic pressure of the body fluids. Thus, if salt pools contribute to the osmo-regulatory mechanism they probably exert a constant effect over the range tested. That is, there is no varying degree of salt fixation or mobilization with increased osmotic stress, a phenomenon suggested by Hukuda (1932) and demonstrated by Gross (1954) in sipunculids.

It should be borne in mind that the above values for solute space are approximate and probably good only for the specific conditions of the experiment, for it is known that blood concentration and total water content change during certain phases of the molting cycle (Baumberger and Olmsted, 1928).

When solute space was estimated on two specimens of *Birgus*, values were 44 and 56% body weight, respectively, data which were subject to considerable error because changes in the external medium by evaporation could not be properly corrected.

2. The Osmo-Regulatory Mechanism

Jones (1941) and Prosser *et al.* (1955) have shown that the green glands of *Pachygrapsus* are ineffective as osmo-regulatory organs. Confirmatory evidence was established in the present investigation when the urine of 5 crabs immersed for 24 hours in 50% sea water was shown to be isotonic to the blood in all cases. Other regulatory mechanisms will be examined in the following section.

Osmotic regulation and permeability of the exoskeleton

Nagel (1934) demonstrated in several decapod Crustacea that non-regulators have more permeable exoskeletons than regulators. An attempt was therefore made to detect a correlation between the ability to regulate osmotically and the permeability of the exoskeleton in *Cambarus clarkii*, *Pachygrapsus*, *Hemigrapsus nudus*, *H. oregonensis*, *Cancer antennarius*, *C. gracilis* and *Pugettia producta*.

Since it was desired to know the role of exoskeleton in osmotic regulation of the different species, it was necessary to test the permeability of equal areas of exoskeleton of animals of about the same size. Discs of exoskeleton approximately three

cm. in diameter were removed from the carapaces of freshly killed animals. Each disc, hypodermis removed, was fitted against a rim of the end of a screw sleeve, then screwed into the end of a glass tube so that the chitinous disc formed the bottom surface (both inside and out) of the glass tube. The opening at the end of the screw sleeve determined the area of the chitin to be exposed, and was uniform for all cases. The tube was then filled with 10 ml. of normal sea water and the chitinous end was immersed in another tube containing 10 ml. of 50 per cent sea water. While the normal sea water inside the smaller tube simulated the body fluids of an animal, the dilute sea water in the larger tube simulated the external medium. The salt change in the dilute medium was then measured by a conductivity bridge after 24 hours to determine the relative permeability. As a check against leaks of water between the rim of the sleeve and disc, a few drops of concentrated dye were placed in the sea water and when such a leak occurred the dye appeared in the outside dilute medium. There was no intentional agitation and conditions for the different species were essentially uniform. Individuals chosen were apparently not close to molt.

Since the salt concentration in the dilute medium increased measurably in all cases, it can be said that the exoskeleton in all species studied is permeable either to salts or to water. If the exoskeleton were permeable only to water, then the volume of fluid in the tube which originally contained 100 per cent sea water should increase; this was never observed. The salinity changes through the exoskeleton of regulating crabs were small. However, if distilled water is used in the outside medium instead of 50 per cent sea water, a 5 per cent change can be effected in the concentration of the inner medium within 24 hours, using samples of *H. oregonensis*. This would afford a detectable volume change were semi-permeability the nature of the exoskeleton, but no volume change was observed. Thus it can be said that the exoskeleton of all non-regulators studied, and at least *Hemigrapsus oregonensis* among the regulators, is permeable to both salts and water. The average permeability of three samples of exoskeleton from each species is expressed in Figure 4. The values should be slightly higher than indicated because the osmotic gradient was reduced as salinity changes occurred. Such differences, however, would be insignificant for regulators.

It can be seen from these results that there is a correlation between regulating ability and exoskeleton permeability. The animal which probably endures the highest osmotic stress in nature, *Cambarus,* shows the lowest permeability value, although sufficient determinations were not made to state that this is significantly different from the values for *Pachygrapsus* and *H. nudus*. The non-regulator which demonstrates the lowest permeability, *C. gracilis*, still shows a value which is three times that of *H. oregonensis*, which has the highest permeability among the regulators.

Pachygrapsus is probably the best regulator among the crabs and it shows the lowest permeability value, but whether the correlation is this good cannot be said on the basis of the evidence available. Too few cases have been used to place weight on the apparent difference between *H. nudus* and *H. oregonensis*. The differences among the non-regulators do not seem to be adaptive, since these forms are stenohaline.

In order to determine whether or not the chitinous exoskeleton shows greater permeability in one direction, a section of the exoskeleton of *Pachygrapsus* was tested as above, first measuring the conductivity changes in the outer medium of

distilled water after four hours when the inner medium is normal sea water, then reversing the media, so that the sea water is on the outside, and measuring the salinity increase in the inner medium of distilled water after four hours. Permeability on three samples seemed approximately equal in both directions.

An interesting question arises as to whether regulating and non-regulating crabs differ in their active mechanisms. Could *Pachygrapsus,* for example, regulate in

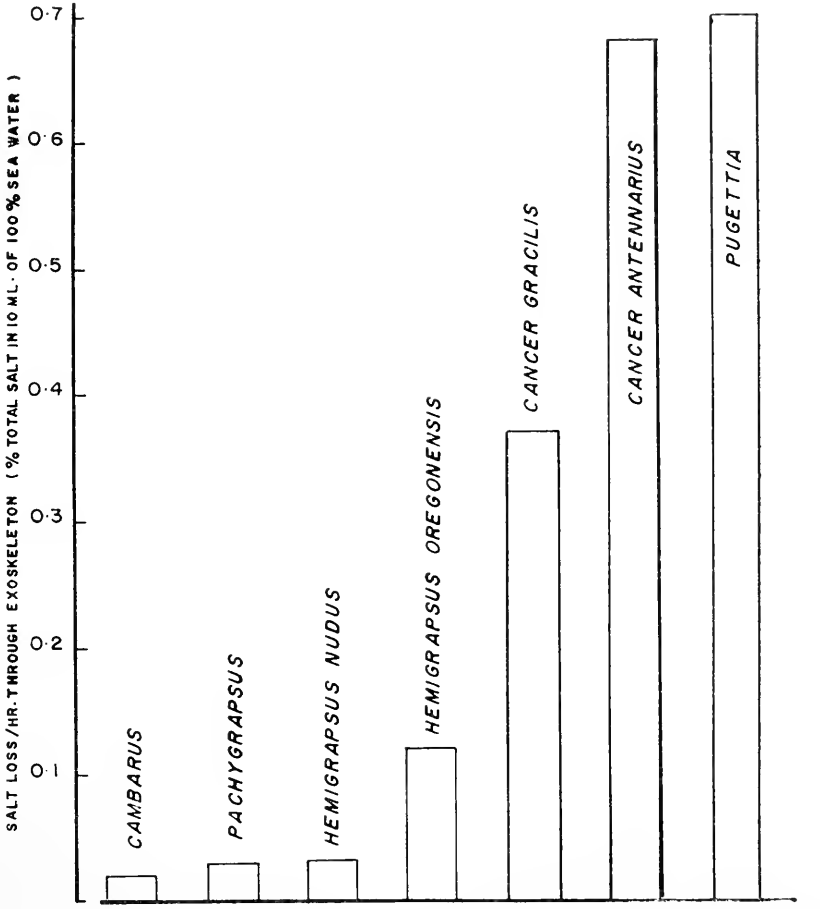

FIGURE 4. The relative permeability of exoskeleton in several decapod Crustacea. Values represent the means of three determinations for each species with a 50% sea water gradient. Variation among the three determinations for each species was in no case more than 25%.

dilute sea water if it were as permeable as *Cancer?* Much evidence is available demonstrating that animals which cannot regulate osmotically are strong ionic regulators (Prosser *et al.,* 1950). It may be that in the case of crabs, the mechanism for active, osmotic regulation is present and functioning in the non-regulators, but the permeability of the exoskeleton is so great that an osmotic gradient cannot be sustained. No evidence has been obtained on this point with respect to crabs but it

has been shown in the case of poikilosmotic sipunculids that salts can be removed from the medium without benefit of a gradient (Gross, 1954).

Osmotic regulation in the gill chamber

It is generally believed that the gills of marine crabs function as osmotic regulatory organs. Koch *et al.* (1954) have produced direct evidence in *Eriocheir* supporting this view. Gross (1955) demonstrated that the gill chamber of *Pachygrapsus* is a site of salt exchange under conditions of desiccation. As one way of testing whether this is more generally true, the salinity changes which occur in the branchial cavity of crabs removed from water were measured after the cavity was filled with water of varying salt content. It was previously shown that no significant changes occur under similar conditions when the gill chambers are filled with normal sea water (Gross, 1955).

First, the volume of fluid held in the chambers was estimated by a method reported previously (Gross, 1955). Crabs whose gill chambers had been pierced for flushing were immersed in tap water, 25% sea water or 150% sea water for a period of one hour; each cavity was then flushed out with 10 ml. of distilled water, the flushings were caught and the salinity of this fluid was measured by means of a conductivity bridge. Assuming that the gill fluid is the same concentration as the medium in which the crab was immersed and knowing the volume and salinity of the flushings, the volume of the branchial fluid can be estimated as well as the absolute quantity of salt in that fluid.

The animal was then immersed in normal sea water for about two hours to permit recovery. Again it was returned to its respective stress medium for an additional period of one hour. The crab then was removed from the water and placed in a closed container which was kept at saturated humidity by a paper towel soaked in the same medium from which the animal had been removed. Such paper was placed where the crab could not reach it. After two to seven days the animal was removed from the container and its gill chamber flushed out with distilled water, the salinity of this fluid being measured. If the latter salinity determination differed from the former for the same crab, it was suggested that salt exchanges occurred in the branchial chamber. Such was the case, for all animals with dilute branchial fluid showed an absolute salt increase after the period in the humid container, while all animals with concentrated branchial fluid showed an absolute decrease. It should be said, however, that the crabs which had been immersed in 150% sea water showed some signs of desiccation.

It is not surprising to find salt exchanges in the gill chamber, for as described above, even the chitinous exoskeleton is permeable to both salts and water. However, if it could be demonstrated that the salinity of the branchial fluid were different from that of the blood when a steady-state had been reached, then it would seem that a dynamic mechanism is present in the gill chamber, permitting an exchange of salt with the body fluids, but also being able to hold an osmotic gradient.

Now, the volume of branchial fluid for each animal was determined with the first flushing operation described above. Thus, the salinity of the second flushing will yield an estimation of the branchial fluid concentration after the period in the humid container. Results suggest that a gradient is sustained in the gill chambers of all animals which had been immersed in dilute media. The mean salinity of such

fluid from two animals which had been immersed in tap water was equivalent to 14% sea water while the blood was estimated to be greater than 75% sea water in concentration. The mean salinity in the gill chamber of four animals removed from 25% sea water was equivalent to 64% sea water while the blood concentrations were estimated to be greater than 85% sea water. Data from the specimens which had been immersed in 150% sea water were considered invalid for this aspect of the experiment.

It might be argued that the apparent gradients described above are not real, but that a great amount of branchial fluid rests on impermeable tissues. In such a case, the fluid in contact with the permeable tissues could become isotonic with the blood and this would affect the total salt content in the gill chamber only enough to give the impression that an osmotic gradient was being held. However, the large apparent gradient sustained when the branchial fluid was initially tap water, and the large salt exchange under conditions at desiccation (Gross, 1955), do not support this argument. Since all animals remained out of the water for at least two days before the salinity of their branchial fluid was measured, there was plenty of opportunity for salt exchanges to take place. It is probable, therefore, that the above described gradients are real, but the fact that more salt change occurred for animals from 25% sea water than for those from tap water raises doubt as to the quantitative value of the data, since the osmotic gradient between blood and gill chamber was greater for the tap water animals.

One more point of interest arises from this experiment. It was previously estimated that the mean branchial fluid volume for 20 *Pachygrapsus* was 1.7% body weight (Gross, 1955). The mean branchial fluid volume of those animals immersed in 150% sea water was only 0.71% body weight. Yet, again, those immersed in dilute sea water averaged 1.7% body weight. This, of course, suggests that *Pachygrapsus* has some control over the water that enters the gill chamber, which would be an aid to osmotic regulation. If such an aiding mechanism for regulation exists, it seems that it functions only for hyper-regulation, but confirmation is needed for this finding.

Metabolic work in increased osmotic stresses

It has been reported that when certain crabs are exposed to osmotic stresses, they respire more rapidly; this increase in metabolism has been interpreted as work accomplished by the regulatory mechanism (Schlieper, 1929; Schwabe, 1933; Flemister and Flemister, 1951). However, Krogh (1939) suggested that such metabolic increases are caused in part by activities of the organism other than osmotic regulation.

I have observed that certain crabs, *e.g., Pachygrapsus,* show violent attempts to escape from a medium which departs much from normal sea water in concentration. Obviously such activities would step up the entire metabolic rate of the crab, making it difficult to show the increased rate due to osmotic regulation alone.

The necessary increase in the osmo-regulatory mechanism cannot be predicted simply by knowing the normal osmotic pressure of the body fluids of an animal and the osmotic stress imposed. Rather, a knowledge of the sustained osmotic gradient is needed to give the relative amount of regulation necessary to withstand a certain stress. It is conceivable that a given amount of osmotic work will main-

tain the body fluids at viable concentrations over a range of osmotic stresses. This could be possible if the animal holds a constant gradient between blood and external medium, although the actual blood concentration alters to permit the constant gradient. Over such a range of stress, then, the constant rate of regulation would be

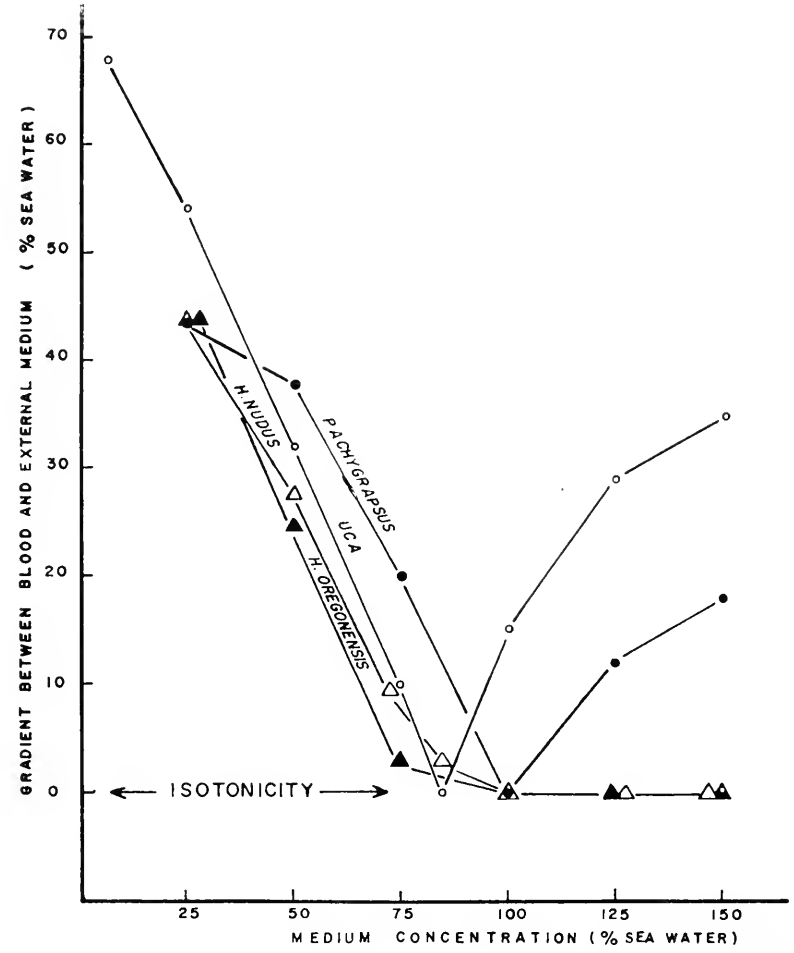

FIGURE 5. Crabs sustain greater osmotic gradients at equilibrium as greater stresses are imposed. Points are approximate mean values and for *Pachygrapsus* are calculated from the present investigation; values for *Uca*, *H. oregonensis*, and *H. nudus*, are calculated from Jones (1941).

expected to cause no variations in the total metabolic rate assuming constant relative roles of the various regulatory organs. On the other hand, if an increasing gradient is established more work would be expected. It has been pointed out recently by Potts (1954) that semi-permeable animals inhabiting fresh water could maintain their blood hypertonic to the medium with less work by excretion of dilute urine than by regulating at the tissue surfaces which are exposed to the external medium.

However, it was also demonstrated that such a mechanism would not be significantly advantageous over the other unless large osmotic gradients were sustained between the blood and external medium (greater than 50% sea water). The possibility should therefore be considered that at certain stresses the mechanism of regulation may shift from one process to another, and that the various processes are not neces-

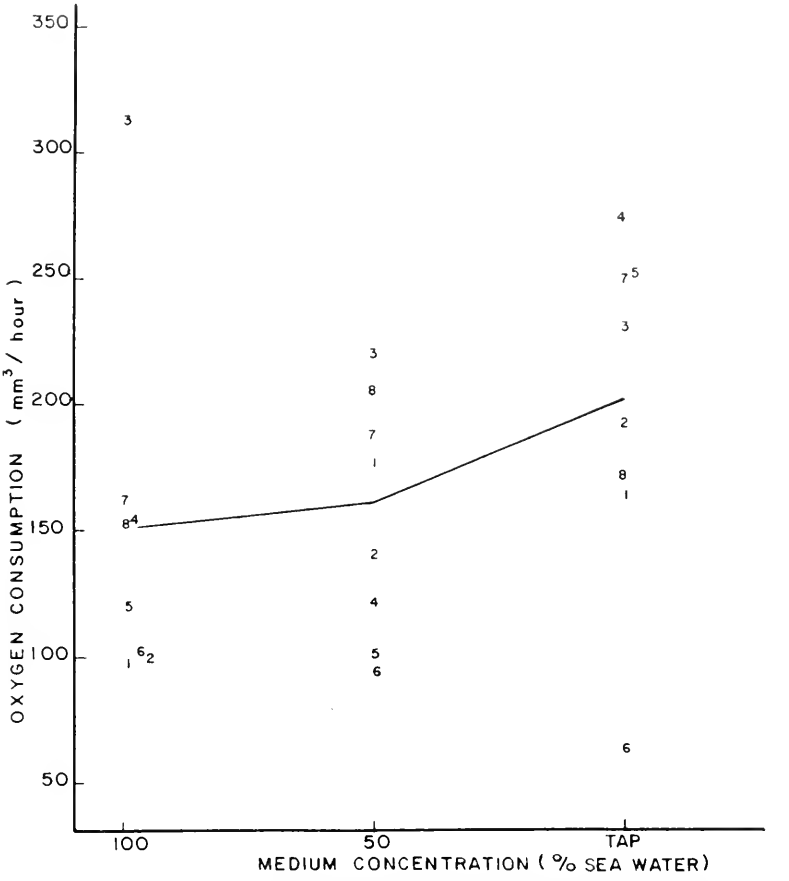

FIGURE 6. Oxygen consumption in *Uca* as a function of osmotic stress. Numerals represent individual specimens. Solid line connects mean values for all cases. Measurements made at 16° C. on crabs of approximately two grams. These could not stand out of the solution.

sarily equally efficient. Nevertheless, it does not seem to be unreasonable to assume that in general more metabolic work is required to sustain a large osmotic gradient than to sustain a small osmotic gradient.

Figure 5 demonstrates that in *Uca, Pachygrapsus, Hemigrapsus nudus,* and *H. oregonensis,* more osmotic work would be expected in greater stresses because the sustained gradients increase with the larger stresses, except for the two species of *Hemigrapsus* when they are immersed in concentrated sea water where they cannot regulate. It can be observed in *Pachygrapsus* that the sustained gradient in-

creases almost linearly with the stress, from a medium of normal sea water up to one of 50% sea water; but in 25% sea water the gradient drops off from the linear relationship with stress. This indicates that the crab must work only slightly faster, and it can be seen that with the same metabolic output an equal gradient could be held in even more dilute water. *Uca* shows almost a linear increase in the gradient up to a medium of 25% sea water and only a slight fall off from this relationship to stress in 5% sea water. *Hemigrapsus nudus* and *H. oregonensis* show behavior in dilute sea water which is somewhat different from *Pachygrapsus* and *Uca,* for they regulate weakly in 75% sea water, sustaining less than a 10% sea water gradient. With like stresses *Uca* and *Pachygrapsus* hold 22% and 20% sea water gradients, respectively. However, as the stress increases both species of *Hemigrapsus* regulate strongly, as illustrated by the increased slopes for these crabs in Figure 5.

If the additional osmotic work could manifest itself, the animals would be expected to consume more oxygen as the medium departs farther from the osmotic pressure of the body fluids. The oxygen consumption of individual specimens of *Uca* was compared when the crabs were immersed in normal sea water, 50% sea water, and in tap water. Since this species may be found commonly in normal sea water, its respiratory rate was measured first in this medium, assuming that harmful effects would be at a minimum here. For the next determination, half the animals were tested in 50% sea water, the other half in tap water. For the third determination the order was reversed so that each specimen was studied in three media. This was a control against permanent injury which might result from the heavy stress imposed by tap water. However, the order of immersion did not seem to make a difference. After determinations in 50% sea water and tap water, the crabs were placed in 100% sea water for 24 hours to allow recovery before the next determination was made. All specimens whose behavior is recorded lived in sea water for at least 48 hours after completion of the experiment. Results in Figure 6 illustrate that the metabolic rate of an individual animal does not necessarily increase with osmotic stress. In fact, of 8 animals, only numbers 2 and 7 show such a behavior. With the exception of two extreme cases, however, number 3 in normal sea water and number 6 in tap water, the respiratory rates for the group of animals seem to be higher in higher stresses. The mean of the 8 specimens shows a value for oxygen consumption in 50% sea water of about 108% of what it is in normal sea water, while in tap water it is about 135% of the value for 100% sea water.

Assuming that the normal osmotic pressure of *Uca* blood is equivalent to that of 85% sea water, there will be a 15% sea water gradient when the crab is immersed in normal sea water; in 50% sea water there will be a maximum gradient of about 35% sea water, and in tap water a maximum gradient of 85% sea water. When the animal is exposed to an osmotic stress, the amount of work should increase by the same factor as does the increased sustained gradient. When immersed in 50% sea water, regulation might increase by 35–15 or 20 units. The mean respiratory rate in 50% sea water shows 8% increase over animals immersed in normal sea water; thus, the fraction of total metabolism responsible for regulation in sea water might be calculated by simple proportion to be 6.0%. In tap water, this value is calculated to be 7.5% of the total metabolism.

These results do not agree with those of Flemister and Flemister (1951) who

studied another crab, *Ocypode*. Flemister and Flemister regarded the increased respiratory rate in hypertonic and hypotonic sea water as a manifestation of greater chloride regulation, and probably osmotic regulation. If this is the case, then about the same amount of metabolic work serves to maintain almost perfect regulation in any medium which establishes a gradient of from 25% to 50% sea water, yet in an isotonic medium, the metabolic rate is at least 20% lower. Could it be that osmotic regulation in *Ocypode* is an all-or-none process which is triggered by a salinity change in the medium? *Uca* is also a member of the family Ocypodidae, and its habitat is similar to that of *Ocypode*. It would seem questionable, that the osmo-regulatory mechanism of these closely related organisms could be essentially different.

It is this author's opinion that the interpretation of increased respiratory rates of animals subjected to osmotic stresses as a manifestation of greater osmotic work is to be questioned. This opinion is based on the inconsistent results found in individual animals in the present investigation (Fig. 6) and on the knowledge from numerous observations on a number of species that crabs are sensitive to osmotic stress and attempt to escape.

Discussion

The osmotic responses of the species studied in this investigation are not always obviously adaptive. The stenohaline forms represented by *Emerita* are presumably limited in their choice of habitat by their tissue tolerances, since they are unable to regulate. Yet, this species can tolerate salinities of from 75% sea water to 125% sea water for at least 24 hours, suggesting that the dilute waters of estuaries and the concentrated waters of tide pools might partially afford a refuge for them. It would seem to be the habits of this animal that dictate that it live near the level of the washing waves, rather than its lack of osmotic regulation.

The strong osmo-regulatory ability of *Pachygrapsus* and *Birgus* superficially seems superfluous in animals of their habits. *Pachygrapsus* generally is found in normal sea water where it does not have to regulate, or on land where it is subjected to desiccation, not direct osmotic forces. *Birgus* is found usually on dry land except for periods when it returns to the sea to reproduce. Gross (1955) discusses the significance of the ability to regulate among terrestrial and semi-terrestrial crabs, and suggests that in nature where osmotic stresses in aqueous media are rarely encountered it is of little importance as such, but appears under the artificial conditions of experimentation, *i.e.*, osmotic stresses, as a secondary manifestation of physiological processes important for life on land. It is interesting that the regulators *Pachygrapsus* and *Birgus* show large tolerances to variations in their blood concentrations.

The immediate resistance to osmotic stresses demonstrated by regulating animals may be a matter of inertia permitted by relatively impermeable exoskeletons, for the present investigation has shown a correlation between osmo-regulatory ability and integumental impermeability. The latter significantly contributes to the time factor which is important to intertidal animals which are subjected to tidal rhythms. In the case of *Hemigrapsus*, hypo-osmotic regulation for 20 hours would be adequate, because it would be rare for this animal to be exposed to the air for such a period.

Pantin (1931) has said that non-regulatory animals may posses the same mecha-

nisms permitting osmo-regulation as do regulators but to a lesser degree. It may be, in view of the above findings, that the difference between a regulator and a non-regulator could be merely a difference in permeability.

Previous investigations have produced evidence that the gills are major osmo-regulatory organs among marine crabs. The present investigation has established that a dynamic flux of salts and water occurs in the gill chamber of *Pachygrapsus*. Although no direct evidence has been produced, it is probable that the gill tissues are capable of actively transporting salts. However, the regulating mechanism may not lie entirely at a tissue level, for there is evidence suggesting that stress media can be partially excluded from the branchial chamber, therefore preventing contact with the gill tissues in large volumes.

The green glands of *Pachygrapsus* are probably not important organs of osmo-regulation, because the urine is close to the concentration of the blood in various concentrations of sea water. Prosser *et al.* (1955), however, have produced evidence that the green glands are important ion regulators. Potts (1954) has shown theoretically that a semi-permeable animal would not gain much regulatory efficiency in excreting a dilute urine, unless extreme stresses were encountered. However, the crabs used in this study are not semi-permeable. In fact, net changes in the osmotic pressure of the blood were shown to be effected mainly by salts, not water.

The relatively constant values for solute space which were calculated from the ratio of concentration changes in the blood to concentration changes in the external medium have interesting implications, namely, that if salt pools are contributing to the blood changes occurring under osmotic stress, they are doing so in a constant manner. The question remains open, whether or not a given alteration in the blood can be accounted for in the external medium.

When regulating crabs are exposed to increasing osmotic stresses, they generally sustain greater osmotic gradients. This would necessitate greater osmotic work. However, increased rates of respiration which are registered by crabs when they endure an osmotic stress cannot be interpreted as the manifestation of that increased work, for other activities, stimulated by the stress, *e.g.*, struggle to escape, cannot be isolated. It may be possible that greater osmotic work alone does not add appreciably to the total metabolic rate.

This investigation was conducted under the direction of Professor Theodore Holmes Bullock, to whom I am grateful for his most helpful encouragement and guidance. I am also indebted to Professor C. L. Prosser and Dr. J. D. Robertson for reading parts of the manuscript and offering helpful suggestions.

Summary

1. The decapod Crustacea, *Emerita, Callianassa, Upogebia, Cancer antennarius, C. gracilis* and *Pugettia* cannot regulate osmotically and from lack of tolerance are generally stenohaline.

2. *Pachygrapsus, Birgus, Hemigrapsus* and *Uca* can regulate osmotically in concentrated and dilute sea water. *Hemigrapsus* is the weakest hypo-osmotic regulator of the four species.

3. Among species where osmotic regulation occurs, it is established immediately and may be long lasting or may grow weaker with time. Although blood concentrations may fluctuate in a given stress, the phenomenon is not common.

4. Equilibrium of the blood concentration, where perfect regulation does not occur, is usually established within 24 hours following immersion; changes occasionally occur later when extreme stresses are imposed.

5. Estimates on the solute space volumes were calculated as 40% for *Emerita*, 54% for *Pachygrapsus* and about 50% for *Birgus*.

6. Concentration changes occurring in the blood of *Pachygrapsus* and *Emerita* are caused mostly by salt rather than water exchanges.

7. There is a dynamic flux of salt and water in the gill chamber of *Pachygrapsus*, thus furnishing further evidence that the gills are osmo-regulatory organs.

8. The osmotic regulating crustaceans *Cambarus, Pachygrapsus, Hemigrapsus nudus* and *H. oregonensis* have less permeable exoskeletons than the non-regulators, *Cancer gracilis, C. antennarius* and *Pugettia* by a factor of at least three.

9. *Pachygrapsus, Uca, H. nudus* and *H. oregonensis* sustain greater osmotic gradients when greater osmotic stresses are imposed. The two species of *Hemigrapsus* are weak regulators in small stress, but the osmo-regulatory activity accelerates as stresses increase. A sensitivity to absolute salinities is suggested.

10. *Uca* averages greater respiratory rates in greater osmotic stresses, but this is not necessarily so for individual specimens and the average differences are small. A discussion of the energetics of osmotic regulation reaches the conclusion that such increases in metabolism are not direct reflections of increased osmotic work but of muscular or other activity.

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STUDIES ON FEEDING, DIGESTION, AND FOOD STORAGE IN FREE-LIVING FLATWORMS (PLATYHELMINTHES: TURBELLARIA)

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Investigations upon turbellarian nutrition (summarized by Hyman, 1951 and Yonge, 1954) have so far dealt mainly with triclads, where intracellular digestion has been demonstrated but the possibility of some supplementary intraluminar digestion not fully explored. Triclad nutrition has therefore been re-examined, using *Polycelis cornuta,* and the opportunity taken to make comparable investigations on representatives of the other turbellarian orders. In each case the nature of the food and the feeding mechanism, the structure of the gut and the course of digestion, and the nature and location of the food reserves have been studied. The various mucoid and rhabdoid secretions have also been examined and an assessment made of their value in feeding.

Materials and Methods

The following Turbellaria, listed systematically with details of their habitat, were examined.

Order Acoela. *Convoluta paradoxa,* Oersted. From rock pools rich in *Ulva* and *Enteromorpha* in Cullercoats Bay, Northumberland.

Order Rhabdocoela. Sub-order Notandropora. *Macrostomum* sp., Oe.

Sub-order Opisthandropora. *Stenostomum* sp., Duges. Both from ponds in the Leeds area.

Sub-order Lecithophora. *Mesostoma tetragonum,* O. F. Müller. From Middlerigg Tarn, Troutbeck, Westmorland.

Order Tricladida. Sub-order Paludicola. *Polycelis cornuta,* Schmanda. From under stones in small fast-running streams in the Leeds area.

Order Polycladida. Sub-order Cotylea. *Cycloporus papillosus,* Lang. On colonial tunicates (*Botryllus* and *Botrylloides*) beneath weed-covered rocks at low-water mark on St. Mary's Island, Northumberland.

Sub-order Acotylea. *Leptoplana tremellaris,* O. F. M. Beneath stones on a predominantly sandy shore at Llanfairfechan, North Wales.

The flatworms were starved to encourage a readiness to feed and to clear the gut lumen and the gut cells of any remnants of previous meals. The animals associated with the flatworms under natural conditions were then presented to them so that the methods of capture and ingestion of the selected prey could be followed in detail. Flatworms were killed where possible in the act of feeding, Steinmann's fixative (equal parts concentrated nitric acid, distilled water and saturated mercuric chloride in 5% aqueous sodium chloride) giving the necessary instantaneous fixa-

tion, and after washing in running water the preparations were stained in borax carmine.

The natural food was used, whenever possible, to determine the site and course of digestion, but in forms with a phagocytic gastrodermis (especially the triclad) both this and the soft foods used by previous investigators (Arnold, 1909; Willier, Hyman and Rifenburgh, 1925; Kelley, 1931) disintegrated during ingestion and it could not be ascertained whether any intraluminar break-up preceded the phagocytosis and intracellular digestion, nor whether the separate constituents of the food (proteins, carbohydrates and fats) were dealt with differentially. Hence the flatworms were fed either on food which reached the gut in a visibly recognizable condition, or on homogeneous food substances whose digestion could be detected by simple chemical tests. Some of these were readily eaten, but with others the natural prey had to be used as a carrier. This particularly useful technique was applied by extracting the body contents of a normal prey with a hypodermic syringe and replacing them with the appropriate food substance. In all cases the flatworms were fixed at intervals after an observed feed, and examined histologically. All fixation (in Susa, saturated mercuric chloride or 10% formalin) was carried out at about 40° C. to prevent rupture or discharge of the gut contents. Sections cut at 10 μ were stained with Ehrlich's haematoxylin and eosin, Heidenhain's iron haematoxylin and Feulgen's stain. Squash preparations were also made, either directly in 0.5% saline or after preliminary maceration in saturated aqueous boric acid, and examined both fresh, and fixed and stained. In cases where ingested food was visible within the living flatworm its fate was also followed by direct observation.

Fat reserves were studied from squashes fixed in osmium tetroxide vapour and from sections prepared after fixation in Flemming's fluid. Carbohydrate reserves were studied after fixation in 95% alcohol, the sections being stained with Best's carmine for glycogen or periodic acid-Schiff's reagent for carbohydrates generally. For protein reserves flatworms were fixed in neutral 10% formalin and the sections stained by the modified Millon method (Bensley and Gersh, 1933).

Special methods for the study of pH changes during digestion and the examination of the mucoid and rhabdoid secretions are described in the text.

Observations

TRICLADIDA. *Polycelis cornuta*

The food and feeding mechanisms

P. cornuta (1–1.5 cm. long and 2–3 mm. broad) feeds upon small annelids, crustaceans and insect larvae, particularly those of Ephemeroptera, Plecoptera and Diptera. It is also a scavenger and feeds upon injured or dead animals, provided they are not too decomposed—juices diffusing from such animals attract all the flatworms in the vicinity and they can be collected in great numbers by anchoring a crushed earthworm in the stream as bait.

The living prey may be seized directly but is often caught after it has become entangled in the mucus produced for locomotion and adhesion by the *Polycelis* population in general. The normal slow movement by ciliary gliding is facilitated by mucus secretions which are laid down in tracts comparable to the slime trail of a snail; this mucus is not particularly sticky but small animals are occasionally trapped

in it. Often, however, ciliary locomotion is supplemented by direct muscular movement in which waves of contraction pass down the body and urge it forward. During such movement, and particularly when the flatworm is facing a current or changing levels upon a plant or stone, mucus of the type usually used for adhesion when at rest is produced from the edges of the body to provide temporary anchorage during the muscular contractions. This mucus is very sticky and as the flatworm moves it is drawn out into strands 2–3 cm. long which stretch between stones and leaves or along a level substratum. The many single strands produced in this way tend to cross or coalesce to form a complex tangle which becomes a most effective trap for small animals. Such deposits occur all over the natural habitat, and can be easily demonstrated in laboratory culture dishes by flooding with weak eosin (Fig. 11). *Polycelis* is gregarious and this habit results in a large amount of mucus being deposited within a given area. Animals with many appendages, such as crustaceans and insect larvae, are easily trapped in these mucus "snares" and become still more entangled in their struggle to escape. The flatworm does not lie in wait near the "snares" but is rapidly attracted by any disturbance in the water created by the struggles of the prey. Several individuals are often attracted to the same prey and their concerted efforts enable them to feed upon animals too large for a single individual. The prey is seized by the flatworm wrapping itself about it and covering it with large amounts of the sticky mucus. In this way even relatively large animals such as gammarids are rapidly immobilized. There is no evidence of any toxic effects of the mucus since prey rescued after complete immobilization show no ill effects. Inert food does not stimulate the formation of much mucus; only the amount necessary for adhesion is secreted when such food is being eaten. After the prey is seized the pharynx is protruded and makes exploratory movements over the body surface. With arthropods it is eventually forced through some weak point in the exoskeleton, as between sclerites or the area of articulation of a limb, and rapidly sucks out the body contents (Fig. 10). The pharynx is very extensible and moves about within the prey, entering the head and larger appendages to draw out all the organs and musculature, so that virtually only the empty exoskeleton is finally left. With annelids the cuticle is breached and the body of the worm extracted, whilst with suitable carrion minute pieces are sucked off by the pharynx. The disintegration of the food on its way to the gut is so rapid that it appears to be due entirely to mechanical disruption during its passage through the pharynx; there is no evidence that digestive juices assist the process since the waves of muscular contraction in the pharynx invariably pass backwards towards the gut, never forwards as would be expected if such juices were being regurgitated. The efficacy of the process is indicated by the homogenized condition of the food as it arrives in the gut lumen.

The structure of the gut and course of digestion

The large cylindrical plicate pharynx lies in the pharyngeal chamber in the posterior half of the body and is directed backwards so that it can be protruded through the posterior central "mouth" by simple muscular elongation (Fig. 1). It is very muscular and contains both eosinophilic and basophilic mucus glands whose secretions help ingestion.

The gastrodermis (Figs. 12 and 14) consists of a single layer of cells resting upon a delicate basement membrane. There are two types of cells, of which the

larger and more numerous are columnar, 35–40 μ in height with basal vesicular nuclei; their distal ends often bulge freely into the gut lumen and their basophilic cytoplasm is finely granular and may contain various eosinophilic inclusions. These inclusions disappear progressively after feeding and hence appear to represent phagocytosed food undergoing intracellular digestion. The second type of gut cell, later referred to as "sphere cells" on account of their appearance, is 20–30 μ high and normally contains intensely staining spheres which appear to constitute a protein reserve.

The finely divided condition and mixed nature of the normal food after ingestion made it difficult to determine whether phagocytosis was preceded by any intraluminar break-up, and experimental feeding by the carrier technique was applied.

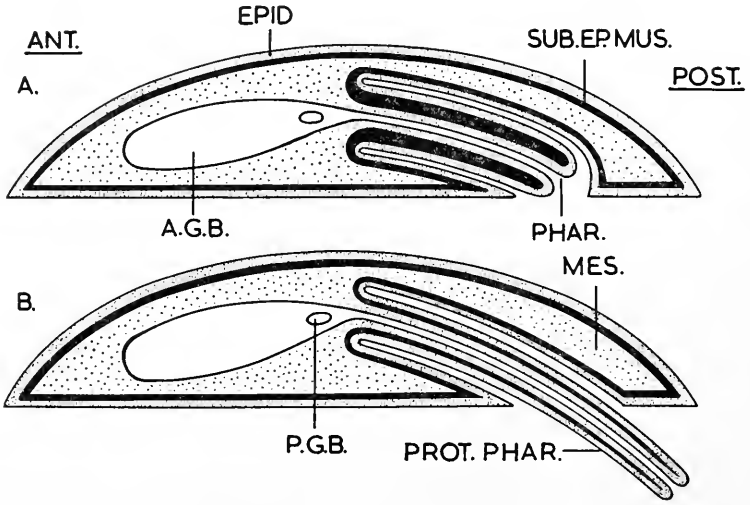


FIGURE 1. Diagrammatic longitudinal sections of *Polycelis* to show the cylindrical plicate pharynx. A, The normal condition with pharynx retracted. B, Pharynx protruded for feeding; ant.: anterior; a.g.b.: anterior gut branch; ev.phar.: everted pharynx; m.g.b.: median gut branch; mes.: mesenchyme; phar.: pharynx; post.: posterior; p.g.b.: origin of posterior gut branches; prot.phar.: protruded pharynx; sub.ep.mus.: subepidermal muscles.

The carriers used in this case were gammarids which were boiled before injection to ensure that no active enzymatic juices of the crustacean remained to attack the test food. The series of test foods was chosen with three objectives: (a) to follow the separate fates of carbohydrates, proteins and fats; (b) to supply unequivocal evidence of the presence or absence of intraluminar digestion; and (c) to determine the maximum size of particle which can be phagocytosed by the gut cells.

(1) Experimental feeding with carbohydrates

Polycelis readily ingested boiled starch paste, and sections prepared immediately after feeding and stained with Lugol's iodine showed the blue and unchanged starch lying in the gut lumen. The paste was quickly phagocytosed and twelve hours after feeding only a small amount remained in the lumen, quite unaltered and still stained blue with Lugol, indicating the absence of any intraluminar digestion of carbohy-

drates. The intracellular digestion of the phagocytosed starch was followed in saline squashes of fed flatworms. Two hours after feeding the columnar gut cells contained large amounts (Fig. 13), which stained with Lugol in various shades of blue and brown. As time passed all stained brown and then eventually disappeared as digestion was completed. Starch was also found in mesenchymal cells so that a certain amount of phagocytosed material passes back into the mesenchyme for digestion. Indicators mixed with the starch paste were unchanged in the gut lumen, giving further evidence of the absence of intraluminar digestion, but as the pH value after phagocytosis was 6.8–7, intracellular digestion of carbohydrates occurs in a neutral to slightly acid medium.

Raw starch grains were ingested and readily phagocytosed if sufficiently small, and could be seen very clearly in sections by polarized light in the columnar cells. They were unaffected by the intracellular diastatic enzymes and were eventually rejected by the cells and returned to the gut lumen. In this case, therefore, no capacity for the selection of suitable food material appears to be possessed by the gut cells themselves. On the other hand, no grains above about 30 μ were taken up and apparently this is the critical size above which phagocytosis is impossible. Later all the starch grains were expelled in the usual manner by taking in water through the pharynx and flushing out the gut contents by violent contractions of the body.

(2) Experimental feeding with proteins

Polycelis fed directly upon clotted frog and chick blood. The erythrocytes were quickly phagocytosed and could be seen in the columnar gut cells in saline squashes made thirty minutes after feeding. The number phagocytosed increased with time and six hours later almost every cell was packed full. Occasionally erythrocytes were also seen in mesenchymal cells. The erythrocytes quickly changed in appearance after phagocytosis and condensed into homogeneous deeply staining spheres which decreased in size and number as digestion and absorption progressed (Fig. 14). They showed no signs of digestion in the gut lumen prior to phagocytosis.

Chopped frog muscle stained with various indicators was fed to the flatworms by means of gammarid carriers. The particles reached the gut in recognizable pieces still showing their characteristic striations and the larger remained unchanged in size, shape and pH value until ejected from the gut some 24 hours later. This indication of the absence of intraluminar proteolysis was confirmed by feeding coagulated albumen, gelatin and fibrinogen, all of which likewise remained unchanged throughout their stay in the gut lumen. The smaller particles of chopped muscle were phagocytosed and neutral saline squashes made one hour after feeding showed many present in the columnar gut cells. Particles stained with brom-cresol green were then a clear blue in color (pH 5.2) but changed with time until six hours after feeding when they had become a clear green (pH 4.6), so that intracellular protein digestion proceeds in a distinctly acid medium. This color faded as the particles themselves became rounded and less distinct. Stained inclusions passing through the same series of color changes were occasionally found in mesenchymal cells. This indicator incidentally proved to be most attractive to these flatworms and they ingested large amounts of food stained by it; particles of the indicator dropped into culture dishes attracted the flatworms to them and stimulated protrusion of the pharynx.

(3) Experimental feeding with fat

Large amounts of cod liver oil suspensions stained with Sudan IV were readily ingested from carrier gammarids, often increasing the body volume of the flatworms by 40–50%. Saline squashes and frozen sections prepared thirty minutes after feeding showed some stained oil globules within the columnar cells, and the number increased with time. Irrigation of the preparations with Nile Blue (Smith, 1907; George, 1951) showed the globules changing from red to deep blue as the stain penetrated the cells showing that much of the phagocytosed oil was undergoing intracellular lipolysis with the production of free fatty acids. Nothing suggesting intraluminar lipolysis was seen. Stained globules appeared early in the mesenchymal cells, and later became much more numerous, but only a few ever showed any color reaction with the Nile Blue. Since the Sudan IV stains the fatty acid radical of fat molecules and remains with it through lipolysis and resynthesis, it appears probable that the red globules in the mesenchyme cells had mainly been broken down and re-formed in the gut cells into the flatworm's own particular type of fat and then passed into the mesenchymal cells for storage. Flatworms with this stored fat had a diffuse pink color which persisted for several weeks and faded only gradually as the fat was metabolized and the Sudan IV excreted. Saline squashes made six weeks after feeding still showed occasional red globules in the mesenchymal and columnar gut cells. It was not possible to determine the pH conditions attending intracellular lipolysis since indicators dissolved in the water of the cod liver oil suspension were not taken up by the cells. Suet particles were also fed. The largest particles remained in the gut unchanged in size and shape until ejected 24 hours later, confirming the absence of intraluminar lipolysis. The smaller particles were phagocytosed and digested intracellularly.

The nature and location of the food reserves

Polycelis forms large reserves of protein and fat, supplemented by some carbohydrate reserve, and these enable the flatworm to survive for at least three months without food.

The gonads, mesenchyme and general body tissues constitute a protein reserve which is drawn upon during starvation, with a consequent reduction in body size, but there are also specific protein reserves in the sphere cells of the gastrodermis. These contain 8–16 small homogeneous concentrations which normally stain deeply with modified Millon, eosin and iron haematoxylin, but their appearance and staining affinity varies with the nutritive state of the animal. In well-fed laboratory individuals, and in those collected out of doors in late summer and well nourished in preparation for winter, the spheres are all compact and densely staining. During starvation they become increasingly indistinct, stain poorly and finally disappear. At the same time a progressive reduction in the volume of both columnar and sphere cells results after about three months in a flattened gastrodermis devoid of any inclusions. The significance of the sphere cells as protein reserve can be demonstrated by comparing the proportion of sphere and columnar cells under a variety of nutritive conditions. These are summarized in Table I which is compiled from cell counts of every third section of a number of histological series.

The fat reserve is laid down as globules in the inner mesenchyme and columnar

gut cells. It decreases only slowly during starvation, and significant amounts still remain after three months without food.

The carbohydrate reserve occurs as small irregular granules of glycogen, scattered throughout the mesenchyme and columnar cells. It decreases rapidly on starvation and disappears from the mesenchyme after about a fortnight. Small amounts persist in the gut cells, however, for much longer than this, but these may possibly result from the conversion of other reserves. A purely carbohydrate diet results in an enormous increase in the amount of glycogen in the mesenchyme and the flatworms, provided they were mature, survived as well on this diet as upon purely protein or fat diets.

The epidermal and subepidermal glands and their relation to feeding

These secretions can be divided into mucoid, giving a positive reaction with P.A.S. and Alcian Blue (Steedman, 1950), and the non-mucoid or rhabdoid, giving a negative reaction.

The mucoid can be further differentiated into eosinophilic and basophilic. The latter is produced by scattered gland cells in the epidermis and outer mesenchyme,

TABLE I

The proportion of cell types in relation to nutrition

Nutritive condition	Ratio of "sphere" to columnar cells
High protein diet	930:3723 = 1:4
Normal diet	625:3957 = 1:6.3
Starvation: 14 days	435:2957 = 1:6.7
Starvation: 28 days	371:3545 = 1:9.5
Starvation: 2 months	176:2668 = 1:15.5
Starvation: 3 months	"Sphere cells" absent and gastrodermis syncytial

and is used in gliding and to a minor extent in trapping prey as already mentioned. There are no large aggregations of basophilic glands as described in some other triclads. The much more adhesive eosinophilic mucus is produced both from scattered gland cells and from concentrations of mesenchymal gland cells, the "marginal adhesive glands" of Hyman (1951), situated along the borders of the ventral surface. During its discharge the epidermis becomes elevated into small temporary papillae so that the secretion is laid down in lines of oval deposits, from which are formed the main elements of the "snares."

The non-mucoid rhabdoids are transparent greenish rods, 15–20 μ in length, produced by mesenchyme cells and passed out to the epidermis. Once outside the body the rhabdoids take up many times their own volume of water ("hydrate") and fuse to form a semi-fluid gelatinous layer which closely invests the body. This material can be collected for examination in two ways. It can be removed from the living flatworm by drawing a fine glass needle a number of times across the dorsal surface of the body. Alternatively, immersion of the worm in strong sodium chloride solution causes the discharge of enormous numbers of rhabdoids and at the same time inhibits their hydrolysis, so that they can be fixed in 95% alcohol for histological examination. Irrigation of unfixed rhabdoids with water causes them to hydrate rapidly (Figs. 15 and 16), producing the same sort of gelatinous sheet as invests

the normal flatworm. The lowest critical concentration of salt to give discharge without hydration is about 3.75% ; possibly hydration within the epidermal cells may be inhibited by some comparable combined effect of the cytoplasmic salts. The rhabdoid material is basic (pH 8.0–8.2), soluble in acids and alkalies, and stains strongly with Millon; apparently it is of a purely protein nature, as all fat and carbohydrate tests were negative. The hydrated material is not toxic to other animals, annelids and crustaceans surviving for long periods in contact with it, and it plays no part in feeding. It is distasteful to fish, pieces of earthworm smeared in it being rejected by sticklebacks, but its main function is probably mechanical, acting as a "fluid cuticle" to protect the epidermis from abrasion and bacterial and fungal attack whilst still permitting ciliary activity, and in life it is probably in a continual state of loss and renewal. There is also a rapid discharge of rhabdoids if a flatworm is injured, to give a protective clot-like effect over the region of the wound.

ACOELA

Convoluta paradoxa (2–3 mm. long and pear-shaped with a pronounced ventral curling of the broader anterior end) feeds upon small marine crustaceans, protozoa, diatoms and similar organisms which are captured by various methods, depending upon their size. Minute prey such as protozoa are captured by the flatworm gliding slowly over algal fronds or stones with the solid syncytial gut partially extruded through the mouth like a large pseudopodium and the food engulfed amoeboid fashion and passed back into the syncytium for digestion. Alternatively the flatworm may spend long resting periods attached to the substratum by the adhesive papillae of the tail and with the rest of the body slightly raised in a "sitting-up" position. If larger prey, such as crustaceans or their larvae, come sufficiently close, the flatworm rapidly extends its body and grasps the prey with the curved anterior margin, which is covered with abundant sticky mucus produced by the frontal and other subepidermal glands. The flatworm then curls up ventrally, starting from the anterior end, so that the prey is drawn beneath it and pressed into the mid-ventral mouth. This is very distensible and the food is ingested whole and intact. More rarely *Convoluta* captures actively swimming creatures whilst it is itself either swimming freely or gliding over the algal fronds, but in all cases capture and ingestion are extremely rapid and occupy only 5–10 seconds. The flatworm shows marked avoiding reactions when dead food is encountered; it feeds only upon living organisms and does not act as a scavenger.

The simple pharynx is a very short ciliated tube leading directly to the solid "gut"—a central compact or vacuolated syncytium, one-third to one-half of which can be protruded through the mouth (Fig. 5). Its cytoplasm is finely granular and contains many vesicular nuclei. All prey when ingested is enclosed in temporary vacuoles which follow no definite path, but merely move back inside the animal to come to rest in any part of the syncytium. Occasionally vacuoles containing digesting prey may be found in the mesenchyme, but the cytoplasm about these vacuoles is always distinct from the surrounding tissue and is apparently a temporarily isolated part of the digestive syncytium. As digestion progresses the food becomes disorganized and breaks up and in Crustacea only fragments of the exoskeleton remain when digestion and absorption are complete, some 18–24 hours after feeding (Fig. 6). These indigestible residues then pass to the mouth to be thrown out.

It was not possible to determine the pH conditions controlling digestion as *Convoluta* refused crustaceans stained with indicator. Attempts to recover food in various stages of digestion for pH examination also failed, as it inevitably became contaminated with mesenchyme, mucus and sea water.

Fat forms the principal food reserve in *Convoluta*, occurring in large amounts as globules of 2–3 μ diameter in the outer, more compact mesenchyme and in the digestive syncytium. Small amounts of glycogen also occur as irregular granules scattered throughout the mesenchyme and digestive tissue. There are no specific protein reserves.

RHABDOCOELA

(1) *Macrostomum* sp. (2–3 mm. long and 0.5 mm. broad) feeds upon minute fresh water crustaceans, annelids, nematodes, rotifers and large ciliate protozoans. The mouth and pharynx are capable of great distension and any small creature moving near the flatworm is seized and ingested whole. Only living food is taken and mucus plays no part in its capture.

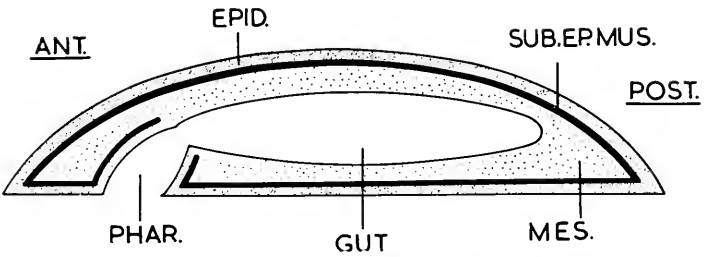


FIGURE 2. Diagrammatic longitudinal section of *Macrostomum* to show the simple pharynx. Abbreviations as in Figure 1.

The ventral slit-like mouth opens into a simple ciliated pharynx longer than that of *Convoluta* but still with no thickening of the underlying muscular layers (Fig. 2). Eosinophilic glands in the mesenchyme open into the pharynx but they are not mucus-producing and their precise function remains unknown. The gut is a simple unbranched sac extending almost the whole length of the body and its wall is made up of two kinds of cells, standing upon a thin basement membrane (Fig. 7). The larger and more numerous are columnar, some 30–40 μ tall and 5–6 μ broad, with basal vesicular nuclei and finely granular cytoplasm which may have inclusions, giving the cell a phagocytic appearance. The second type of cell is smaller and bears a close resemblance to the "sphere-cell" of the triclad gastrodermis. It is club-shaped, 15–20 μ tall and 3–4 μ broad distally, with the nucleus in the narrower basal region where the cytoplasm is often strongly basophilic. These cells contain 10–15 eosinophilic spheres which also stain with iron haematoxylin and modified Millon.

Sections of *Macrostomum* fixed 15 minutes after feeding on *Daphnia* showed the intact crustaceans lying free in the gut. In others fixed 10 hours later digestion was well advanced, with the muscles and organs greatly disorganized, whilst 24 hours after feeding only the exoskeleton remained, but as the latter retained its general shape there can be little movement or contraction of the gut during digestion.

This was confirmed by observation of living flatworms. During digestion the columnar gut cells became shortened, swollen and indistinct from one another, probably as a result of secretory activity or absorption of digested food, or both. The "sphere cells" remained unchanged. These observations confirmed the presence of intraluminar digestion in *Macrostomum* but after feeding upon *Paramecium* or small annelids almost all the gut cells showed discrete inclusions which were clearly particles phagocytosed from the disintegrating food mass in the gut lumen (Fig. 7). Thus digestion in *Macrostomum* must be a combination of intraluminar and intracellular processes, the latter occurring when the food in the lumen is sufficiently disintegrated for the particles to be phagocytosed. The small amount of phagocytosis and intracellular digestion seen with crustaceans may be due to the exoskeleton retaining most of the disintegrating material until it is rendered completely soluble. The pH conditions of digestion could not be determined as the flatworms refused to ingest prey stained with indicator.

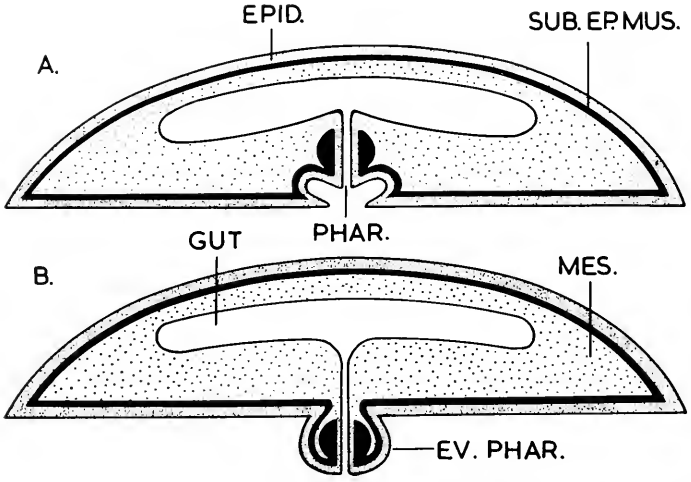


FIGURE 3. Diagrammatic longitudinal sections of *Mesostoma* to show the bulbous pharynx. A, The normal condition with the pharynx retracted. B, Pharynx everted for feeding. Abbreviations as in Figure 1.

Small amounts of glycogen and fat occur in the columnar gut cells and in the mesenchyme, whilst the "sphere-cells" of the gut epithelium appear to constitute a definite protein reserve, since they disappear after 10–14 days' starvation.

(2) *Stenostomum* sp. (1.5–3 mm. long and very slender) feeds mainly upon large ciliates and rotifers, which are seized in the anterior ventral mouth and engulfed whole. These animals are usually found by chance but the flatworm may search actively for them, crawling slowly forwards with the anterior end slightly raised and the mouth wide open. The latter is capable of great distension and is fringed with large cilia which probably help to sweep the prey back into the pharynx, where it is retained a few seconds for examination. If unsuitable it is rejected, but if acceptable the mouth is closed and the pharynx contracts violently to force the food into the gut. The pharynx is a simple ciliated tube, similar to that of *Macrostomum*, with some basophilic gland cells producing mucus to help ingestion. The

gut is a simple blind sac extending almost to the posterior end of the body, its anterior end is contracted and open communication with the pharynx exists only during actual ingestion. The gastrodermis is of unciliated club-shaped cells, 20–25 μ tall and 6–8 μ broad distally with basal nuclei and granular inclusions.

When ingestion is complete, and the pharynx again closed off, the gut contracts violently and the food is driven to and fro in the lumen (Fig. 9). Ciliates are broken up within fifteen minutes, and indeed are often disintegrated by the pharynx during ingestion. Rotifers resist longer but eventually their body contents are squeezed out through the mouth or some weak spot in the integument and the empty cuticle ejected. The resultant particles are phagocytosed by the gut cells and show for a time as eosinophilic inclusions until intracellular digestion is completed. Occasionally *Paramecium* stained with indicators covering a pH range of 3.5–8.4 were accepted but during their disintegration the pH remained unaltered, showing the absence of intraluminar digestive activity.

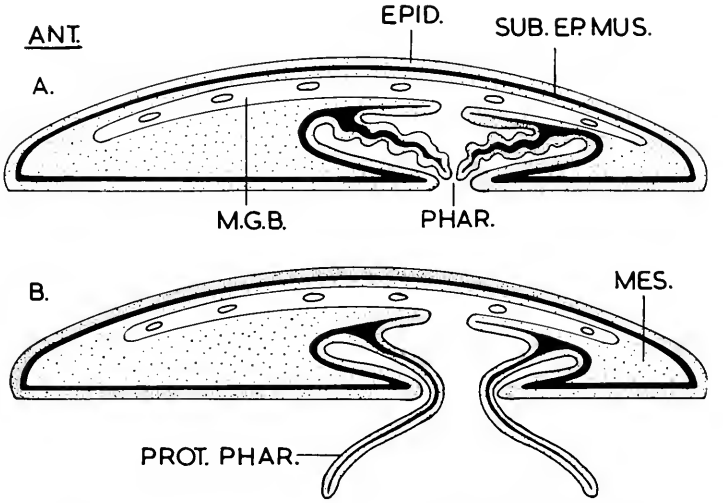
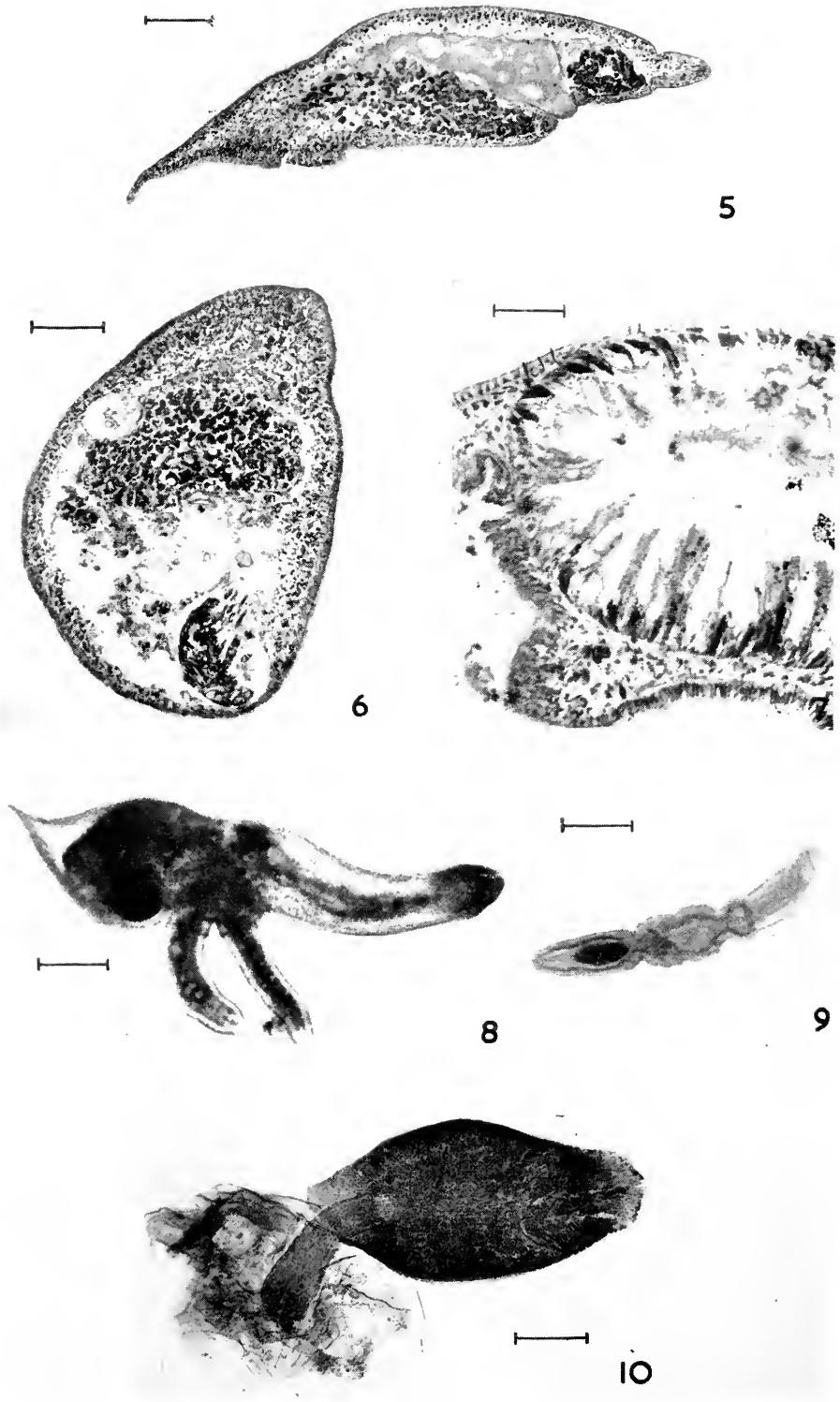


FIGURE 4. Diagrammatic longitudinal section of *Leptoplana* to show the ruffled plicate pharynx. A, The normal condition with pharynx retracted. B, The pharynx protruded to envelop food. Abbreviations as in Figure 1.

Fat forms the only food reserve in *Stenostomum*, occurring as small globules in the gut cells. The amount decreases rapidly and little remains after a week, which is about the maximum period the flatworm can endure without food.

(3) *Mesostoma tetragonum* (5–8 mm. long and 1–2 mm. broad) feeds upon small oligochaetes, crustaceans and insect larvae, which are captured directly by the flatworm wrapping itself around them. The bulbous pharynx is then protruded through the median ventral mouth (Fig. 3) and applied to the prey's body wall. Strong sucking movements made by alternate contractions and expansions of the radial musculature of the pharynx draw small prey bodily into the gut (Fig. 8). Larger animals are held until the body wall ruptures and the pharynx can be thrust into the body to suck out the contents, but in a somewhat inefficient manner so that frequently only the body fluids are withdrawn. *Mesostoma* is also a



FIGURES 5–10.

scavenger and will feed upon any sort of dead prey, if not excessively decomposed. The gut is a simple sac and the gastrodermis is syncytial, 15–20 μ thick, often with spherical inclusions.

The ingestion of whole prey implied that digestion in *Mesostoma* is at least partially intraluminar and this was confirmed by experimental feeding with clotted frog blood. The erythrocytes are largely broken up within thirty minutes, though their freed nuclei are still distinct, but there is no evidence of phagocytosis by the gut cells during this initial period. After three hours the material in the gut lumen has become a homogeneous mass, though a light reaction with Feulgen indicates that the ruptured nuclei are not yet entirely decomposed, and as this stage is reached material of similar appearance begins to show as small spheres within the gut cells. After six hours the lumen was empty and the gut cells packed with spheres, which in turn decreased and disappeared within 24 hours. Intracellular digestion in *Mesostoma* is therefore preceded by some degree of intraluminar digestion, but details are unknown as no successful method of feeding with indicators could be found.

POLYCLADIDA

(1) *Cycloporus papillosus* (1–2 cm. long and broadly oval) is usually found attached by its single ventral sucker to the surface of the encrusting colonial tunicates *Botryllus* and *Botrylloides*, and it appears to feed exclusively upon these animals. The anterior cylindrical plicate pharynx, shorter but otherwise resembling that of *Polycelis*, is protruded forwards and thrust down into the colony to suck out individual zooids. The pharynx leads into the median gut branch which gives off 6–8 pairs of lateral diverticula, subdividing in turn to give the typical polyclad arrangement. The distal gut branches lead up to large "anal chambers," ranged along the margins and opening by pores through the epidermis. In histological preparations the branches and anal chambers always appear closed off from each other, and no actual passage outwards of gut material has ever been found; possibly temporary openings may develop for the passage of excess water taken in with the food.

The majority of the gastrodermal cells are columnar, 35–40 μ tall and 5–8 μ wide, with their free margins ciliated, the cilia being particularly strong in the median diverticulum (Fig. 17). There is, however, an anomalous region of columnar cells at the posterior end of this diverticulum where the cilia are replaced by what appears to be a "brush border." Scattered amongst the columnar cells are smaller

FIGURE 5. Longitudinal section of *Convoluta* showing the central vacuolated syncytium. Haematoxylin and eosin. Scale 0.25 mm.

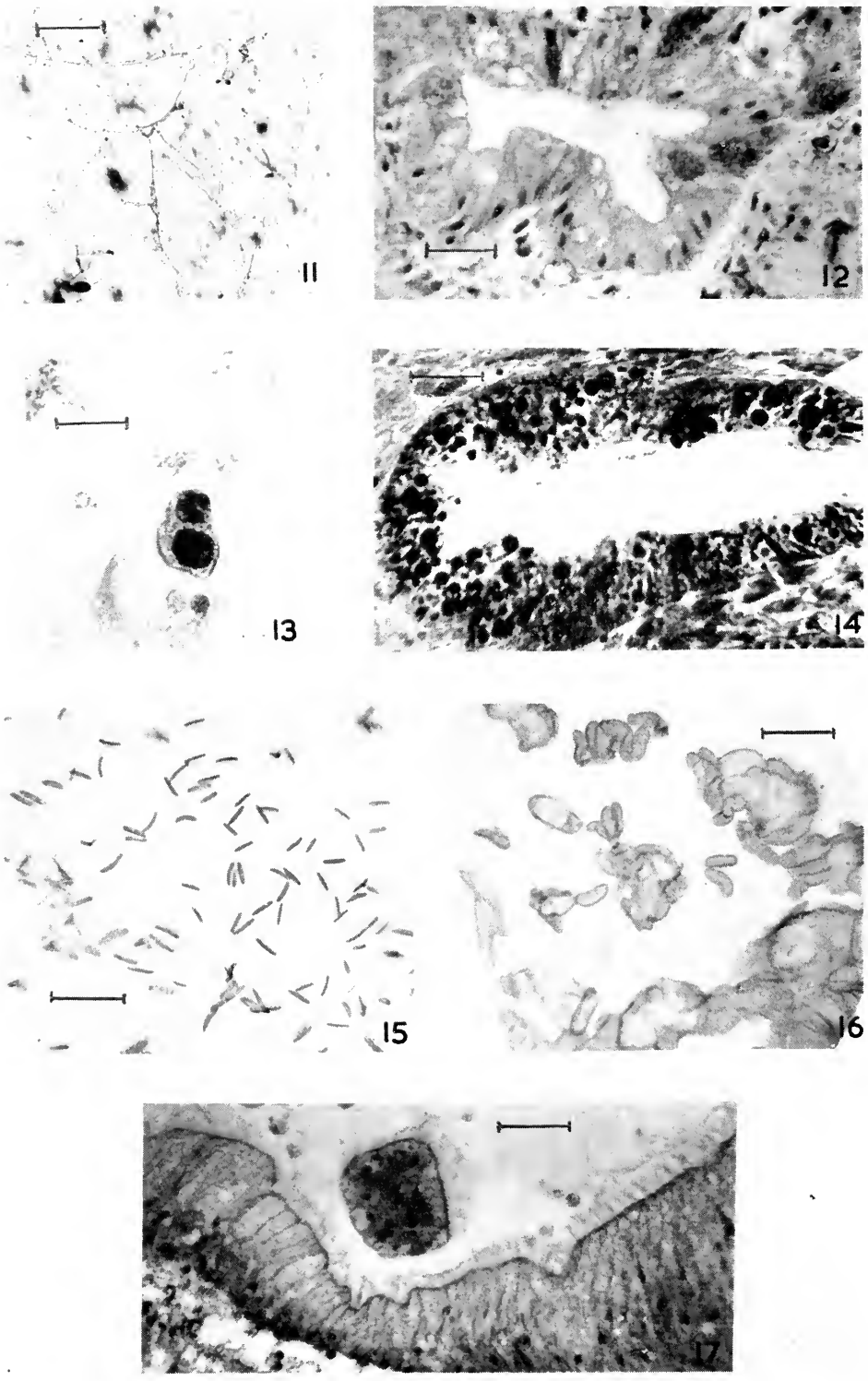
FIGURE 6. Transverse section of *Convoluta* showing a recently ingested intact crustacean and one completely digested except for the exoskeleton. Haematoxylin and eosin. Scale 0.1 mm.

FIGURE 7. Longitudinal section of *Macrostomum* showing part of the simple pharynx (left) and the gastrodermis with small, club-shaped "sphere cells" between the long, projecting phagocytic cells. Haematoxylin and eosin. Scale 20 μ.

FIGURE 8. *Mesostoma* ingesting an annelid. From life. Scale 1 mm.

FIGURE 9. *Stenostomum* immediately after ingesting a *Paramecium* stained with neutral red. The gut is beginning to contract to break up the ciliate. From life. Scale 0.4 mm.

FIGURE 10. *Polycelis* killed whilst feeding on a large *Daphnia*. Note the protruded pharynx. Steinmann's fixative and borax carmine. Scale 1 mm.



FIGURES 11–17.

numbers of spherical glandular cells, up to 45 μ in diameter, their cytoplasm laden with tiny granules. These cells occur in various sizes, the smaller appearing between the bases of the columnar cells and the larger nearer the lumen; occasionally they were seen free in the lumen, and their disintegrated remains were found both here and between the bases of the columnar cells. Their phases of growth and disintegration, however, could not be related in any way to the condition or amount of food in the gut and it is not at all certain that they are concerned in digestion. The columnar cells themselves, on the other hand, do appear to secrete juices into the lumen, becoming swollen and vacuolated soon after feeding.

In feeding, the tunicate zooids reach the gut almost undamaged; histological examination at intervals after an observed feed shows a progressive homogenization of the food, leading to complete absorption after about twelve hours. At no time is there any indication of other than intraluminar digestion. Confirmation came from feeding starved *Cycloporus* on *Botryllus* colonies injected with starch paste; Lugol sections showed digestion confined within the lumen, with unchanged starch never appearing within the gut cells. Food containing indicators, however, was consistently refused. Fat globules in the ciliated cells and mesenchyme constitute the only significant food reserve. Only minute amounts of glycogen occur in the mesenchyme and there are no specific protein reserves. The flatworm cannot withstand more than about seven days' starvation.

Only basophilic mucus glands are present, scattered throughout the epidermis and ventral mesenchyme, and mucus plays no part in feeding. Rhabdoid-producing cells are common in the mesenchyme, particularly between the posterior gut diverticula, and their cycle of activity can be followed in its entirety. Immature cells contain small eosinophilic granules and rods which enlarge into the fully formed rhabdoids. As these are discharged the cells shrink to a fraction of their former size and migrate into the nearest gut diverticulum; there they disintegrate and are presumably expelled with the unwanted food material. The discharged rhabdoids themselves follow one of two courses. Some pass to the epidermal cells and so to the exterior in the usual way. The experimental removal and examination of these rhabdoids gave results identical to those reported for *Polycelis*. Others aggregate to form conspicuous tracts, greyish white in the living flatworm, which converge upon the gonopore. The rhabdoids pass through or between the cells of the gonopore wall and are discharged with the eggs. Sections of the egg masses show the individual eggs to be embedded in a gelatinous matrix which has properties similar to those of hydrated rhabdoids. The rhabdoids are not concerned in any

FIGURE 11. Mucus "snares" produced by *Polycelis*. Alcian blue. Scale 0.5 mm .

FIGURE 12. *Polycelis*. Transverse section of gut diverticulum showing columnar phagocytic cells and smaller heavily staining "sphere cells." Iron haematoxylin. Scale 40 μ.

FIGURE 13. *Polycelis*. An isolated columnar cell with phagocytosed starch. From a saline squash. Lugol. Scale 40 μ.

FIGURE 14. *Polycelis*. Transverse section of the gut diverticulum 24 hours after feeding on chick blood, showing phagocytosed erythrocytes undergoing intracellular digestion. Iron haematoxylin. Scale 40 μ.

FIGURE 15. *Polycelis*. Rhabdoids discharged after immersion in 5% aqueous sodium chloride. Scale 50 μ.

FIGURE 16. *Polycelis*. Rhabdoids discharged as in Figure 15, then irrigated with tap water. Scale 50 μ.

FIGURE 17. *Cycloporus*. Longitudinal section of the median gut branch showing ciliated gastrodermis. Haematoxylin and eosin. Scale 40 μ.

way in the formation of the egg membranes since these stain strongly with P.A.S. and are already fully formed in the oviduct well above the point of entry of the rhabdoids.

(2) *Leptoplana tremellaris* (2–2.5 cm. long and pear-shaped) feeds upon polychaetes, isopods and amphipods which are captured directly without any trapping devices. The prey is seized by the anterior part of the flatworm's body and wrapped round by the expanded body margins. The flatworm then curls up and passes the prey back along the ventral surface to the median mouth. The pharynx is of the ruffled plicate type (Fig. 4), consisting of a large thin convoluted oval curtain suspended from the roof of the peripharyngeal chamber, and is protruded through the mouth to envelop the prey. Small animals are ingested whole, either by simple peristalsis or by the pharynx being drawn back within the mouth, a process requiring five minutes. Larger organisms are enveloped as far as possible and the pharynx is retracted slightly so that the prey is partially ingested; the flatworm then presses itself down on the substratum and the pharynx makes strong sucking movements which are often supplemented by movements of the whole body. This process may continue for an hour or more, whilst the prey is disintegrated and ingested piece by piece or has its integument ruptured and the contents squeezed out. *Leptoplana* also feeds upon dead animal material provided it is not too decomposed.

The gut is of the usual polyclad form but the gastrodermis here consists of unciliated vacuolar columnar cells. In well-fed individuals these are indistinct in outline or even syncytial and the cytoplasm loaded with eosinophilic spheres which disappear on starvation. This appearance of phagocytic activity was confirmed by experimental feeding with frog blood and small pieces of *Littorina* muscle. The former were rapidly taken up by the gut cells and their intracellular digestion and disappearance easily followed. The small pieces of muscle entered the gut entire but subsequent changes in their histological appearance showed that they were partially broken down by enzymatic activity in the gut lumen before entering the gut cells, where their fate follows that of the usual eosinophilic inclusions. In this case, therefore, intraluminar digestion is incomplete and is succeeded by phagocytosis and intracellular digestion.

The possibility that enzymes from the gut could be regurgitated to assist disintegration in the protruded pharynx was investigated by using pieces of *Littorina* foot muscle too large to be ingested whole. These pieces were removed from the flatworm's pharynx after a series of time intervals and checked against control muscle for pH and enzymatic changes. Control muscle was slightly acid (pH 6.6–6.8), but after ten minutes decreased to 5.4 and after twenty to 4.5. For proteolytic activity the muscle was laid on gelatin-coated slides and half an hour allowed for any enzyme to diffuse out. The gelatin below and around a piece of muscle which had been in the pharynx for a quarter of an hour was dissolved away and the holes formed in the gelatin film showed clearly after staining the slide with eosin. Control muscle had no such effect. Corresponding acidity and enzyme production in the gut lumen can therefore be reasonably inferred.

Fat again forms the only significant food reserve, and what has been said of *Cycloporus* about mucus and rhabdoids applies also to *Leptoplana*.

Discussion

The general pattern of flatworm nutrition consists more of a series of intimate relationships between the nature of the food, the structure and function of the pharynx, and the course of digestion, than of a simple increase in complexity from primitive to more elaborate forms. Possibly the "amoeboid" method of feeding in the Acoelan *Convoluta* is primitive to the group (*cf.* Hyman, 1951), and no longer found in other forms. Certainly the alternative method in *Convoluta* of using the body as a whole to envelop the prey is still retained in one form or another by the majority of flatworms, with its effectiveness enhanced by elaboration of the pharynx. The limitation of the simple pharynx, found also in the rhabdocoels *Macrostomum* and *Stenostomum,* is that prey has to be engulfed whole and its size is thereby restricted. In contrast, the bulbous pharynx of the rhabdocoel *Mesostoma* can not only ingest small animals but can be forced into larger animals to suck out their contents, though little triturating effect is possible. The cylindrical plicate pharynx of the triclad *Polycelis* is used in a similar but much more effective manner, the whole contents of the prey being withdrawn and discharged into the gut in a finely divided condition. The comparable pharynx of the polyclad *Cycloporus,* however, is used to deliver largely undisrupted food into the gut, the difference being reflected in the subsequent course of digestion. The ruffled plicate pharynx of the polyclad *Leptoplana,* though developmentally akin to the last, is used rather as an extension of the gut in which preliminary breakdown is enzymatic instead of mechanical. Thus the elaboration of the pharynx has made available to the flatworms an increasing range in the size and variety of their food. Mucus plays a minor part in the feeding of the majority, but does serve to hold the prey and facilitate ingestion. In *Polycelis* alone it is used for the actual capture of prey.

The course of digestion is linked with the feeding mechanism, since this determines the condition of the food on entry to the gut. In *Convoluta* the position is anomalous in that digestion in its gut syncytium might be looked upon either as intracellular or as occurring within temporary lumina. In *Polycelis* the food is very finely divided by the pharynx and the particles taken in by phagocytosis for intracellular digestion. No evidence of intraluminar digestion was found in this species, either of mixed food or of the three food elements fed separately, in agreement with Willier, Hyman and Rifenburgh (1925) and Kelley (1931); the contrary opinion of Arnold (1910) was based on the erroneous supposition that the "sphere cells" of the gut were glandular and discharged into the lumen. Some evidence of intracellular digestion in mesenchyme cells was observed, of food apparently received indirectly from the endoderm cells. In striking contrast is the purely intraluminar digestion of *Cycloporus,* which shows that the flatworms are capable of evolving this more advanced process. In all the other types examined some preliminary breakdown of the food, either by mechanical or enzymatic means, occurred in the pharynx or in the gut lumen, to be followed by phagocytosis and intracellular digestion; this preliminary breakdown should perhaps be interpreted as an adaptation to allow the persistence of the more primitive form of digestion.

Experimental feeding with proteins, carbohydrates and fats given separately showed that fully grown *Polycelis* can not only utilize and store all these food elements but can survive upon any one indefinitely. *Polycelis* also forms normal food

reserves of all three types: specific protein reserves in the "sphere cells" of the gut, fat and glycogen in the gut and mesenchyme cells. Glycogen is the first to be used up and appears the least important. In the others fat forms the main food reserve, supplemented by protein in *Macrostomum,* but they are less prominent than in the triclad, and the capacity to withstand starvation is correspondingly reduced.

I wish to thank Professor E. A. Spaul for suggesting this problem and for assistance, and Dr. T. Kerr for constant guidance and encouragement.

Summary

1. A study has been made of the food, feeding mechanisms, digestion and food storage in the triclad *Polycelis cornuta,* supplemented by observations on representatives of the other three flatworm orders.

2. Each form has a characteristic method of feeding, but in general the range of available prey has been greatly increased by elaborations in the structure and use of the pharynx. Mucus plays a minor part in feeding, except for the "snares" of the triclad. Rhabdoid material has no function in feeding. It forms a temporary cuticle and in polyclads a covering for the eggs.

3. In *Polycelis* mechanical breakdown of the food is followed by phagocytosis into the gut cells and intracellular digestion. Elsewhere the preliminary breakdown may be wholly or in part enzymatic; only in *Cycloporus* is there purely intraluminar digestion with absorption in place of phagocytosis.

4. *Polycelis* can make use of all three food elements, and stores of each type normally occur. In the other forms food storage is less well developed though reserve fat at least is usually to be found.

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VARIATION OF NITROGEN AND CARBOHYDRATE CONSTITUENTS DURING THE DEVELOPMENT OF HIMANTHALIA ELONGATA (L.) S. F. GRAY

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Seasonal variations in the chemical composition of brown algae have been studied by various workers (Lapicque, 1919; Lunde, 1940; Black, 1948a, 1948b, 1949, 1950a, 1950b, 1954a, 1954b; Channing and Young, 1952, 1953; Øy, 1951; Smith and Gordon Young, 1953; Wort, 1955). Black and Dewar (1949) correlated the physical and chemical properties of the sea water with the chemical constitution of algae. In all these investigations, however, no indication of the age, stage of development or sex of the plants is given.

Moss (1950) found that marked changes in the chemical composition of *Fucus vesiculosus* were associated with the development of the reproductive structures. In later work Moss (1952) noted that during the Spring a variation in chemical constituents occurred in several developmental stages of *Himanthalia elongata* collected at the same time from the same habitat.

Black (1954a) has shown that at certain times of the year gradients of carbohydrates, proteins and mineral matter occurred along the frond of *Laminaria saccharina*. Studying the variation of nitrogen in *F. vesiculosus* and *L. saccharina*, Jacobi (1954) observed horizontal and vertical gradients of nitrogen, water content and fresh weight. During the time of sporulation the frond of *L. saccharina* showed a higher percentage of soluble nitrogen than during the time of vegetative growth. Jacobi suggested that ontogenetic factors were more decisive in bringing about variations in chemical composition of brown algae than the availability of nutrients in the sea water as postulated by Black and Dewar (1949). For *Himanthalia elongata* similar variations in chemical constituents occur along the length of the plant (Jones, 1956). The same author noted that the mature vegetative tissues of the plant were lower in nitrogen content than the actively growing reproductive tissues, and that the male receptacles were higher in nitrogen content than the female receptacles.

No work appears to have been carried out to determine the variation of chemical constituents during the growth and development of brown algae from germination to maturity and senescence. For such a study, therefore, the brown alga *Himanthalia elongata* has been selected, for it enables an independent study to be made of the variation in chemical composition of both vegetative and reproductive tissues during the growth and development of the plant.

Materials and Methods

Collection of material

Samples of *Himanthalia elongata* were collected from St. Mary's Island, Northumberland, at low water as the tide receded. All samples were returned to the

laboratory in large glass vessels, thus ensuring that the plants, prior to analysis, were fully turgid. During 1954, the following major stages of growth and development of the plant were investigated:

1) The development of the young vegetative buttons until they produced receptacles at the end of the year. At St. Mary's Island, the young receptacles made their appearance during September to October.

2) The maturation of the plants in their second year of growth. During this period the receptacles grow rapidly in the summer months and develop conceptacles containing the gametes.

For analysis, samples were sorted into their respective stages of development. Each sample contained at least 100 individuals. Wherever possible the vegetative buttons were separated from the receptacles and analyzed separately so that variations in chemical composition throughout the development of both vegetative and reproductive tissues could be studied. Because of the difference in nitrogen content of male and female receptacles of *Himanthalia* (Jones, 1956), for the mature plants only female ones were used.

Analytical procedure

Methods of analysis were those described previously (Jones, 1956). Fucoidin as combined L-fucose, however, was estimated by the improved method of Black, Cornhill, Dewar, Percival and Ross (1950).

Expression of experimental results

For the purpose of this study it was considered that the results would lend themselves best for interpretation if they were expressed on a unit plant basis.

Results

1. *Young developing plants*

During the development of the young vegetative button there is a gradual increase in fresh and dry weight until the young receptacles make their appearance in October (Table I). Thereafter the weight of the button remains comparatively steady. The receptacles, however, increase in both fresh and dry weight after their initial appearance.

The total nitrogen of the young plants gradually increases throughout the year. In the button the protein content steadily increases, while the non-protein nitrogen increases with the growth of the button until October, when, at the time of receptacle initiation, it falls appreciably (Fig. 1). The relationship between the protein and non-protein is illustrated in Figure 2. During the period January to August the ratio rapidly decreases, suggesting a building-up of non-protein constituents. From August to the end of the year, the ratio increases indicating a synthesis of protein. This synthesis precedes the development of the young receptacles, which are themselves rich in protein and non-protein nitrogen. The build-up of a soluble non-protein reserve, of which peptide nitrogen is the chief constituent, is therefore clearly indicated during the early stages of the development of the button.

TABLE I

Variation of constituents during the development of young plants, 1954

Month		Weight of plants gm./unit plant		Nitrogenous constituents mg./unit plant						Carbohydrate constituents mg./unit plant				Total ash mg./unit plant
		Fresh	Dry	Total N	Protein N	Non-protein N	Volatile base N	Free amino N	Peptide N	Alginic acid	Mannitol	Laminarin	Fucoidin	
January	B	0.54	0.07	1.02	0.87	0.15	0.04	0.04	0.07	13.9	3.9	1.4	5.1	28.0
February	B	0.72	0.11	1.48	1.25	0.23	0.06	0.06	0.10	—	12.1	—	—	—
March	B	—	—	—	—	—	—	—	—	—	—	—	—	—
April	B	0.78	0.14	2.02	1.68	0.34	0.08	0.09	0.15	26.1	13.6	2.7	10.0	56.0
May	B	—	—	—	—	—	—	—	—	—	—	—	—	—
June	B	1.69	0.24	3.71	2.79	0.92	0.31	0.15	0.35	43.8	32.4	5.1	18.6	91.0
July	B	2.00	0.32	4.62	3.58	1.04	0.45	0.24	0.61	56.0	41.5	7.0	20.5	112.0
August	B	1.80	0.31	4.31	3.06	1.25	0.38	0.15	0.50	58.3	43.1	12.3	28.0	108.0
September	B	1.96	0.38	4.67	3.67	1.00	0.36	0.19	0.50	64.8	47.8	34.5	31.4	110.0
October	B	1.62	0.33	4.44	3.69	0.75	0.21	0.25	0.31	71.5	36.6	27.0	20.7	97.0
November	B	1.76	0.36	5.16	4.24	0.92	0.17	0.14	0.33	82.0	25.1	21.5	22.5	120.0
November	R	0.15	0.03	0.56	0.33	0.23	0.02	0.02	0.03	4.0	2.0	—	1.7	8.0
December	B	2.00	0.36	5.32	4.58	0.74	—	—	—	83.0	24.4	11.1	26.0	127.0
December	R	0.32	0.04	0.91	0.53	0.28	—	—	—	6.6	2.4	0.5	2.6	13.0

B = Button.
R = Receptacles.

Throughout the growth of the young plant the alginic acid content increases even after the production of the receptacles. The other carbohydrate substances, namely mannitol, laminarin and fucoidin gradually increase during the early months of the year, then become more concentrated during the summer months, no doubt the result of active photosynthesis. Between August and October the vegetative but-

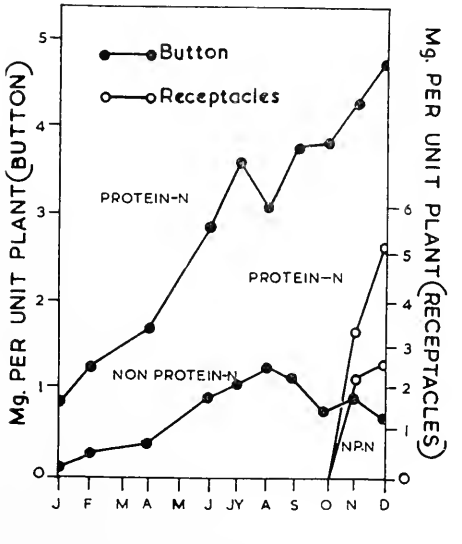

FIG. 1

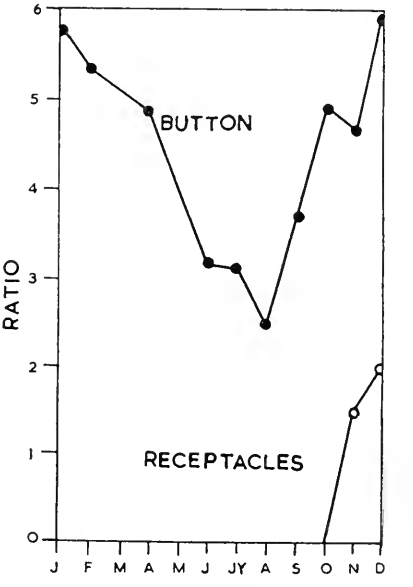

FIG. 2

FIGURE 1. Variation in nitrogen during development of young plants.
FIGURE 2. Ratio protein : non-protein nitrogen during development of young plants.

tons exhibit a fluctuation in carbohydrates associated with the production of the receptacles. It is seen that the laminarin falls to a much lower level after receptacle formation in October. A similar fall in mannitol and fucoidin also occurs at this time. In the young receptacles the mannitol and fucoidin increase during the last two months of the year.

These latter results indicate the building-up of a reserve of carbohydrates throughout the early months of the year, which, with the increased photosynthesis during the summer, reaches a high concentration in the button. However, with the advance of the winter months this reserve is utilized for the rapid development of

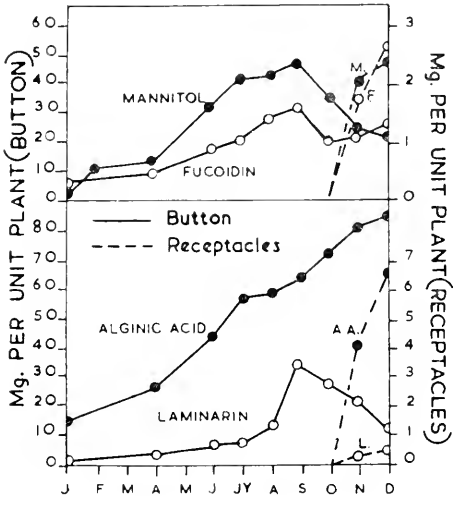

FIG. 3

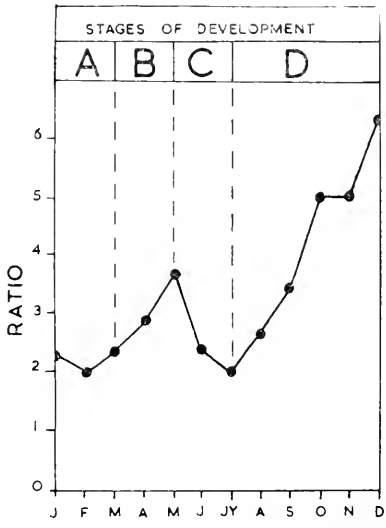

FIG. 4.

FIGURE 3. Variation in carbohydrates during development of young plants.

FIGURE 4. Variation in the ratio protein : non-protein nitrogen during development of receptacles in their second year of growth. A: Period of slow growth; conceptacles absent. B: Period of steady growth and development of conceptacles. C: Period of rapid growth and development of gametes. D: Cessation of growth, extrusion of gametes, and breakdown of receptacles.

the receptacle initials which, by repeated apical growth, give rise to the reproductive thongs. Concomitant with this there is, in the button, a fall in mannitol, fucoidin and laminarin (Fig. 3). The receptacles, after their appearance, carry on their own metabolic activity and synthesize their own carbohydrates.

The ash content of *Himanthalia*, like that of most marine algae, is considerably higher than that found for land plants. With the growth of the young plants there is a relatively high rate of accumulation of minerals from the surrounding sea water, particularly during the early months of development.

2. *The mature plants*

In the mature plants, during the second year of growth, the button remains relatively constant in fresh and dry weight (Table II). The receptacles, however, ex-

TABLE II

Variation of constituents during development of mature plants, 1954
(*The vegetative button*)

Month	Weight of plants gm./unit plant		Nitrogenous constituents mg./unit plant						Carbohydrate constituents mg./unit plant				Total ash mg./unit plant
	Fresh	Dry	Total N	Protein N	Non-protein N	Volatile base N	Free amino N	Peptide N	Alginic acid	Mannitol	Laminarin	Fucoidin	
January	2.19	0.39	4.95	4.07	0.88	0.21	0.21	0.53	83.3	39.0	18.0	42.4	125.0
February	2.51	0.45	5.68	4.77	0.71	0.15	0.17	0.42	—	37.8	—	42.6	162.0
March	2.17	0.40	5.40	4.47	0.83	0.16	0.16	0.38	88.4	22.1	—	23.5	147.0
April	1.88	0.38	4.58	4.00	0.58	0.12	0.12	0.33	88.7	25.6	8.1	—	136.0
May	2.17	0.40	5.02	4.29	0.73	0.13	0.22	0.40	88.7	28.5	—	23.3	144.0
June	1.97	0.37	4.92	4.29	0.63	0.13	0.20	0.31	83.4	32.8	—	27.9	138.0
July	1.95	0.40	5.60	5.06	0.54	0.12	0.21	0.32	88.0	30.6	—	—	116.0
August	1.95	0.38	5.41	4.85	0.56	0.10	0.19	0.25	84.0	29.1	9.5	33.9	105.0
September	—	—	—	—	—	—	—	—	—	—	—	—	—
October	1.81	0.35	4.74	4.23	0.51	0.11	0.26	0.20	77.5	21.5	—	—	100.0
November	1.62	0.33	4.81	4.37	0.44	0.13	0.18	0.18	75.2	20.9	7.3	30.5	95.0
December	1.64	0.33	4.77	4.32	0.45	—	—	—	—	—	—	—	—

hibit marked changes. During the early months of the year, growth of the receptacles, as determined by the fresh and dry weights, is slow, but steady. In the spring and early summer there is an increase in both fresh and dry weights, reaching a climax during the month of July, when the fresh weight of the receptacles has increased some five-fold. This increase is not due to water uptake and turgor extension of the cells alone because a similar five-fold increase in the dry weight also occurs (Table III). After the month of July, with the approach of the winter months, the fresh and dry weights of the receptacles begin to fall. From October to December this decrease is rapid. The receptacles undergo several anatomical changes during this period of growth. For the first two months of the year the receptacle tissue is non-fertile. From March to May, conceptacles develop. From May to July there is a rapid development of conceptacles, terminating in the pro-

TABLE III

Variation of constituents during development of mature plants, 1954
(*The receptacles*).

Month	Weight of plants gm./unit plant		Nitrogenous constituents mg./unit plant						Carbohydrate constituents mg./unit plant				Total ash mg./unit plant
	Fresh	Dry	Total N	Protein N	Non-protein N	Volatile base N	Free amino N	Peptide N	Alginic acid	Mannitol	Laminarin	Fucoidin	
January	0.67	0.08	2.24	1.56	0.68	0.07	0.08	0.23	—	—	—	—	—
February	1.19	0.14	3.85	2.50	1.35	0.10	0.15	0.42	—	11.2	—	—	—
March	1.58	0.19	5.32	3.73	1.59	0.14	0.22	0.60	30.6	18.7	2.2	6.3	81.0
April	2.14	0.27	7.63	5.65	1.98	0.17	0.32	0.60	34.6	23.2	2.6	10.8	134.0
May	9.76	0.87	24.57	19.11	5.46	1.04	1.16	1.91	143.9	59.4	6.9	28.5	446.0
June	26.24	2.81	78.51	55.38	23.13	7.64	3.85	8.46	477.4	152.5	31.8	82.9	1324.0
July	112.80	14.97	347.00	231.14	115.86	40.00	21.26	41.62	2776.5	938.5	308.4	473.0	6719.0
August	101.50	13.37	251.49	182.50	68.99	14.97	11.17	23.40	2535.0	1458.8	266.0	764.8	6000.0
September	95.80	13.89	243.91	189.46	54.45	12.83	10.69	21.81	2868.3	830.1	314.3	923.5	6376.0
October	89.50	12.28	203.47	169.21	34.26	7.00	9.70	11.79	2773.5	746.6	391.7	—	5511.0
November	58.62	7.60	126.38	105.81	21.20	3.57	3.80	8.44	2009.4	266.0	195.3	—	3405.0
December	16.00	2.05	34.60	29.86	4.74	—	—	—	540.8	51.3	43.3	—	906.0

duction of antheridia and oogonia. By August the receptacles are "ripe" throughout their entire length, except for some 5 to 6 cm. above the button which remains sterile. Growth ceases and there is a gradual extrusion of the gametes in a basipetal sequence. Disintegration from the tips of the receptacles then follows. This occurs comparatively slowly over the months of August to October, but thereafter very quickly, so that by January of the next year only a few plants remain, the majority having completely disintegrated.

Very little change, if any, occurs in the nitrogenous constituents of the button in the second season after it has reached maturity. Most of the nitrogen in the button is in the form of protein; very little non-protein nitrogen is present. The receptacles show great fluctuations in their nitrogen content (Table III). The protein and non-protein nitrogen increases steadily during the early months of the year, but from March to July there is a rapid increase, particularly in protein. From August to the end of the year the nitrogen content decreases. The ratio protein : non-protein nitrogen (Fig. 4) shows a correlation between the ratio and the stage of development reached by the receptacles. During the early months of the year when growth of the receptacles is slow but steady, the ratio gradually rises in favor of protein synthesis within the receptacles. Between May and July the ratio falls sharply and this is coincident with the period of rapid growth and development of gametes within the conceptacles. This suggests that the early synthesis of protein is associated with extra conceptacle tissue, and that, at a later date, this protein is broken down to soluble constituents and transported to the conceptacles where re-synthesis takes place in the production of the gametes, resulting in the increase in the ratio from August to the end of the year. From October onwards the gradual extrusion of the gametes and the wearing away of receptacle tissue keep pace with one another and the ratio continues to rise. It is seen that the peptide nitrogen exceeds the free α-amino nitrogen in the soluble non-protein constituents of the mature receptacles.

During the production of the receptacles there was a proportion of the soluble non-protein nitrogen which could not be accounted for. Nitrate nitrogen could not be detected in the plants at any stage of development. It was therefore considered to be some nitrogenous substance associated with reproduction such as nucleic acid.

In the button the carbohydrates remain relatively constant throughout the year. Like the young vegetative button the "old" tissue remains high in alginic acid content. The mannitol is low, similarly laminarin and fucoidin, the latter being higher than the laminarin and similar in magnitude to the mannitol. Throughout their growth the receptacles are particularly low in fucoidin and laminarin. Alginic acid is the major constituent of the receptacle tissue, mannitol being slightly lower. During the early months of the year there is little change in the content of alginic acid and mannitol. However, with increased photosynthesis in the spring and summer months both these constituents increase rapidly. After the extrusion of the gametes the alginic acid and mannitol are considerably reduced.

With the increased growth the accumulation of inorganic ions by the developing receptacles occurs during the months of January to July and thereafter falls following the disintegration of the tissue. The mature button remains comparatively uniform in mineral ash content throughout the year, although there is a tendency toward a falling-off during the latter part of the plant's existence.

General Discussion

During the present investigation, variations in chemical constituents have been followed throughout the development of *H. elongata*. Other workers have generally selected their plants at certain times of the year, or have used well established and fully developed plants for their investigations, failing to include the young stages. This is particularly the case where the grapnel has been used for collection, for this method selects only the larger algae. The number of individuals analyzed from one habitat is also of great importance. The results obtained by Black (1949, 1950b) for the Laminariaceae were based on results of the chemical analysis of two plants per month. Baardseth and Haug (1952) have since shown that certain constituents of marine algae vary considerably from one plant to another in a uniform

Table IV

Expression of results obtained for the receptacles of mature plants during the months of April and August 1954

Month	Protein N	Ash	Alginic acid	Mannitol	Laminarin	Fucoidin
a. Results expressed as mg. per gm. dry weight						
April	21.04	500.0	128.0	86.6	13.4	39.9
August	13.65	445.7	189.6	109.6	19.9	32.2
Month	Protein N	Ash	Alginic acid	Mannitol	Laminarin	Fucoidin
b. Results expressed as mg. per unit plant						
April	5.63	134.0	34.6	23.2	2.6	10.8
August	182.50	5959.0	2535.0	1458.8	266.0	430.5

population, even when collected from the same habitat. The ash and alginic acid contents of 55 *L. digitata* plants which they collected at one time showed variations as great as those obtained by Black throughout the whole year.

The majority of studies concerning the chemical composition of marine algae have been carried out by chemists concerned with the possible utilization of algae for industrial purposes. These studies, based on the variation of chemical constituents expressed as a percentage of the dry weight, have lead to considerable misconceptions of the physiological problems of growth and development in marine algae. For example, Black (1948a, 1948b, 1949, 1950b) found that the Laminariales and the Fucales exhibit a decrease in "crude protein" content expressed as a percentage of the dry weight, during the period of rapid growth. He attributed this decrease of protein to the lack of nitrate in the sea water at that time of the year. One can hardly accept the statement made by Black (1950b, p. 52) that "rapid growth of the plant results in a decrease in the crude protein content." Where an increase in growth occurs, increase in protoplasm must be the determining factor. An increase in protoplasm with a decrease in protein is difficult to imagine, particularly as protein is one of the major components of protoplasm.

At the time of maximum growth in *Laminaria* and *Fucus* the carbohydrates and other substances, particularly ash, are high. An increase in these other substances will result in an *apparent* decrease in the protein content expressed on a dry weight basis. Had Black expressed his results on a unit plant basis a truer picture of growth and the variations accompanying growth and development would have been made, as the examples for the receptacles of *Himanthalia* illustrate (Table IV). A decrease in protein, ash and fucoidin content between April and August occurs when the results are expressed on a dry weight basis. In the actual plant, however, these constituents have greatly increased over this period (Table IVb). As a result of such an increased synthesis of metabolic products the plant has increased in size, form and reproductive development.

Parke (1948), in her studies on the growth of *L. saccharina,* found that the plant reached its maximum weight during June to July after the period of rapid growth. Plants were found reproducing at all months of the year, but the greatest number were found during October and March. Black (1948b, 1949) found that for the fucoids the maximum dry weight of the plants occurred during the period May to June. For *Himanthalia*, the present author recorded maximum fresh and dry weight values during the months July and August. At all these times of maximum weight the plants commence their reproductive phase, producing either spores or gametes. The development of the reproductive tissues will therefore exert a considerable influence upon the chemical composition of the plants.

Moss (1950, 1952) showed that for *F. vesiculosus*, and later for *Himanthalia elongata,* reproduction definitely influenced the chemical composition of the plants. Jacobi (1954) found that when *F. vesiculosus* and *L. saccharina* were grown under favorable conditions, seasonal variations in nitrogen similar to those published by Black occurred, and that prior to reproduction a mobilization of protein was evident. In *L. saccharina* a higher percentage of soluble nitrogen was recorded for the reproductive tissue than the adjacent vegetative tissue. This lead Jacobi to conclude that the increase in nitrogen content of the plant was most probably due to the development of the reproductive tissues which are higher in nitrogen during spore production or gamete formation.

The present work on the change in chemical composition during the growth and development of *Himanthalia* substantiates the findings of Moss (1952) and Jacobi (1954). During the first year of growth the young vegetative plants gradually build up a reserve of nitrogen and carbohydrate constituents which are utilized for receptacle production. The young receptacles, after their initiation in October, continued to grow steadily until the following year, when, during the months of April to July, they grew very rapidly and reached maturity. The fucoidin, mannitol and laminarin, accumulated during the summer months in the young vegetative buttons, on the appearance of the young receptacles, were greatly depreciated, suggesting they were utilized in the production of reproductive tissues. Associated with the receptacle formation there was a rapid synthesis of protein from the non-protein constituents. In the button and also in the developing receptacles the alginic acid content continued to increase. After the establishment of the receptacles the composition of the vegetative button throughout the second year of growth remained relatively constant.

With the increase in the sea temperature and the increased photosynthesis

during the early summer months active metabolism resulted in the extensive growth of the receptacles. The two polysaccharides, fucoidin and laminarin, were very low during this period. Alginic acid, mannitol and mineral ash, however, continued to increase. Utilizing the inorganic nitrogen sources of the surrounding sea water, the receptacles synthesized amino acids and peptides which were later utilized in protein synthesis during the formation of the gametes in the conceptacles. The plant by August reached maturity and the gametes were extruded in a basipetal sequence. This process was accompanied with one of disintegration of receptacle tissue, resulting in a reduction in the chemical composition of the receptacle, until, by December, little remained other than the sterile bases attached to the senescent button. The whole plant then quickly decomposed.

The high ash content of the tissues, particularly of the receptacles, is of interest for the ash content of land plants is usually only represented by about 5 per cent of the dry matter of the plant (Thomas, 1949). Unlike land plants, marine algae are immersed in their nutritive medium, the sea water. In many cases seaweeds concentrate elements several thousand times the concentration found in the surrounding sea water (Black and Mitchell, 1952). The mineral ash consists of inorganic salts, presumably in solution in the cell sap, and cations in combination with such organic substances as alginic acid and fucoidin. Again the reproductive tissues exhibit differences from the vegetative tissues of the plant, insofar as they were found to be considerably higher in ash content. The receptacles contained as much as 50 per cent of their dry weight in mineral ash. Similar high results were found for the receptacles of *F. vesiculosus* (Moss, 1950), particularly when the receptacles were ripe.

The effect of the onset of reproduction upon the chemical composition of plants is not only to be found in marine algae. Variations in nitrogen with development of reproductive tissue have been found to occur in land plants. McCalla (1933), growing wheat in water cultures, found a decrease in nitrogen in the vegetative parts of the plant after the ears had emerged. These findings were later confirmed by Miller (1939) who showed that from the end of May onwards the inflorescences of winter wheat were the only aerial parts of the plants to gain in nitrogen, whereas the stems and leaves lost their nitrogen progressively. Blanck, Giesecke and Heukeshoven (1933) and Blanck and Giesecke (1934) found that maturation of the oat plant was accompanied by a redistribution of nitrogen, which first decreased in the roots and later in the stems and leaves while, on the other hand, it accumulated in the inflorescences. In the barley plant Richards and Templeman (1936) also indicated a pronounced movement of nitrogen from the vegetative parts to the developing grain.

It appears, therefore, that in the life history of *Himanthalia elongata* from germination to maturity and senescence separate parts of the plant undergo their own ontogenetic changes which greatly affect the chemical composition of the plant.

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Summary

1. Monthly variations in chemical constituents have been followed throughout the development of *Himanthalia elongata*.

2. Nitrogen, laminarin, mannitol, alginic acid and fucoidin increase during the first year's growth of the vegetative button.

3. Associated with receptacle initiation, the carbohydrate and nitrogen reserve of the button is utilized and there is a synthesis of protein from the non-protein constituents.

4. The young receptacles build up a reserve of non-protein materials which are later utilized in protein synthesis during the formation of the gametes.

5. It was concluded that during the life history of *Himanthalia elongata* from germination to maturity and senescence, separate parts of the plant undergo their own ontogenetic changes which are correlated with changes in the chemical composition.

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STUDIES ON SHELL FORMATION. VI. THE EFFECTS OF DINITROPHENOL ON MANTLE RESPIRATION AND SHELL DEPOSITION [1, 2]

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Shell formation in mollusks concerns the elaboration of an organic framework or matrix and the orderly growth of calcium carbonate crystals within this matrix. This matrix is composed of three fractions: a water-soluble protein, a scleroprotein, and a polypeptide (Grégoire, Duchâteau and Florkin, 1955). Synthesis and secretion of matrix and any active transport of inorganic ions involved in shell deposition will require metabolic energy. Glycolysis (Humphrey, 1950) and the utilization of tricarboxylic acid cycle substrates (Humphrey, 1947; Humphrey and Jeffrey, 1954; Cleland, 1951; Jodrey and Wilbur, 1955) have been demonstrated in oyster tissues and would lead to the formation of high energy phosphate compounds. If these high energy phosphate compounds are utilized in shell deposition, then dinitrophenol (DNP) by preventing phosphorylation (Hunter, 1951; Lardy and Wellman, 1953; Shacter, 1955) or reducing the tissue concentration of the compounds already formed (Hunter, 1951; Shacter, 1955) may be expected to retard shell deposition. This problem has been examined in the oyster *Crassostrea virginica*. The action of dinitrophenol was studied with respect to (1) respiration of shell-forming tissue; (2) calcium deposition using radioactive calcium as an indicator; and (3) shell regeneration in the whole oyster.

Methods

Measurements of the effect of DNP on respiration were carried out by the Warburg method using a strip of tissue about one cm. wide taken from the posterior portion of the mantle. Endogenous respiration was measured in sea water for 30–60 minutes. DNP was then added from the sidearm, and measurements were continued for periods of one to six hours. DNP solutions were buffered at pH 8 with 0.03 *M* glycine.

The isolated mantle-shell preparation (Hirata, 1953) was utilized for measurement of Ca^{45} deposition as described by Jodrey (1953). These mantle-shell preparations were placed in one liter of sea water containing DNP for one hour. Ca^{45} (one ml. of high specific activity) was then added and the preparations remained in this solution for an additional six hours. Reversibility of DNP action on calcium deposition was tested by first immersing mantle-shell preparations in DNP solu-

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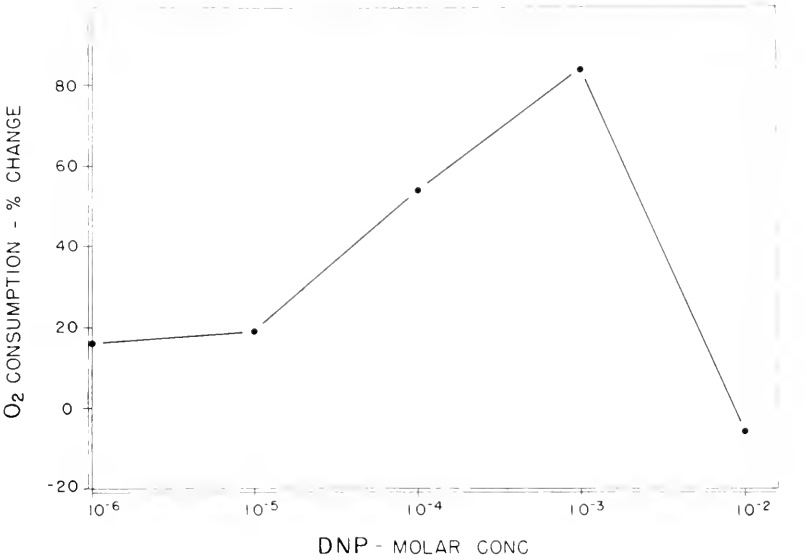

FIGURE 1. Effect of DNP on mantle respiration. Tissue weight, 180–220 mg. wet weight; temperature, 25.6° C.; pH 8.0; DNP, 0.2 ml. in sidearm; total volume 2.2 ml.; gas phase, air; salinity, 33.6–35.4 ‰; mean oxygen consumption of control, 15.6 μl O_2 per 100 mg. wet weight per hour.

tions for seven hours and then washing them in running sea water for two hours. The preparations were then placed in sea water containing Ca^{45} for six hours and the deposition of Ca^{45} was measured. Control preparations were carried through the same procedures but without DNP.

TABLE I

DNP effect on Ca^{45} deposition

DNP conc.	*n*	DNP added counts/min.*	*n*	No DNP counts/min.	P
10^{-3} *M*	5	35 ± 24	5	323 ± 179	<0.01
10^{-4} *M*	12	69 ± 37	12	193 ± 143	<0.01
10^{-5} *M*	9	376 ± 171	9	481 ± 205	0.3 > P > 0.2

Figures in columns three and five show mean calcium deposition by isolated mantle-shell preparations with standard deviations; *n* gives the number of cases. The concentration of Ca^{45} added per liter varied from 5.25 μc to 12.75 μc. For uniformity of presentation calcium deposition is expressed as counts per minute per 10 μc added per liter sea water per 6.2 cm.² of shell. Temperature 25–26° C.; salinity 31.7–34.0 ‰. The experiments were not all carried out at the same time which probably accounts for the differences between the three mean values in column five.

* Shells without mantle were included along with the mantle-shell preparations in these experiments in order to obtain a measure of Ca^{45} exchange. However, it is not certain that the concentration of calcium for exchange is the same for both (Wilbur and Jodrey, 1952), and accordingly exchange corrections have not been made in the figures in Table I. When exchange values are subtracted from the deposition in the mantle-shell preparations the statistical significance is essentially that given.

Table II

Reversibility of DNP effect on Ca^{45} deposition

n	DNP added, then washed	*n*	No DNP, then washed	P
12	129 ± 93	12	87 ± 34	$0.2 > P > 0.1$

Details given in Methods and footnote of Table I. DNP concentration, 10^{-4} *M*.

The action of DNP on shell regeneration was studied on intact oysters with shells notched at the posterior edge and placed in 200 ml. or 1000 ml. of sea water containing various concentrations of DNP. All solutions were aerated and were changed daily. The extent of regeneration was determined microscopically.

Oysters were collected near Beaufort, N. C., and maintained for two weeks prior to use in natural waters or in tanks with running sea water supplied through hard rubber lines.

Results

DNP caused a stimulation of mantle respiration at concentrations of 10^{-3} *M* and 10^{-4} *M* (Fig. 1). This increased respiration remained constant for at least six hours. The concentration of DNP which caused maximum respiratory stimulation (10^{-3} *M*, Fig. 1) gave essentially complete inhibition of Ca^{45} deposition by mantle-shell preparations (Table I). The same relationship of respiratory stimulation and inhibition of Ca^{45} deposition held true for 10^{-4} *M* DNP (Fig. 1 and Table I). In further experiments the inhibitory action of 10^{-4} *M* DNP on Ca^{45} deposition was found to be reversible (Table II). When the concentration of DNP was lowered to 10^{-5} *M* and 10^{-6} *M*, there was no statistically significant effect on mantle respiration (Fig. 1). Likewise, at 10^{-5} *M*, DNP had no significant effect on the deposition of Ca^{45} by the mantle-shell preparations (Table I).

Shell regeneration in the presence of DNP was inhibited as the concentration of the inhibitor was increased (Table III). The inhibitory concentrations of DNP were about the same for both calcium deposition by the mantle-shell preparation and for regeneration in the whole oyster (*cf.* Tables I and III). The last column in Table III indicates that DNP is toxic. However, of 20 oysters which were dead by the eleventh day of DNP treatment (all DNP concentrations), 10 showed regeneration. In those cases in which regeneration occurred, the structure of the

Table III

Effect of DNP on shell regeneration

DNP conc.	*n*	Oysters showing regeneration in DNP	Comments
0	10	10	No deaths in 14 days
10^{-5} *M*	10	8	1 dead after 14 days
5×10^{-5} *M*	10	7	9 dead after 11 days
10^{-4} *M*	10	3	9 dead after 5 days

Temperature 24.0–29.5° C.; pH 7.7–8.1; salinity 18.0–35.99 ‰. Regeneration occurred in both valves in all but one case.

shell as observed microscopically was the same in DNP-treated oysters and oysters in sea water.

Discussion

The effects of DNP on the respiration of oyster mantle followed the general pattern as seen in many animal and plant tissues (Simon, 1953). Mantle respiration increased with increasing concentration of DNP, reaching a maximum 87% above the endogenous level. This degree of stimulation was considerably higher than that produced in the mantle by the addition of citric acid cycle intermediates (Jodrey and Wilbur, 1955). At concentrations of DNP which caused a respiratory stimulation, Ca^{45} deposition by the oyster mantle was reversibly inhibited.

The action of DNP may involve one or more of the following mechanisms concerned with shell deposition: (1) transport of calcium and carbonate ions across the mantle; (2) transfer of $CaCO_3$ crystals from the interior of mantle cells; (3) synthesis and secretion of the shell matrix; or (4) amoebocyte activities and shell regeneration. If the transport of the calcium and carbonate ions across the mantle requires the expenditure of energy, this active transport could well be inhibited by DNP (Taggart and Forster, 1950; Mudge, 1951; Levinsky and Sawyer, 1953). The transport of calcium carbonate crystals from the cell interior, as suggested by Bevelander (1953), might utilize an energy mechanism susceptible to the action of DNP. In the regeneration experiments failure of intact oysters to deposit matrix alone in the presence of DNP may be due to an effect on matrix synthesis since peptide bond and protein synthesis are known to be inhibited by DNP (Borsook, 1954; Tarver, 1954; Siekevitz, 1952). However, in view of the toxicity of DNP (Table III) one should not conclude that failure of regeneration reflects only a direct effect on matrix synthesis or secretion.

Another possibility arises from the fact that in the snail *Helix aspersa*, amoebocytes play a part in shell regeneration and are thought to be responsible for the deposition of matrix and calcium carbonate crystals (Wagge, 1955). However, the role of amoebocytes in oyster shell regeneration has not been studied and accordingly we do not know their significance with respect to the inhibition of regeneration by DNP.

Summary

1. Dinitrophenol stimulated oyster mantle respiration at 10^{-3} *M* and 10^{-4} *M*. Respiration was not significantly different from the endogenous rate at 10^{-2} *M*, 10^{-5} *M* and 10^{-6} *M* DNP.

2. Calcium deposition, as measured by Ca^{45}, was inhibited in isolated mantle-shell preparations at 10^{-3} *M* and 10^{-4} *M* DNP, the same DNP concentrations which stimulated respiration. Inhibition was found to be reversible.

3. Shell regeneration in whole oysters was inhibited by DNP. DNP concentrations which inhibited regeneration were toxic.

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TEMPERATURE DEPENDENCE OF BREATHING RATE IN CARP

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Temperature is an essential factor in the aquatic environment; it exerts a profound influence on the morphologic and physiologic characteristics of fishes. To infer from this the importance of temperature in adaptation, in its genetic meaning, seems to be logical.

An adequate study of this adaptation presupposes easily recognizable adaptive variants within a population. Our original intention was to work out an easy method to detect such physiologic variants which would not involve necessarily the death of the experimental objects, for breeding purposes. For several reasons—among which figure the data of Fox (1939) concerning the frequencies of respiratory movements with regard to the geographical distribution of poikilotherms—we have studied the same frequencies in *Cyprinus carpio* L. in relation to temperature. It appeared soon that the breathing rate in this fish is highly dependent on size. As a first contribution to the problem we intended to solve, this paper reports the influence of age and size on the mentioned physiologic trait.

Material and Methods

Our experiments have been performed on *Cyprinus carpio,* belonging to the race of glass-carps from the temperate warm waters. The best temperature for development for *Cyprinus carpio* would be situated, according to Huet (1953), between 20° and 25° C.; according to Schaeperclaus (1949) the optimum is nearer to 27° C. but at 15° C. a good production is still obtained.

As experimental objects we used fishes of three months, one year, two years and four years old, having live weights from 20 to 2000 grams. The fishes have been obtained from a single commercial stock.

A starvation period of at least 48 hours preceded all experiments.

Each individual of the different weight and age classes has been examined at several temperatures, from 4° C. up until death occurred, at about 38° C. Transfers always took place from lower to higher temperatures. Intervals can be read from the graphs to be discussed presently.

During measurements each temperature was kept constant within 0.1°, and the water abundantly aërated to provide a constant saturation with oxygen.

For practical purposes the fishes were put in small wire baskets. Measurements have been performed only on perfectly quiet animals.

Results

The dependence of the frequency of respiration upon the temperature in a single individual

1. Temperature acclimation

By temperature acclimation we mean the temporary adaptive alteration of the phenotype after an external temperature change.[1] It is known from Wells (1935a)

[1] We wish to reserve the term *adaptation* for a similar alteration of the genotype, which is included in the definition of *acclimation* or *acclimatization* by Prosser (1955) and by Bullock (1955).

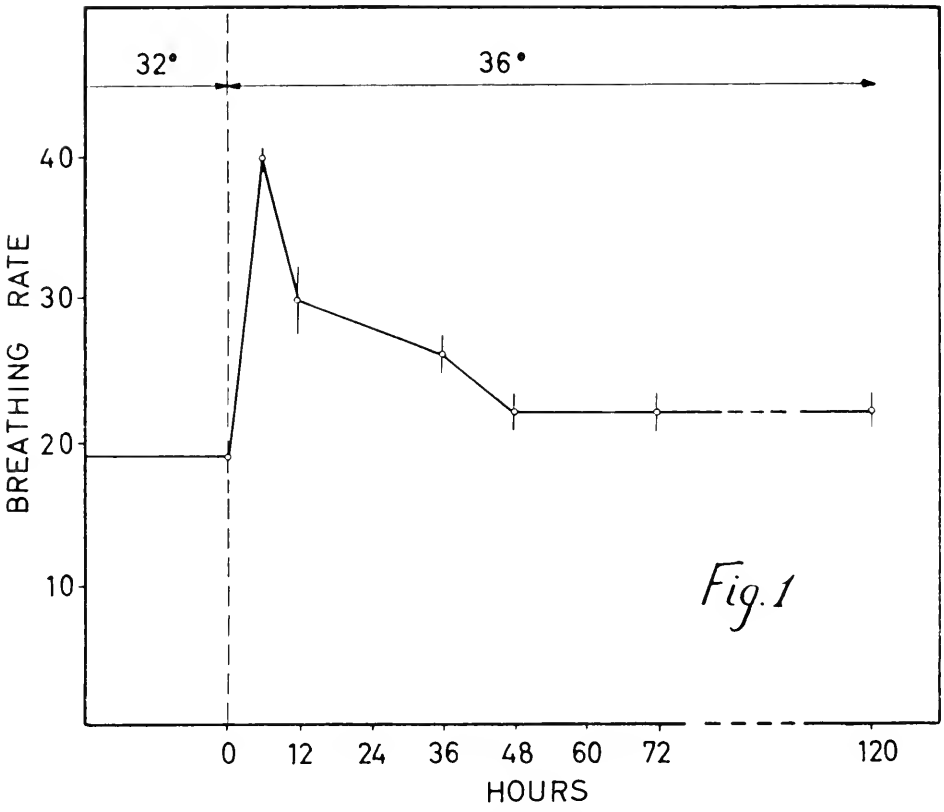

FIGURE 1. Successive mean respiration frequencies after transfer from 32° to 36°, plotted against acclimation time (carp No. 16). Vertical bars added to the points cover the range $M \pm \sigma$.

TABLE I

Frequency of respiration as a function of temperature
(Carp No. II)

Temperature (° C.)	Duration of respiratory pauses	Number of pauses per minute	Breathing frequency
4	1–2 mins.	1	2
8	½–2 mins.	2–1	6
12	15 secs.	3	9
18	—	—	24
15	16 secs.	3	13
20	10 secs.	5	24
26	10 secs.	5	25
29	10 secs.	5	24
34	10 secs.	5	24
35	—	—	24
37.4	3 secs.	15	32

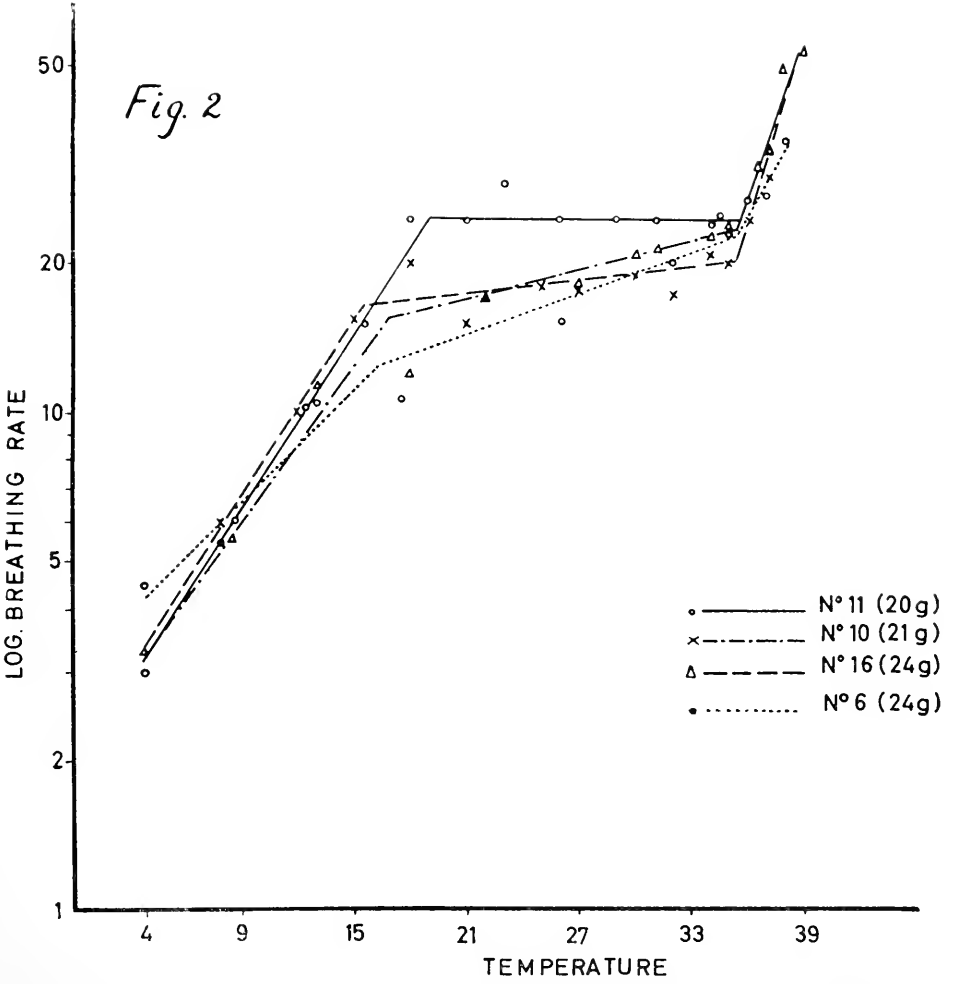

FIGURE 2. Rate–temperature curves for breathing frequency in carps of 20 to 24 grams.

and Schlieper (1950), that, in fishes, this acclimation takes from 24 hours to a few days after the transfer from one temperature to another. Only after this lapse of time, constant and consistent results, on metabolism at least, have been obtained. Before acclimation is completed, the oxygen consumption is generally higher than normal.

Figure 1 shows that similar phenomena characterize the accommodation of the frequency of respiratory movements. This frequency, after an initial rise of 100%, reaches a stable value after 48 hours following a transfer from 32° to 36° C. Usually a steady-state is reached within three to four days, although rarely it may last fourteen days. Acclimation is generally shortest between 16° and 30°. All fishes which maintained this steady-state for 48 hours were considered as completely acclimated. Only measurements obtained on such fishes are considered further in this paper.

At given high temperatures the fishes die before having attained complete acclimation as defined. These temperatures are indicated in this paper as the upper lethal temperatures.

2. Respiratory pauses and frequency of respiration at a given temperature

Typical for the respiration of fishes, especially of small ones, are the so-called pauses, during which no respiratory movements occur. After such a pause, which may be variable in length, the fish breathes a few times at a rapid rate and then pauses again. The duration and the frequency of the pauses vary with the temperature (Table I).

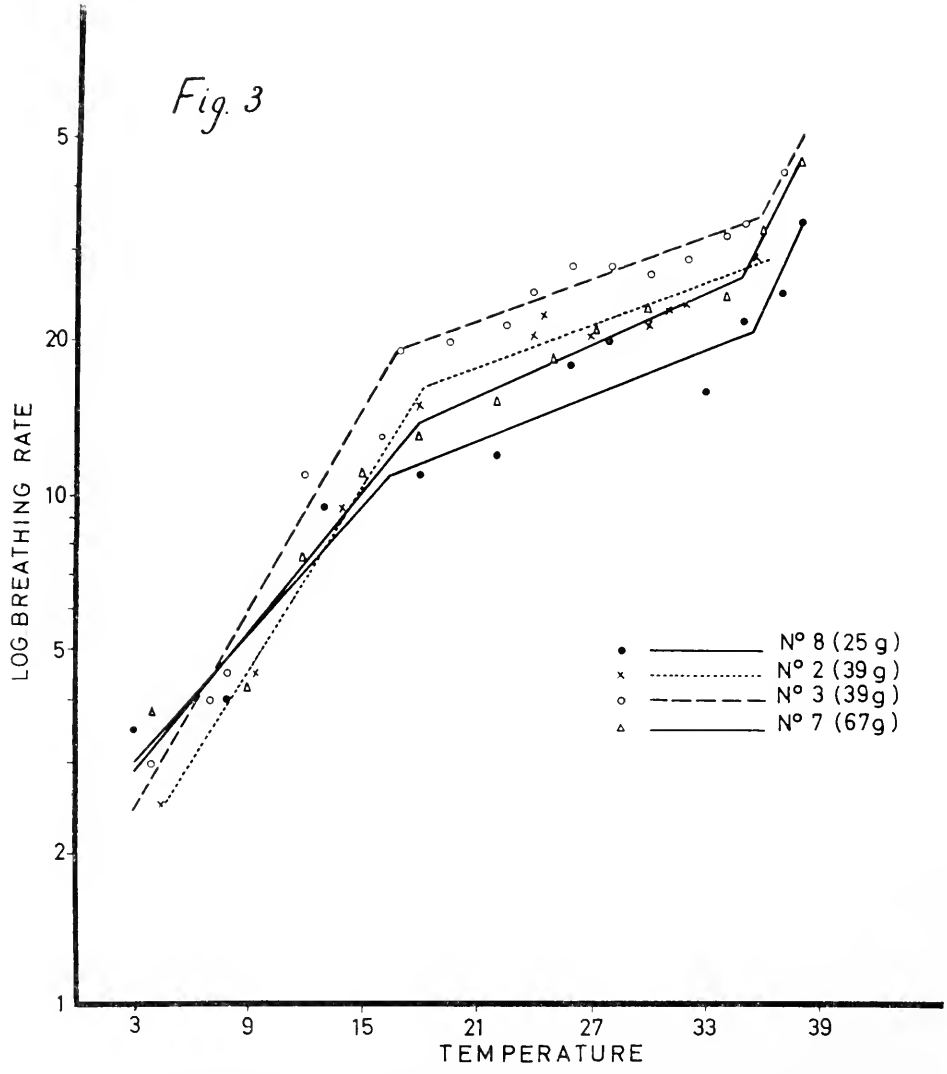

FIGURE 3. Rate–temperature curves for breathing frequency in carps of 25 to 67 grams.

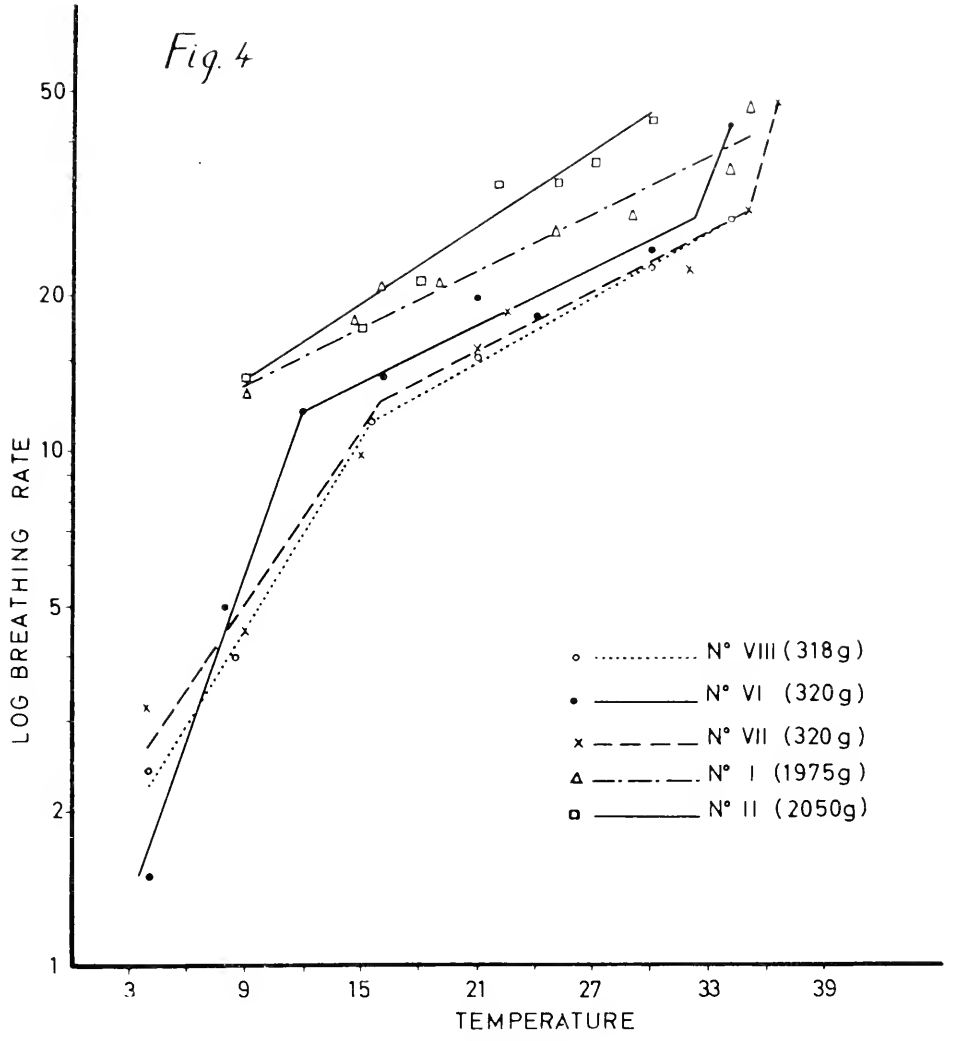

FIGURE 4. Rate-temperature curves for breathing frequency in carps of 318 to 2050 grams.

In this paper breathing frequency is expressed as movements per minute, ignoring the pauses. The time intervals used for one count were chosen long enough to eliminate the disturbing influence of the pauses. As a rule these intervals were five minutes at low, and two minutes at high temperatures, the counts being repeated at each temperature as many times as needed for adequate statistical treatment. Immediately after transfer to a higher temperature no respiratory pauses may occur, making for lower standard deviations (Fig. 1). Otherwise no trends in standard deviations could be detected during acclimation, with respect to time, or after acclimation, with respect to temperature.

3. Respiration frequency as a function of temperature

The variations of the mean frequencies of respiratory movements with respect to temperature, show, in the case of the smaller fishes, a typical pattern: first, at lower temperatures, a progressive increase of the frequency with a rise in temperature; further a less steep or even horizontal central part, where the frequency in-

TABLE II

Q_{10}-values, breathing rates at 24° and lethal temperatures for carps, arranged according to weight and age*

Serial number	Weight in grams	Age in years	Q_{10}			Rate at 24°	Lethal temperature
			Low	Intermediate	High		
11	20	$\frac{1}{3}$	2.10 (4–19)	1.00 (19–35.4)	2.82 (35.4–37)	24.6	38
10	21	$\frac{1}{3}$	2.13 (4–15.6)	1.10 (15.6–35.4)	3.84 (35.4–38.4)	18.0	38.5
6	24	$\frac{1}{3}$	1.98 (4–17)	1.22 (17–35.2)	3.36 (35.2–38)	18.2	39
16	24	$\frac{1}{3}$	1.69 (4–16.3)	1.30 (16.3–35.3)	2.28 (35.3–37.8)	15.9	38
8	25	$\frac{1}{3}$	1.84 (3–16.5)	1.31 (16.5–35.4)	2.31 (35.4–37.5)	14.2	38
2	39	$\frac{1}{2}$	2.07 (4.7–18.3)	1.26 (18.3–35)	—	19.5	39
3	39	$\frac{1}{2}$	2.16 (4.7–17)	1.31 (17–35.5)	2.66 (35.5–37)	14.0	39
7	67	2	1.89 (4–18)	1.37 (18–35)	2.66 (35–37.7)	17.6	38
VIII	318	2	2.19 (4–15.5)	1.40 (15.5–34)	—	17.4	36
VI	320	2	2.83 (3.5–12.3)	1.35 (12.3–32)	—	20.2	36
VII	320	2	2.08 (4–16)	1.34 (16–35)	4.20 (35–36.5)	18.2	37
IX	325	2	—	—	—	—	35.8
I	1975	4	—	1.39 (9–35)	—	25.9	36
II	2050	4	—	1.51 (9–30)	—	33.0	36

* The temperature limits, in degrees C., are indicated in brackets.

creases much less with temperature, and finally a sudden rise of the respiratory rate at a point which is near to the lethal temperature.

This pattern is particularly well recognizable when the data are plotted on semilog coordinates, showing the proportionate response of the breathing rate to the temperature (Figs. 2, 3 and 4). This proportionate response appears to show, over broad temperature ranges, practically constant values, which change abruptly to other such values, at given critical temperatures. From eye-fitted curves through the data for individual fishes Q_{10}-values for each of these ranges can be readily estimated, while their intersections localize, with a fair approximation, the critical temperatures. The lower critical temperatures appear to be situated between 12.3° and 19°, the higher between 32° and 34.5° (Table II). The lower critical temperatures delimit a first zone of high Q_{10}-values from 1.69 to 2.83. Within the range extending from the low to the high critical temperatures Q_{10}-values vary from 1.00 to 1.51. Within the range of high temperatures Q_{10}'s reach values between 2.28 and 4.20. Because of the short interval separating the high critical and the lethal temperature, only a few data can be obtained in this range, making for a limited significance of these last Q_{10}'s for most fishes.

Nevertheless it is perfectly clear that for the population of carps studied, typical regulation phenomena characterize the respiratory rates over given homeostatic zones between 12.3° and 34.5°. However, within these zones a considerable individual variation, as to the Q_{10}'s, as well as to the situation of the critical temperatures, is showing up.

a. The influence of weight

A closer inspection of the log R-T curves in connection with the weight variants, leads readily to the recognition of the following facts.

First, all small fishes (39 grams and lower) exhibit more effective regulatory mechanisms than larger fishes. In the first category all Q_{10}'s, within the homeostatic zone, fall between 1.00 and 1.31, and, moreover, the correlation between weight and the respective values is very close (Table II). All larger fishes, on the other hand, have Q_{10}'s between 1.34 and 1.51 and, again, the largest fishes have very low regulatory capacities.

Concomitant with the occurrence of lower Q_{10}'s within the homeostatic zones of larger fishes, is the lowering of the upper lethal temperature threshold of the same fishes, with respect to the small fishes. Fishes of 67 grams and less have lethal temperatures at 38° and 39°, while those of higher weights have lethal temperatures between 35.5° and 37° On the other hand, the largest fishes (± 2000 grams) fall easily into a lethargic state at 4°, where the measurement of barely recognizable respiratory movements is no more possible. Therefore, a subdivision of the R-T curves of large fishes into three, with differential regulatory characteristics, seems to be impossible. Their narrowed viable range of temperatures can be characterized only by a single, high Q_{10}-value.

A last remarkable feature is the variation of the mean respiratory rate. The different individual R-T curves are clearly situated at unequal over-all rate levels. It is difficult to express this shift of the curves along the ordinates. We have tried to do this by indicating the estimated rate for all fishes at 24° (Table II). It appears, then, that the largest fishes have very high rates, smaller fishes attaining

only about half of these rates. The smallest fish, however, shows again a high over-all rate.

b. The influence of age

On the basis of our data it is not possible to differentiate clearly the effect of age from the influence of size. A certain indication is obtained from fish No. 7 (Table II). This fish was intentionally starved during one year in the laboratory. During that year its weight practically remained constant (67 grams), and was far below the weight of normally fed fishes of the same age (± 320 grams). For as far as the lethal temperature is concerned (38°) this fish reacted according to its weight class. Its Q_{10} within the homeostatic zone, however, falls within the range of its age group (1.37).

c. Residual variation

The number of individuals studied within each weight and age class does, of course, not allow an estimation of the individual variation of breathing rate within a homogeneous group. From our data we can, however, infer its existence. Most interesting, in this respect, is the fact that the R-T curve of fish No. VI is, in its whole, shifted to lower temperatures, for a distance of about 3°, when compared to fish No. VII, belonging to the same weight and age group (Fig. 4 and Table II). This might indicate a differential adaptation to temperature of both fishes.

Individual variation, with respect to the efficiency of the breathing regulation within homeostatic zones of equal extension, seems to exist as well. This is indicated by the detected differences in Q_{10}'s within age and weight classes.

Discussion

Two main points of interest are revealed by our analysis. The first is the shift of the upper lethal temperatures from 38°–39° for small fishes, to 35–36° C. for large-sized individuals. The second is the gradual disappearance of homeostasis for breathing frequencies with increase in size.

Decreased resistance to high temperature with size, if we restrict the survey to post-embryonic life, is not a general trend among fishes. The literature concerning the subject is reviewed by Hart (1952). All three types of fish species seem to exist: those with increasing, those with stationary, and those with decreasing resistance with increasing size. In most cases the separate effects of size and age have not been studied. In *Salvelinus fontinalis* no size effect was recorded for yearlings of the same age (Fry *et al.*, 1946), indicating seemingly that the age effect on temperature resistance would be preponderant. However, Spaas (unpublished results) found a size effect in one-year-old trout and a mean increase in high temperature resistance in two- and three-year-old fishes of the same species, which, however was lower than expected on the basis of the size differences for temperature tolerance within yearlings. Both factors, age and size, seem to interact in some way. The few data recorded in this study are not opposed to such a view.

A second main point, disclosed by our analysis, is the progressive increase of dependence of the frequency of respiratory movements upon the temperature. It

is illustrated by the gradual disappearance of a homeostatic zone of practical stability of this frequency at several temperatures in small fishes, and by a correlated increase of the over-all Q_{10}-value of this process in the course of life history.

By reputation, poikilotherms are animals without homeostatic mechanisms regulating their most essential physiologic functions, such as oxygen consumption, heat production, activity and development. More specifically, for oxygen consumption, they have been generally assumed to follow Krogh's "Normalkurve."

However, a certain number of workers have shown that the type of response to temperature of such functions is not only a specific trait among poikilotherms, but even that it varies intraspecifically as well.

According to recent reviews of the data (Rao and Bullock, 1954; Bullock, 1955), it seems justified to admit that in most cases a higher dependence on temperature is characteristic for animals from warmer habitats. A survey of the data on speed of development leads to the same conclusion with regard to specific and to racial geographic variation (Heuts, 1956). More effective homeostatic mechanisms seem to be a distinctive trait of inhabitants of colder areas, especially when intraspecific comparisons are made. Poikilotherms are poikilostatic organisms, but they are such to varying, genetically determined, degrees.

On the other hand, it is clear enough that the homeostatic mechanisms of the homoiotherms also are operating only within a given temperature range in a given individual (the thermoneutral zone), and, further, that the ranges of this zone are equally subject to specific variation in relation to the habitat, northern animals possessing more efficient homeostatic mechanisms operating at low temperatures (Scholander *et al.,* 1950).

The conclusion is obvious, and has been drawn, that a homeotherm is clearly adapted to the temperature range where the Q_{10} for biological processes appears to be equal to 1.00, and where, consequently, its behavior is most homeothermic.

In poikilotherms such homeostatic zones, with Q_{10}'s approaching 1.00, are generally not found or not recognized.

Only recently Bullock (1955) strongly called attention to the existence of homeostatic mechanisms for a number of rate processes among several poikilotherms. In addition to the cases cited by this author a few other examples may be mentioned here.

A clearly delimited homeostatic zone for oxygen consumption is indicated, although not recognized by the author, in *Fundulus parvipinnis* between 18° and 22° for small fishes (Wells, 1935a, 1935b). Its existence shows up due to almost continuously increasing experimental temperatures between 10° and 24°. A definite fall of Q_{10} values for oxygen consumption between 15° and 20° in *Salvelinus fontinalis* is demonstrated by Job's data (1955). Unfortunately no measurements have been made beyond 20°. The same author mentions the results of Edwards (1946) on the click beetle *Melanotus communis,* which, between 17° and 27°, shows a definite decrease of Q_{10}-values for oxygen uptake. Job, however, is inclined to regard this phenomenon as due to an experimental error.

A number of indications seem thus to point to a possibly widespread phenomenon of homeostatic mechanisms among poikilotherms, which may be brought to light by more accurate experimental procedures. These zones being presumably narrow, their absence in current observations might be due to the experimental

procedure making use of too discontinuous temperature gradients. Still another source of error might be an insufficiently long acclimation to the experimental temperatures.

If, however, homeostatic zones can be demonstrated to exist in poikilotherms, then it seems logical to admit, as is done in homeotherms, that they delimit adaptive zones, and to conclude that a poikilotherm is adapted to such conditions, where it is least poikilostatic. Such a situation exists clearly in young carps. In the smallest fishes Q_{10}-values approach 1.00 over a temperature range from 16° to 35°. Within this range they are homeostatic for the studied trait, and to this range they are probably best adapted.

How far the frequencies of respiratory movements reflect the oxygen consumptions at different temperatures remains, of course, an open question. They will not truly reflect oxygen consumption, if, principally, the utilization coefficient of the oxygen present in the water, flowing over the gills during each breathing movement, would be different according to temperature. For trout and eel, at least, van Dam (1938) has shown that the utilization percentage is not affected by a temperature difference of 8°.

For obvious reasons, the complete disappearance of homeostatic zones in carps, with increasing size, cannot be asserted. They certainly are narrowed in the course of life, though this progressive alteration is not, or not directly, dependent on age. A similar narrowing of homeostatic zones is obvious in *Fundulus parvipinnis* (Wells, 1935b), concomitant with the over-all higher dependence of oxygen uptake on temperature with increasing size.

In other cases, however, the individual evolution is exactly the opposite. Job (1955) recognizes a flattened proportionate response to temperature, a higher temperature independence, in large-sized *Salvelinus fontinalis*. Similarly Spaas (unpublished results) finds an increased dependence of oxygen consumption in large versus small yearlings of brook- and sea-trout.

The click beetles already mentioned (Edwards, 1946) show, on the other hand, a response of oxygen uptake to temperature per unit wet weight, which is independent of size. The case is, however, not strictly comparable, because all click beetles, whatever their dimensions, have reached final sizes.

Between the first fact disclosed by our analysis (the shift of upper lethal temperature to lower values in the course of life) and the second (the decrease in homeostatic efficiency) there seems to be a close relation in our material, as one would logically expect. Lack of adequate data on post-embryonic development prevents the generalization of this observation for individual life cycles. Some data on embryonic development, however, indicate clearly that a similar relationship does not hold in racial and interspecific comparisons.

Races or species with a higher temperature independence for speed of embryonic development can nevertheless have a narrower temperature tolerance range during development than another race or species with a more dependent development. If the relationship between temperature tolerance limits and regulation holds in individual cycles, then this cycle can be labelled as physiologically regressive (with respect to the general trend of the phylogenetic record) in *Cyprinus carpio* and in *Fundulus parvipinnis*. Several Salmonidae seem to follow a physiologically progressive ontogeny.

A last point of interest is the fact that the methods used seem to be adequate to

detect individual adaptive differences to temperature ranges, as well as individual differential degrees of homeostatic attributes, without necessarily killing the experimental objects. This permits genetic studies of the mentioned characteristics, and creates thus the possibility of filling gradually the evolutionary gap between poikilotherms and homeotherms.

Summary

1. The dependence of the frequencies of respiratory movements upon temperature has been studied in *Cyprinus carpio* L.

2. The degree of dependence of these frequencies upon temperatures is primarily determined by the size of the fishes. Large fishes are highly, small fishes only slightly, dependent.

3. Small fishes are characterized by a broad homeostatic zone of independence of the breathing frequency on temperatures. This homeostatic zone disappears in large fishes. The upper lethal temperatures decrease concomitantly with the disappearance of the homeostatic zones.

4. Individual variants, as to the degree of homeostasis and as to the adaptive temperature ranges, can be detected by the method presented. It can be used for studying genetic determination of these characteristics.

5. A survey of the data in the literature allows one to distinguish poikilotherms with progressive and with regressive ontogenies, with respect to homeostatic behavior.

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OXYGEN UPTAKE IN INSECTS WITH CYCLIC CO_2 RELEASE

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The periodical release of carbon dioxide (CO_2), as first found by one of us in bugs (Punt, 1944), is well established now in several insects in rest (Punt, 1950, 1956a) and in diapausing pupae of Lepidoptera (Punt, 1950; Schneiderman and Williams, 1953, 1955; Buck, Keister and Specht, 1953; Buck and Keister, 1955).

As to the oxygen uptake, there is some controversy among the above mentioned authors. Punt described in *Carabus nemoralis* (Müll.), *Locusta migratoria migratorioides* (Rch. and Frm.), *Meloë proscarabaeus* L. and *Rhodnius prolixus* (Stål.) a diaferometrically recorded periodical oxygen uptake, which ran parallel to the periodical CO_2 bursts.

In diapausing pupae, on the other hand, a continuous oxygen uptake was found by Schneiderman and Williams and by Buck and Keister using the Warburg technique.

The aim of this paper is to give more details about our investigations concerning the oxygen uptake in carabids and in the pupae of the cecropia silkworm. Some results of experiments on the influence of oxygen tension on the burst frequency will be mentioned. A possible explanation of the oxygen records will be given and the preliminary results of the estimation of the CO_2-binding power of insect haemolymph will be discussed.

Material and Methods

The insects under investigation were *Carabus nemoralis* (Müll.), *Hadrocarabus problematicus* Hrbst., *Periplaneta americana* L., pupae of the cecropia silkworm (*Hyalophora cecropia* (L.)) and pupae of *Sphinx ligustri* L.

The cecropia pupae were received from Dr. Schneiderman to whom we want to express our acknowledgment here.

A single animal was put in a glass tube, through which a very constant current of CO_2-free outdoor air was sucked. The gas exchange was continuously estimated by means of two diaferometers as described earlier (Punt, 1950, 1956a). In these instruments the gas which is to be analyzed passes through a copper tube, in the center of which an electrically heated platina wire is suspended. The temperature of the wire, and as a consequence the electric resistance, depends on the CO_2 and O_2 percentage, respectively, of the passing gas. The wire is connected in a Wheatstone bridge with a galvanometer. In all experiments two parallel diaferometers were used to record the CO_2 production and the O_2 uptake simultaneously. The gas coming from the animal container was dried and equally divided among both machines; the portion for the O_2 diaferometer was absolutely freed from CO_2 by soda lime. As the O_2 reading is slightly influenced by the removal of the CO_2, a valid R. Q. cannot be extracted directly from the curves. Both galvanometer

responses were recorded on one paper strip in a rotating-drum camera, the slit of which was perpendicular to the direction of rotation. Care was taken that the diaferometers had the same "latent period" in order to get synchronous points on the ordinate of the graphs. The galvanometers were connected in such a way that O_2 uptake and CO_2 release were recorded in the same direction.

In other experiments, for comparison, the direct Warburg method was used to estimate the O_2 uptake. The animals were placed in standard Warburg flasks (16 ml.) containing filter paper drenched in 10% KOH. The temperature was kept constant at 20° C.

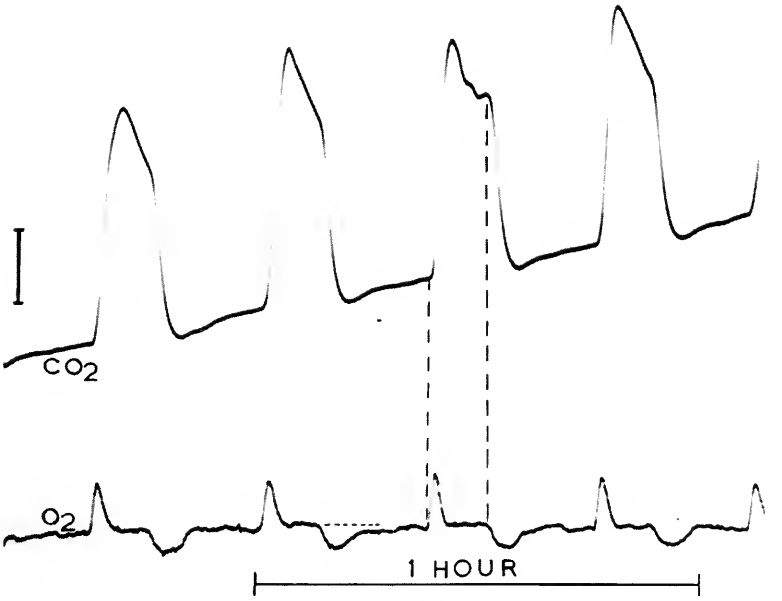

FIGURE 1. Photographically recorded CO_2 production and O_2 uptake of one specimen of *Carabus nemoralis*. The index on the left side indicates approximately 10 μL per hour (O_2 and CO_2). The inclination of the CO_2 line is caused by galvanometer drift; 20° C.

The CO_2 dissociation curve of haemolymph was estimated by means of the Haldane method for blood gas analysis. In order to work with small quantities, a special apparatus was built with a 10-ml. reaction vessel, the thermo-barometer vessel being of the same size. Gas mixtures were analyzed in the Haldane gas analysis apparatus.

RESULTS

1. Oxygen uptake in carabids

In a previous paper (Punt, 1956a) the discontinuous oxygen uptake in *Carabus nemoralis* (Müll.), *Locusta migratoria migratorioides* (Rch. and Frm.), *Meloë proscarabaeus* L. and *Rhodnius prolixus* (Stål.) was described.

The oxygen uptake was found to be (at any rate partly) periodical; the spikes in the photographic records were exactly synchronous with the CO_2 bursts. But

there could be noticed a marked difference between the forms of the O_2 and CO_2 curves, which difference was most evident in *Carabus* (Figs. 1 and 2).

Both the opening and the closing moments of the spiracles are rather distinct in the CO_2 line: a steep curving up of the galvanometer record indicates the moment of opening and a more or less sharp notch in the line, followed by a decline to zero level (or so close to zero that the difference was within the range of experimental error), marks the moment of closing. Sometimes, in *Carabus* and *Periplaneta*, the line rises somewhat in the interburst period (Fig. 1), probably due to small interburst CO_2 release (*cf.* Schneiderman and Williams, 1955).

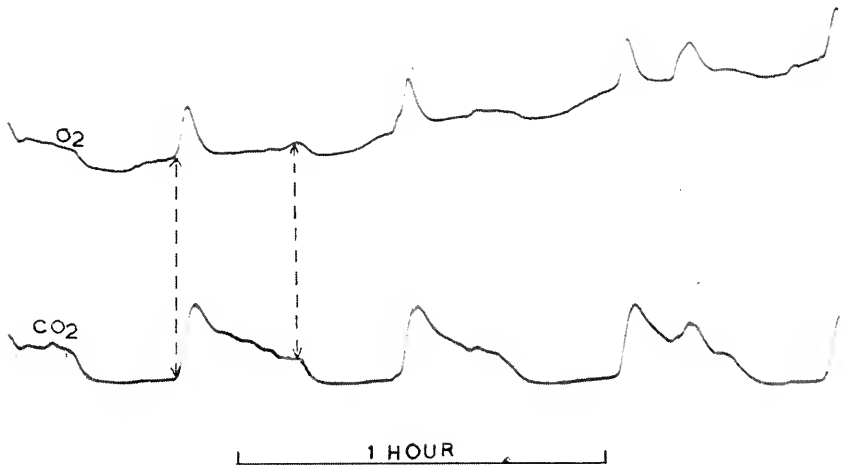

FIGURE 2. As Figure 1, but from a beetle in which the ratio between the open period and the closed period is discrepant from the mean value (1:2) as is occasionally found.

The O_2 line, too, shows these peculiarities, but while the CO_2 spike shows a maximum just after opening of the spiracle and declines rather regularly till the moment of closing, the O_2 line goes down much steeper nearly to the interburst level. After the moment of closing of the spiracles (corresponding to the sharp bend in the CO_2 line) the O_2 line declines still more to a minimum, to curve upwards again after some time to come to a "steady-state" which is maintained till the next burst. This steady rate of oxygen consumption is slightly lower than the minimum O_2 uptake in the burst period. As at that time we only meant to determine the type of respiratory activity and not the total gas exchange, there were unfortunately no zero readings recorded in the above mentioned paper. The respiration was recorded over periods of 6 hours continuously and it is rather inaccurate to interpolate a zero line over so long a period, particularly as there may have been some galvanometer drift due to external circumstances.

Next we performed some experiments of much shorter duration, preceded and followed by zero readings in order to be able to interpolate the zero line in our

records. Though there was considerable individual variation, the O_2 uptake in the interburst periods could be calculated from planimeter measurements to be approximately 60% of the total O_2 consumption. So in *Carabus* the oxygen uptake is both continuous and periodical. In a great number of experiments with *Periplaneta americana* we came to the same conclusion. It has already been shown that these O_2 spikes could not be caused by CO_2 interference in the O_2 diaferometer (Punt, 1956a).

As was mentioned, Schneiderman and Williams, and Buck and Keister reported the O_2 uptake in diapausing pupae to be continuous only. In order to determine whether this discrepancy between our results in *Carabus* and the results of Schneiderman and Buck was the consequence of the use of different techniques, the gas ex-

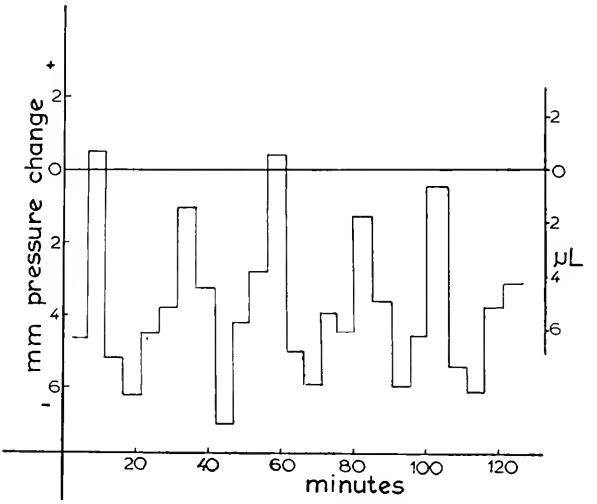

FIGURE 3. Manometrically estimated O_2 uptake in *Hadrocarabus problematicus* (440 mg.). Warburg flasks provided with KOH-drenched paper; 20° C.

change of carabid beetles was estimated in a Warburg apparatus. There were performed a great number of experiments with specimens of *Hadrocarabus problematicus* of a weight of 250–650 mg. The insects were placed in a normal side-arm flask with gas vent. The side-arm contained filter-paper, which protruded into the flask above the beetle. The paper was drenched in 10% KOH. After half an hour of temperature equilibration the stopcocks were closed and manometer readings made at intervals of three or five minutes. The change in pressure in this interval as compared with the foregoing reading was plotted against time (Fig. 3). In this way the oxygen consumption could be calculated from these figures, taking the flask constant into account. As the insect's volume was not exactly known, the index on the right of Figure 3 represents an arbitrary value only. The results show that O_2 consumption in *Hadrocarabus* is at any rate partly periodical, which is in accordance with the diaferometer experiments. Sometimes the curve rises above the zero line, which would indicate that there is in this interval an increase in pressure in comparison with the foregoing reading. This can only mean that at these mo-

ments the CO_2 bursts occur and the CO_2 absorption is not yet completed. But as the CO_2 is readily absorbed, the pressure is considerably decreased in the following five minutes, accentuating the apparent O_2 uptake in this period. From a large number of experiments with the diaferometer we know that at this temperature (20° C.) in carabids the open spiracle period can be sharply distinguished from the closed period, the interburst period being mostly twice as long as the burst. As this ratio cannot be clearly seen in the manometrical curves it may be concluded that in *Hadrocarabus* O_2 uptake is partly continuous as well.

When instead of containing KOH-drenched filter paper the side-arms were empty, the line represented in Figure 4 was found. Here, too, the moment of opening of the spiracles could be found in the curve as an increase in pressure,

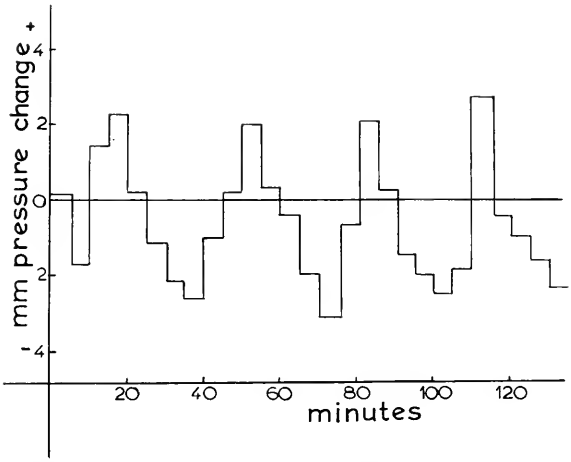

FIGURE 4. *Hadrocarabus problematicus* (500 mg.). Variations in pressure in a Warburg flask, without KOH; 20° C.

reaching a maximum in about ten minutes. This was followed by a slow decrease in pressure, lasting for about fifteen to twenty minutes by which the cycle was completed.

The decrease in pressure in the closed period is interpreted as a continuous O_2 consumption, but Buck, Keister and Specht (1953) considered the possibility that telescoping of the insect was involved, which in this manometric method could not be distinguished from some other phenomenon causing pressure loss. Our results, however, in the "open circuit" with the diaferometer made this assumption doubtful. Still we thought that in this beetle the space between the abdominal tergites and the elytra, which can be rather well closed and into which eight out of nine pairs of spiracles open, had something to do with the cyclic gas exchange. So we perforated the elytra. This had not the slightest influence on the periodical respiration. The sealing of three pairs of the spiracles with melted bee's wax made the periods more irregular and increased their frequency. It did not matter very much which pairs of spiracles were sealed. Introduction into the spiracles of very tiny glass capillaries, which were fixed with bee's wax, made the O_2 uptake more con-

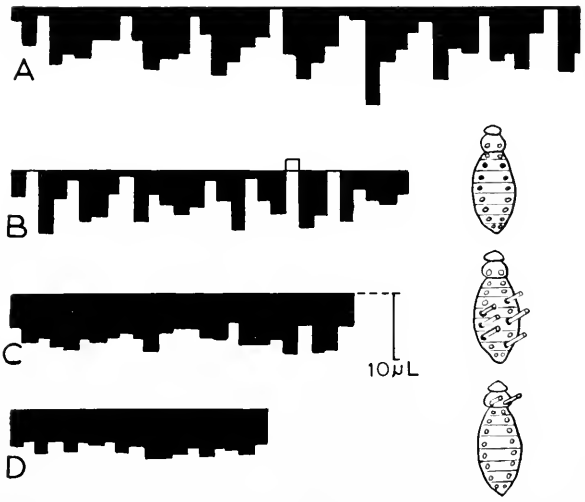

FIGURE 5. Oxygen uptake in *Hadrocarabus*, manometrically estimated at three-minute intervals; 20° C. A: normal insect at rest, the elytra being removed. B: Some of the spiracles sealed with bee's wax. C: Some of the abdominal spiracles kept open with glass tubes. D: The same with the thoracic spiracles.

tinuous. The clearest effect resulted here from placing the capillaries in the large thoracic spiracles (Fig. 5).

2. The oxygen uptake of Hyalophora cecropia pupae

Two years ago, in the Physiological Laboratory, University of Utrecht, the following experiments were performed with cecropia pupae. The gas exchange was

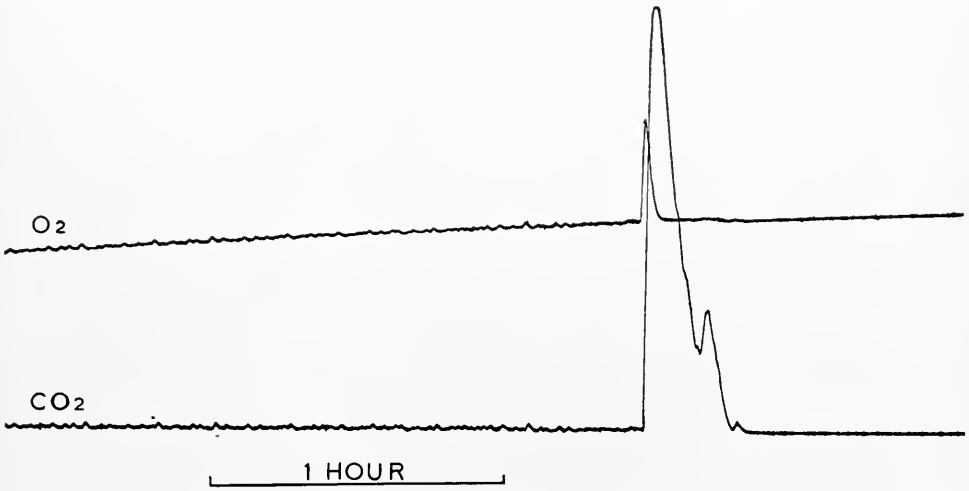

FIGURE 6. Photographically recorded CO_2 release and O_2 uptake in a pupa of the cecropia silkworm; 20° C.

estimated with two diaferometers simultaneously, one for recording the CO_2 production, the other for the O_2 consumption. In Figure 6 the photographically recorded galvanometer responses are shown. At 20° C. these diapausing pupae showed one CO_2 burst in about four hours. The form of the CO_2 line is exactly the same as described earlier in pupae of *Sphinx ligustri*. Just after the CO_2 spike the line falls to approximately zero level and is very smooth, but after about one hour small perturbations return in the line, which go on and increase until the next CO_2 burst. Exactly synchronous with the CO_2 bursts small O_2 spikes were found. This O_2 line falls after some minutes but remains slightly above the interburst level. In the interburst period the O_2 line, too, is at first very smooth, but shows after some time the same small perturbations as the CO_2 line (in fact running exactly parallel to them). It looks as if the spiracles are no longer hermetically closed but are leaking or fluttering from time to time. Unfortunately we did not esti-

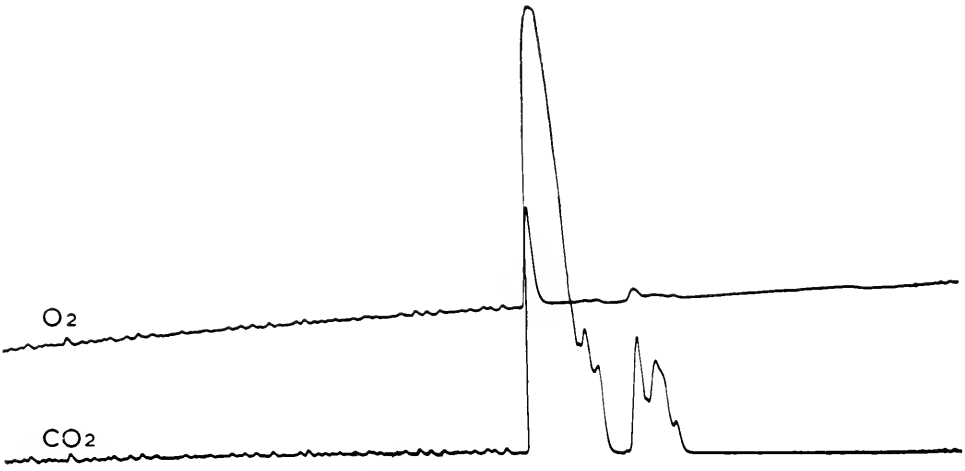

FIGURE 7. The same pupa as in Figure 6. A double burst is recorded.

mate the exact value of the O_2 uptake in these prolonged experiments, as was pointed out in the foregoing section. But as the O_2 diaferometer was adjusted to be approximately as sensitive as the CO_2 apparatus in order to get the same response for the same percentage of gas, it is clear from these graphs that O_2 uptake must take place in the interburst periods as well. The area of the O_2 spike was much smaller than that of the CO_2 burst, whereas the interburst perturbations were of the same size in both lines. The impression which we have from some zero readings of the O_2 line and from calculations of the O_2 spike area is that there must be a relatively large interburst O_2 uptake which was considered to have a mean value of 92% of the total O_2 uptake. This confirms the findings of Schneiderman and Williams and of Buck *et al.* But still a periodic O_2 uptake is found as well. Perhaps of interest in this connection is what we find on page 147 in the paper of Buck and Keister (1955) where we can read: "Small but statistically significant perturbations were in fact seen in some of our O_2 uptake records. . . ."

Occasionally a double burst was recorded as was already described for pupae of *Sphinx* and *Papilio* (Punt, 1950; Fig. 7).

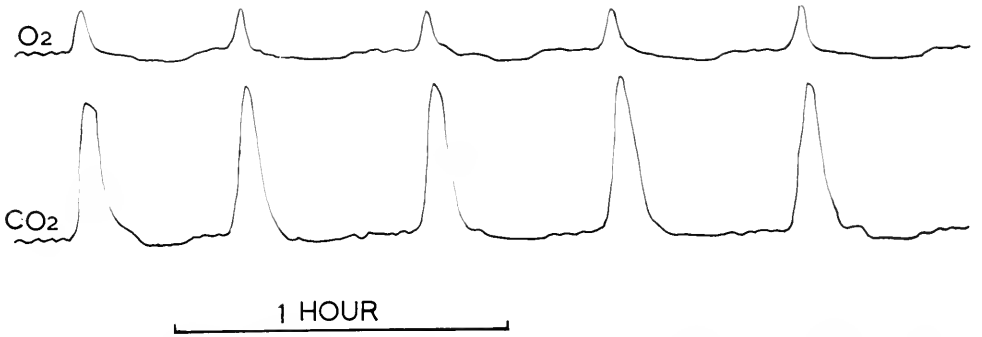

FIGURE 8. Gas exchange of a cecropia pupa, a few weeks before hatching (May, 1954). Drawn from photographic records.

In Figure 8 we find a record of simultaneously estimated CO_2 release and O_2 uptake of the same cecropia pupae made a few weeks before hatching. The graph is on the same amplification and on the same time scale as Figure 6. The burst frequency has increased to two per hour. The O_2 uptake in the bursts is relatively larger.

3. Influence of oxygen tension on burst frequency

The influence of CO_2 tension on the burst frequencies in *Carabus* has been previously described (Punt, 1955, 1956b). Increasing pCO_2 caused a prolonged open-

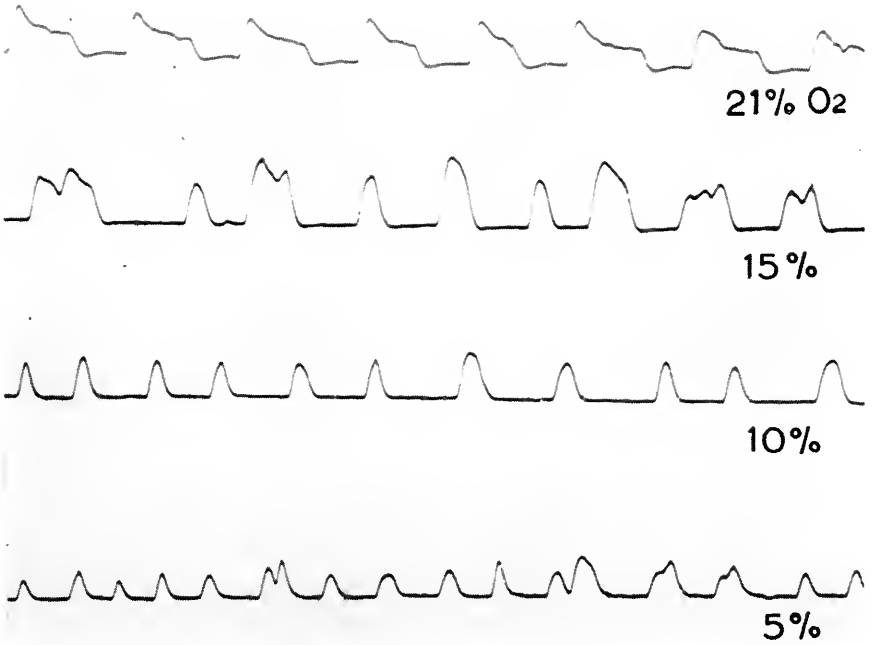

FIGURE 9. *Hadrocarabus problematicus;* CO_2 burst frequency as influenced by different O_2 tensions.

ing of the spiracles, until at about 1½% of CO_2 the closing of the spiracles was inhibited. In the present investigation experiments were performed on the influence of decreasing oxygen tension on burst frequency in *Hadrocarabus problematicus*. In Figure 9 a summary is given of the results. A decrease of pO_2 caused an increase in CO_2 burst frequency and consequently a decrease in burst volume. This is in accord with Wigglesworth's observation on spiracular movement in fleas (Wigglesworth, 1953).

4. The CO_2 dissociation curve of haemolymph

For reasons given in the discussion, we thought it worthwhile to know something about the CO_2 dissociation curve of insect haemolymph. Much work has been done on the composition of the body fluid of insects, but as to the possibility of buffering the CO_2 our knowledge is scanty (Reali, 1955; van Asperen and van Esch, 1956; Levenbook and Clark, 1950). We therefore tried to estimate the CO_2

TABLE I

The CO_2-binding property of haemolymph of Sphinx ligustri pupae

Quantity of haemolymph (mL)	pCO_2 (mm.Hg)	Total CO_2 (volume %)
0.50	0	2.2 ± 0.8
0.70	0	1.7 ± 0.6
0.69	0	2.2 ± 0.6
0.49	19	5.1 ± 0.9
0.23	31	10.9 ± 1.0
0.50	38	9.2 ± 1.1
0.53	38	8.9 ± 0.9
0.38	73	18.2 ± 1.4
0.24	84	17.9 ± 2.2
0.33	105	20.0 ± 1.5

saturation of haemolymph under different tensions of CO_2. The haemolymph of diapausing pupae of *Sphinx ligustri* was gathered by puncturing the pupae with a syringe which was previously moistened with tromboliquine (heparin), in order to prevent clotting. The exactly measured quantity of haemolymph was put in the small bulb-flask of the Haldane blood-gas analyzer and saturated with CO_2 under a certain tension by rotating the flask mechanically for one hour. After that time the analysis was performed in the normal way, tartaric acid being used to expel the CO_2 from the haemolymph. The results are to be found in Table I and in Figure 10. The data are corrected for dissolved CO_2 at the different tensions and at the temperature used (20° C.) so that the total amount of CO_2 could be plotted against the pCO_2. Though only a few experiments were performed (our stock of pupae being exhausted for this season), it proved that the CO_2-binding capacity exceeds the line which represents the dissolving of CO_2 in water at the same temperature (the solid line in Figure 10; data from Umbreit *et al.*, 1949). Our data are too few in number to allow drawing a correct line through the points.

Insect haemolymph probably contains some CO_2-binding principle but in our pupae the capacity was rather disappointing as only twice as much CO_2 could be extracted from the haemolymph as would dissolve in pure water. In certain other

insects (*Gastrophilus* and *Hydrophilus; cf.* Florkin, 1937) the quantity of bound CO_2 was found to be much larger, but these may perhaps represent special cases. More work on this subject is in progress.

Discussion

We will try to give a possible interpretation of the recorded O_2 uptake and CO_2 release in *Hadrocarabus*. This interpretation is, of course, only hypothetical, but

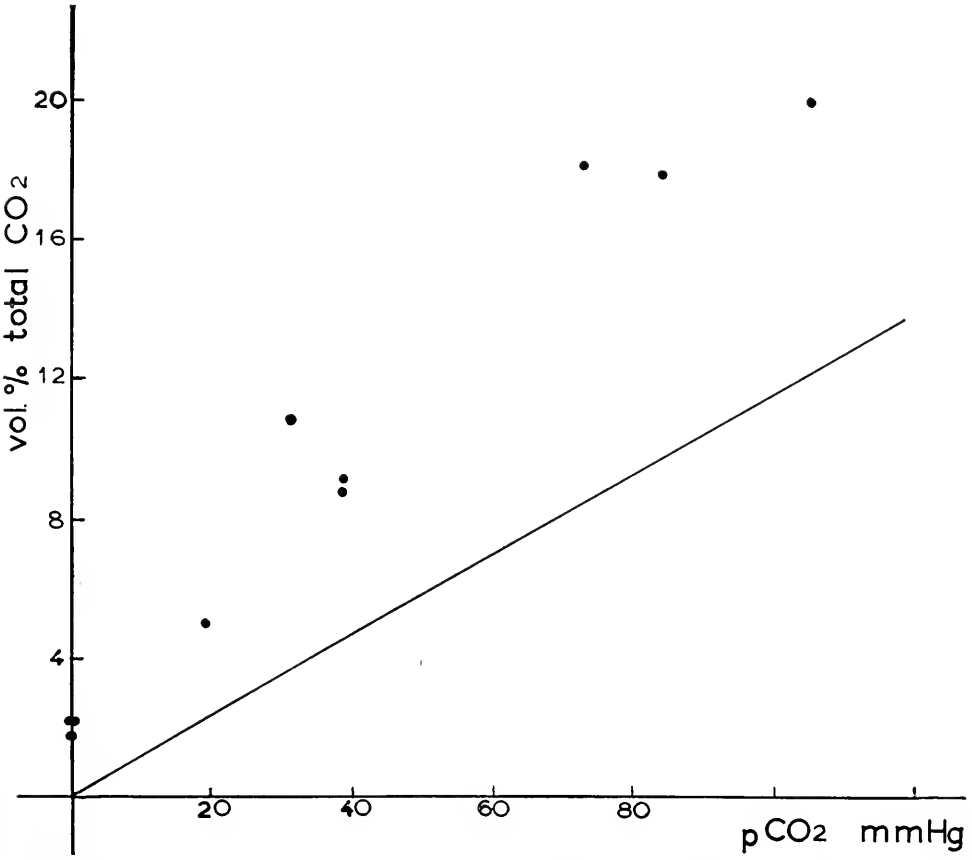

Figure 10. The CO_2 capacity of haemolymph of *Sphinx ligustri* (20° C.). Solid line: CO_2 capacity of pure water.

is in agreement with the results of our and other investigations and partly based upon assumptions from Wigglesworth (1953) and from Buck and Keister (1955, p. 162). Probably in other insects, including diapausing pupae, the same phenomena may occur.

We may assume that oxygen is taken up continuously in some way or another. But the quantity which can penetrate into the insect body in the "closed spiracles"

period is insufficient to cover the metabolic oxygen want. So an oxygen debt is incurred, oxidation being incomplete, and acid metabolites are formed and accumulated in the body fluid. This is in accordance with the theory of Wigglesworth, who attributes the withdrawal of fluid from the tracheal endings to increased osmotic value of the tissue fluids, due to the increasing amount of acid metabolites.

Carbon dioxide is formed by metabolic processes and stored in the body fluid, not only as dissolved gaseous CO_2 but probably bound in some buffer system or CO_2-binding principle. But as soon as the pO_2 in the tracheal system is diminished and an oxygen debt develops in the tissues, the acid metabolites drive the CO_2 from the bound phase into the gas phase and as a consequence the pCO_2 in the tracheae will increase. As soon as the pCO_2 has reached a certain threshold the spiracles open and the CO_2 burst takes place. In the open period the CO_2 diffuses out, not only from the tracheae but from the tissue fluid as well, until the moment when the pCO_2 reaches a level at which the spiracles may close. In the meantime O_2 has entered the tracheae, which is seen as the initial spike in the O_2 uptake line. But after this initial spike the O_2 line falls down to a level slightly above the steady state of the interburst period. Probably this part of the O_2 line represents the real oxygen consumption of the animal per unit of time.

When the spiracles are closed at last, the amount of O_2 in the tracheal system is sufficient to cover the metabolic want for some time (the O_2 line going down to nearly zero), but as soon as the pO_2 inside the trachea is low enough, a leakage of O_2 starts (continuous O_2 uptake). This leakage is not sufficient, however, to cover the real oxygen want and acid metabolites are formed again, by which the described respiratory cycle is completed. This hypothesis is in accordance with the assumption that CO_2 controls the sudden opening of the spiracles (Buck and Keister, 1955; p. 161). The action of decreased pO_2 on the triggering of the spiracular opening may be seen as an indirect one: decreased pO_2 increases the degree of hypoxia and hence the amount of gaseous CO_2 liberated into the tracheae. Similarly Wigglesworth's assumption that in pure oxygen, opening is induced by a large amount of CO_2, and in 5% O_2 by a very small amount of CO_2, could be interpreted to mean that in pure oxygen the CO_2 remains bound in the body fluid and that in both cases (100% of O_2, and 5% of O_2) the tension of free CO_2, triggering the spiracles, may be the same. Probably this threshold CO_2 tension is not very high, for there is evidence that in about 1½% of CO_2 the spiracles do not close at all (Punt, 1956b).

So it looks as if in all cases it is the pCO_2 which controls spiracular movement. When oxygen tension is lowered, oxygen debt develops more readily and the pCO_2 will reach the critical level sooner. As a consequence the burst frequency is increased. The burst volume is decreased, as no large quantities of CO_2 can be accumulated in the short interburst period. It would be worthwhile to determine the pH of the haemolymph under these circumstances. When oxygen tension is raised, acid metabolites are not formed so soon and more metabolic CO_2 may be bound in the body fluid, the intertracheal pCO_2 only reaching the critical value after a longer period; burst frequency is decreased, burst volume increased (Punt, 1956b). As to the experiments in pure oxygen, when the spiracles are opened the tracheae are filled with pure oxygen and as a consequence the oxygen leak in interburst period is much less. Schneiderman and Williams (1955; p. 134) stated that the "interburst CO_2 output," too, may become undetectable in pure oxygen.

Summary

1. The simultaneous determination of CO_2 production and O_2 uptake in *Hadrocarabus problematicus* Hrbst. is described. Both CO_2 release and O_2 uptake are cyclic, but there is some interburst O_2 uptake.

2. The diaferometrically recorded O_2 uptake is discussed, and compared with the results as obtained with the Warburg technique.

3. In diapausing pupae of the cecropia silkworm (*Hyalophora cecropia*) the continuous O_2 uptake forms a larger percentage of the total amount of O_2 consumption than in *Hadrocarabus*. Still there was found an initial maximum of O_2 uptake of short duration at every CO_2 burst.

4. Some experiments on the CO_2 dissociation curve of haemolymph of pupae of *Sphinx ligustri* are mentioned. There is evidence that in haemolymph a CO_2-binding principle is present.

5. The interaction of O_2, haemolymph and CO_2, resulting in a certain intertracheal pCO_2 controlling spiracle movement, is discussed. Probably the pO_2 is only indirectly involved in the triggering of spiracle opening.

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STUDIES ON MARINE BRYOZOA. X. HIPPADENELLA CARSONAE, N. SP.

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The writer wishes to express her most sincere appreciation to the National Science Foundation for research grants which have greatly aided this and other studies, and to the Smithsonian Institution, U. S. National Museum for the loan of Bryozoa collected by Comdr. David C. Nutt on the U. S. Navy's 1947–48 Antarctic Expedition.

The purpose of this paper is to give a detailed description of the morphological features of *Hippadenella carsonae*, new species and to note other species which are ecologically associated with it.

Hippadenella carsonae is a lepralioid Ectoproct, Order Cheilostomata, Suborder Ascophora, of the Family Hippoporinidae of Bassler (1953). It is named in memory of Louisa Carson, a beloved teacher of my early school years.

Species Data

Hippadenella carsonae, n. sp.

Diagnosis: Colony calcareous, twig-like; branching open. Twigs slender, cylindrical, with usually nine to twelve, sometimes more, wedge-shaped zooecia in cross-section. Mural rims raised, crinkled. The smooth to tubercled frontal is a pleurocyst with three to five pairs of oblique, tubular pores. Orifice lepralioid, rounded. Lyrula absent but two slight cardelles are placed low. Operculum with lateral parenthesis-like sclerites. Very broadly oval median suboral avicularium placed at a varying angle to the frontal plane and mounted on a wide porous avicularial chamber that contains prominent avicularial glands, muscles and vestigial polypide. Hyperstomial ovicell very salient, globose, but slightly flattened, tubercled and non-porous. Its arched rim is a continuation of the zooecial mural rims. Two communication areas (a multiporous pore chamber and corresponding opening) in lateral wall. Distal wall a sieve plate of numerous pores.

Measurements: The first figures are the minimum, the next the maximum and the last (in parentheses) the average of ten readings (or occasionally more) for each structure. Readings are in millimeters. L is for length, W for width, D for diameter, H for height.

1.073–1.406	(1.247)	L	Zooecia
0.407–0.555	(0.444)	W	Zooecia
0.115–0.173	(0.146)	L	Avicularial apertures (rostal and back areas)

0.101–0.173	(0.137)	W	Avicularial aperture
0.058–0.086	(0.072)	L	Mandible
0.094–0.173	(0.124)	W	Mandible
0.144–0.202	(0.173)	L	Zooecial orifice
0.144–0.216	(0.179)	W	Zooecial orifice
0.158–0.187	(0.170)	L	Operculum
0.173–0.202	(0.192)	W	Operculum
0.432–0.475	(0.452)	L	Ovicell
0.389–0.490	(0.422)	W	Ovicell
0.675–0.705	(0.690)	L	Tentacles (two readings only)

The zooecial polypide measurements below are based on only one reading each:

0.147	D	Tentacular bundle within the tentacular sheath
0.264	L	Esophagus
0.132	D	Esophagus, just below the tentacles
0.073	D	Esophagus, just above the cardia
0.029	H	Esophageal epithelial cells near tentacles
0.014	H	Esophageal epithelial cells near cardia
0.132	D	Stomach caecum (empty)
0.250	L	Rectum (empty)
0.088	D	Rectum (empty)

Zoarium: The zoarium or colony consists of openly branching twigs which sometimes fuse or anastomose with other twigs at point of contact. The twigs are fragile and brittle, breaking into shorter twigs, so that no true picture of the extent of a colony, nor the ultimate pattern of branching, whether dichotomous or irregular (Figs. 11, 13) can be gotten from the mass of short fragments at hand. Some of the broken fragments were up to 51 mm. long and about 2 or 3 mm. in diameter. There was about a pint of material in the collection, but not one of the stalks was a complete, intact colony.

To the naked eye the twigs appear smoothly cylindrical (Figs. 13, 21) except in the ovicelligerous region where they are covered with bumps (ovicells) as in Figure 11. The usual number of zoids around a branch is about nine to twelve, although occasional stalks may have almost twice that number just before a bifurcation (Figs. 14, 21). Zoids face outward around the usually cylindrical branches radially.

Dead twigs are white, living twigs yellow. In practically all the living twigs, *i.e.*, twigs collected when they were in the living state, the polypides were confined to the tips or distal part of the stalk while the basal, proximal part of the twig was dead, polypideless. The yellow color of live twigs is due to the presence of yellow polypides within the zooecia.

Zooecia: The outside calcareous skeletal case in which the soft zoid parts (digestive tract, musculature, tentacles) are housed is the zooecium. Zooecia are wedge-shaped in cross section (Fig. 21), mostly rectangular in frontal or face view and quincuncially arranged (Figs. 6, 12) like bricks in a wall. In side view the end wall (Figs. 8, 10) slants a bit obliquely downward and backward. In some zooecia

it is nearly horizontal. The frontal wall is of variable thickness, sometimes being twice as thick as the side wall.

Zooecial boundaries are well defined by mural rims (Figs. 3, 12, 17, 18, 23). The height of these partitions varies with age and with exposure to molar forces. Sometimes these rims are high and very crinkled. Other times they are nearly level with the zooecial front. In early secondary calcification the distal mural rims send slender calcified trabeculae across the operculum (Figs. 18, 23). Later, the orifice may be incompletely obliterated by more extensive calcification over the operculum (Figs. 6, 17).

The frontal wall is flat to convex, porcellanous to sparsely tubercled, non-porous centrally but completely perforated by about three to five pairs, usually three pairs (Fig. 10), of obliquely directed marginal tubular pores or tubes whose diameter varies (Figs. 30, 32). These tubes end in slight elliptical craters above the frontal (Fig. 32). Viewed from the front, the top pair of tubes slants diagonally, converging downward toward the zooecial mid front. The middle pair slants toward the zooecial mid line. The bottom pair slants diagonally upward toward the zooecial mid front, as in Figure 30. In other words, the marginal tubes lean toward the zooecial center front.

The front may be perfectly smooth except for the raised pores or it may be roughened by a few tubercles (Figs. 6, 17).

The side walls are straight and have two widely spaced, multiporous, blister-like interzooecial communication areas. These areas are variously known in bryozoan literature as rosette plates, septula, pore chambers, corresponding openings. The distal one is a pore chamber and the proximal one is a corresponding opening (Figs. 8, 10, 19, 26). The pore chambers have six to twelve small pores (Fig. 19). The corresponding opening has a single large hole rimmed about by an irregular annulus (Fig. 26). The quincuncial arrangement of zooecia brings the pore chambers of one vertical row of zooecia against the corresponding openings of the left or right vertical rows of neighboring zooecia, and vice versa. Silén (1944) has given an excellent and extensive account of pore chambers, corresponding openings, pore plates and various types of interzooecial communications for various species. Conditions in *Hippadenella carsonae* are in agreement with his findings for related species.

The end wall is oval to pear-shaped (Figs. 10, 21). Its smaller, medial part is punctured by numerous (about forty, more or less) small, closely spaced pores and slants downward more than the broader peripheral non-porous part. Sometimes the slant is rather steep, sometimes deviating little from the horizontal.

The calcareous frontal walls are a bit too opaque usually for satisfactory study of soft internal parts as tentacles, gut, gonads, musculature and avicularial apparatus. Decalcification was not attempted because of the nature of the colony—a number of zooecia radially arranged, very close together. Crushing the stalk gently in a drop of Euparal on a slide usually gave well dispersed material quickly and in reasonably satisfactory condition for microscopic study. Attempts were made to clear the youngest, slenderest, polypide-containing tips in glycerine and others in dioxan. Some clearing was accomplished with the glycerine but none with the dioxan. So, the description of the soft internal parts is based on crushed, dispersed material and also on what could be seen through the walls of the younger, less calcified zooecia.

For a study of the exoskeleton or zooecial walls calcining (burning off the chitinous and membranous coverings with a small blow pipe) is the most satisfactory method of preparation. However, calcining must be halted before the specimen becomes too fragile and disintegrates to powder.

Autozooecial polypide: The autozooecial polypide consists of the tentacles, gut and associated musculature (Figs. 2, 7, 16, 29). All polypides are in a retracted position, the polypides withdrawn into the body cavity, none with tentacles extruded through the orifice, so characteristics of the retractor muscles, parietal body wall muscles and tentacle number could not be studied. As far as can be deduced from retracted specimens there seem to be about twelve to fourteen tentacles. The tentacles are ciliated, rather stout and not of excessive length. They are withdrawn into a transparent membranous tentacle sheath which is closed at the top near the operculum (Fig. 16) by a sphincter or diaphragm. Two flask-shaped "oral" or "sub-oral" glands of unknown function are part of the sheath, just beneath the diaphragm. Attached to the diaphragm are two bundles of muscle fibers, the parieto-diaphragmatics, one on each side (Figs. 2, 16, 29). Their muscle fibers extend diagonally back and upward to attach to the zooecial wall. A short distance below the parieto-diaphragmatics are two membranous bands, the parieto-vaginals, which contain a few delicate fibers that are deviated from the tentacle sheath. The parieto-vaginals connect the tentacular sheath to the lateral zooecial walls.

The gut consists of mouth, esophagus, stomach (which has three divisions: cardiac, caecal and pyloric), rectum and anus. In some bryozoa there is a ciliated pharynx between mouth and esophagus but not in *H. carsonae*. The esophagus of *H. carsonae* is short and tapers slightly. Its epithelial mucosa cells are tall columnar, hyaline and not ciliated. Those near the mouth are twice as tall as those near the cardia, the diminution in height being gradual. A sphincter separates the esophagus from the cardia. Retractor muscles attach the lophophore (region at the base of the tentacles and around the mouth) to the body wall, so there originates a curtain of muscle fibers just at the beginning of the esophagus. The epithelial cells of the cardia are low, cuboidal, non-ciliated and not much different in diameter

List of Abbreviations Used on the Plates

A Abductor mandibuli muscle fibers
B Adductor mandibuli muscles
C Avicularial back area
D Avicularial chamber
E Avicularial gland
F Avicularial polypide
G Avicularial rostral area
H Avicularium
I Cardelle
J Cardia
K Esophagus
L Mandible
M Mural rim
N "Oral" gland
O Orifice or aperture
P Ovicell
Q Parieto-diaphragmaticus muscle
R Parieto-vaginal band
S Pore chamber or rosette plate
T Pylorus
U Rectum
V Sclerite
W Stomach
X Tentacles
Y Tentacular sheath

All figures, except Figures 4, 8, 10, 11 and 13, were drawn with the aid of a camera lucida, and are of *Hippadenella carsonae,* new species, from Antarctic type locality, Sta. 104. Figure 11 is from the holotype, the others from paratypes. Measurements for structures are given in text.

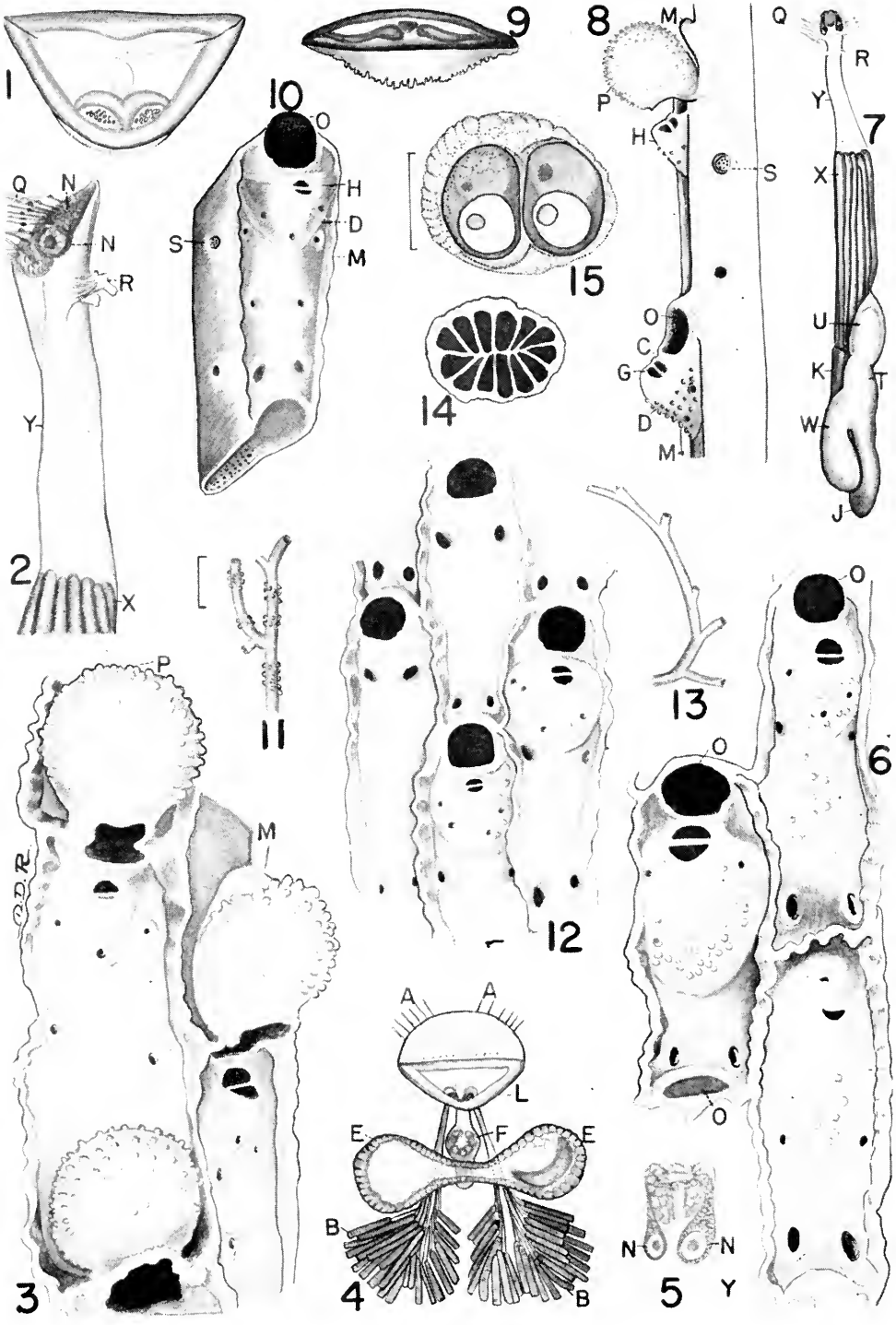
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PLATE I

from those of the esophagus but their cytoplasm is granular rather than hyaline. The cardial wall is thin. The cardia is J-shaped, parallelling the esophagus. The stomach caecum originates at the upper side of the cardia, near the pylorus, and has a highly granular cytoplasm. The pylorus is ciliated, internally. The different parts of the stomach and also the rectum are all rather thin-walled. Diatoms must be a part of the *H. carsonae* diet because diatom shells are in some of the recta.

The gut and tentacles amply fill the body cavity.

The ovary is attached to the side wall, behind the tentacular sheath and below the operculum. It is present in non-ovicelled zoids and presumably also in ovicelled ones as well as in zoids with or without an avicularium. If an avicularium is

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Explanation of Plate I

Figure 1. Mandible, top surface view. Transparent oval in center is the lucida. Adductor muscle fibers attach as individual dots in the two semicircular areas in front of lucida. Transparent rimming membrane (*cf.* Figs. 9, 25) here barely visible about rounded tip.

Figure 2. Side view of upper third of polypide. Compare with Figures 7, 16, 29.

Figure 3. Frontal view of three calcareous ovicelled zooecia. The upper two have avicularia whose chambers are small. From calcined specimen.

Figure 4. Diagram showing contents of avicularial chamber, as seen from top and front. One of the avicularial glands contains a hardened or coagulated crescent-shaped secretion. The delicate abductor muscle fibers are shown bent upward, out of their natural position, for the sake of clarity. They should extend downward and backward toward the base of the avicularial chamber, some distance away from and back of the adductor tendons.

Figure 5. Side view of "diaphragm" or sphincter and the two "oral" glands which contain some secretion. The sphincter is slightly relaxed, so is evident a narrow central passageway that must widen considerably to permit extrusion of tentacles.

Figure 6. Frontal view of three calcareous zooecia. The upper two have fully developed and functional orifices and avicularia. The bottom one has a nearly obliterated orifice and avicularium due to secondary calcification. Avicularial chambers are large as compared with those of Figure 3.

Figure 7. Retracted polypide. The slender upper third corresponds to Figure 2 but is from a different side. The esophagus is partly hidden by the stomach caecum. The anus is at the uppermost tip of rectum.

Figure 8. Diagrammatic side view of zooecia, front wall to the left, side wall facing observer. Ovicelled zooecium complete, the non-ovicelled one only partly shown. The end walls slant, sometimes less obliquely than shown, may even be nearly horizontal in some zooecia. Side walls are perforated by two communication areas. Upper, distal multiporous area (S) is like an internal blister (*cf.* Fig. 19) and is called a rosette plate or pore chamber. Lower, proximal single opening fits against a rosette plate of a neighboring zooecium which is not here shown. Single opening bears the inadequate name of "corresponding opening."

Figure 9. Mandible, edge view. The delicate serrated membrane hangs vertically down from front edge. More heavily cuticularized parts are darkened.

Figure 10. Diagram of a single, non-ovicelled, wedge-shaped zooecium. Its side wall (with two communication areas) is at left. Its frontal wall (with six frontal pores) is at right. A multiporous end wall is at bottom. Three pores are shown on the avicularial chamber.

Figure 11. Colony fragment, drawn to the one-cm. scale at immediate left. Bumps along stalk are ovicells.

Figure 12. Frontal view of four zooecia, two with avicularia and two without. From a calcined specimen.

Figure 13. Another colony, with more branches. Drawn to same scale as Figure 11.

Figure 14. Cross-section of a slightly flattened branch which has thirteen wedge-shaped zooecia at this level.

Figure 15. Four eggs in ovary. Nucleoli prominent, excentric. Nuclei clear, vesicular. Cytoplasm homogeneous and denser. Drawn to the 0.06-mm. scale at left.

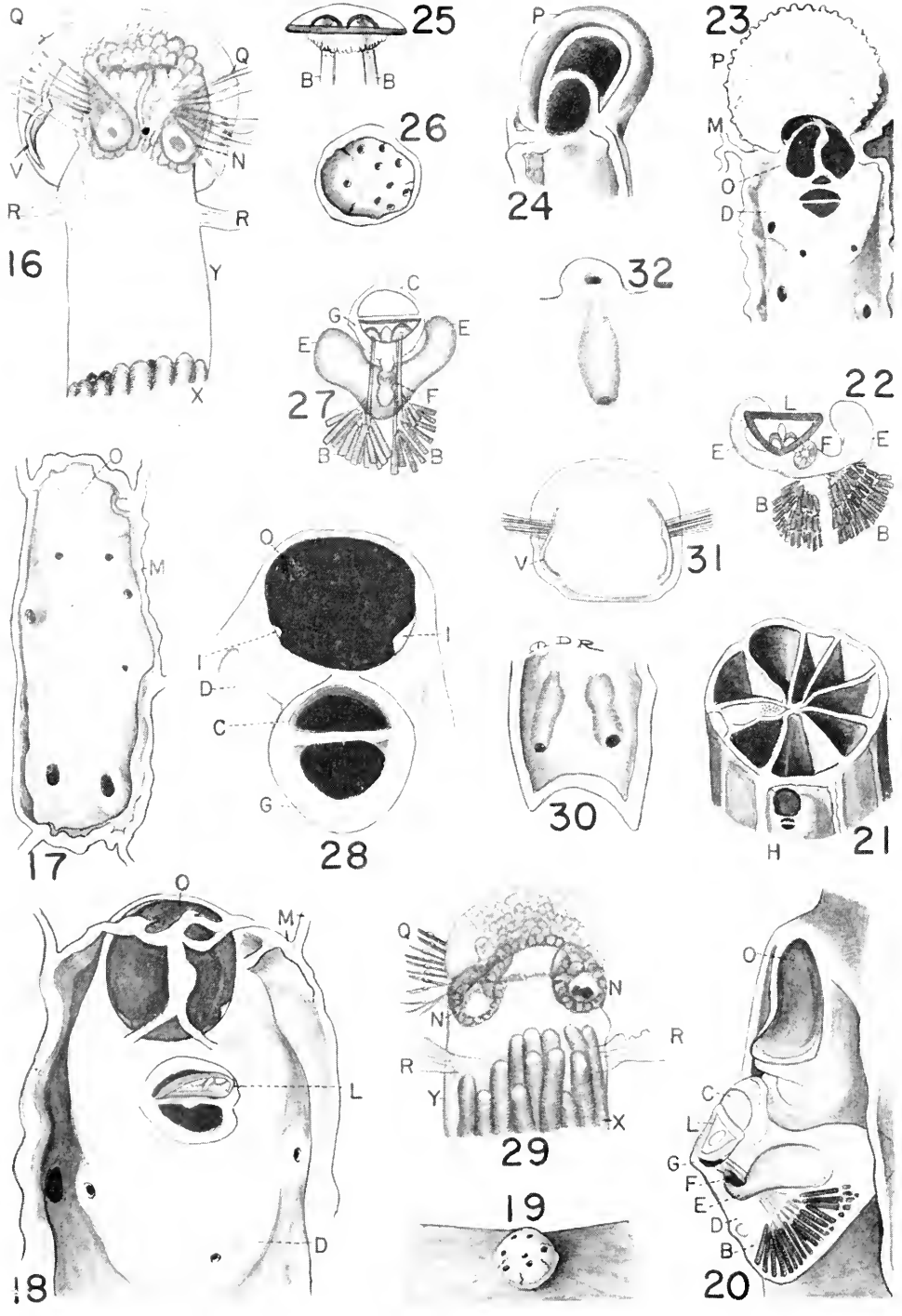
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PLATE II

present the ovary is about half-way between the operculum and the avicularial chamber, back of the tentacular sheath. It contains several eggs of different sizes. The ova have a large excentric nucleolus and an almost clear nucleus (Fig. 15). Whether an ovary is present in every zoid cannot be ascertained because of the opacity of zooecial walls. Whether this species is hermaphroditic or dioecious is unknown at present. It was not possible to determine the exact site of origin of spermatogenic tissue although some material, apparently spermatogenic, was found near the stomach in rather sizable patches in the crushed specimens.

Orifice: The *H. carsonae* orifice resembles those of the genera *Cryptosula* and *Hippodiplosia*. It is rounded, lepralioid. Part of its distal wall is formed by the next distal zoid. A median tooth, lyrula, is absent. Two slight cardelles or ledges for operculum articulation are placed low at the sides, dividing the orifice into two areas of unequal size. The larger distal area is formed by the anter (distal orifice lip) while the smaller proximal area is bounded by the poster (proximal orifice

Explanation of Plate II

Figure 16. Operculum, "diaphragm," "oral" glands, tentacle sheath, tentacle tips and anchoring musculature shown from the back surface. The channel for protrusion of tentacles is more contracted than in Figure 5.

Figure 17. Frontal view of zooecium with completely sealed-over orifice in a more advanced stage of calcification than that of Figure 6.

Figure 18. Frontal view of the upper half of a zooecium in an early stage of secondary calcification where calcareous trabeculae extend from the mural rims across the orifice. A partly opened mandible articulates with the pivot.

Figure 19. Multiporous rosette plate of side wall.

Figure 20. Profile of zooecial orifice and avicularium with operculum and mandible, respectively, in place. The avicularial wall is left more transparent than it normally would appear to show the position of the avicularial contents. Compare with Figures 4, 22, 27.

Figure 21. Stereogram of typical cylindrical stalk. Orifice and avicularium on bottom zooecium. Porous end wall tops another.

Figure 22. Oblique top view of avicularial apparatus. Glands curve around adductor tendons to fit the avicularial chamber.

Figure 23. Front of a zooecium which has an ovicell and avicularium, and shows secondary calcification trabeculae spanning the orifice. Ovicell opening is completely plugged up by domed calcareous lamina which occupies about half the ovicell interior and which is here faintly outlined, *cf.* Figure 24.

Figure 24. Ovicell whose front wall has been broken away to show the double outer wall and the third (innermost) lamina which has sealed off the ovicell cavity. The lamina resulted from secondary calcification.

Figure 25. Edge view of another mandible with serrate membrane and adductor muscle tendons which attach to semicircular areas (*cf.* Fig. 1).

Figure 26. Looking through a "corresponding opening" with its irregular annulus or ledge, into the multiporous blister-like rosette plate (*cf.* Fig. 19).

Figure 27. Avicularial contents from the under side. Abductors are not shown.

Figure 28. Detail of orifice, cardelles and avicularium. The bands leading from orifice to avicularium represent differences in thickness of frontal calcification and are also the distal limits of the avicularial chamber.

Figure 29. Frontal view of "oral" glands, operculum and tentacle tips which are very close to extrusion. The "diaphragm" opening is wider than in Figures 5 and 16.

Figure 30. Interior of frontal zooecial wall showing shape and direction of tubular frontal pores. The interior openings have been blackened. The exterior openings are faintly rimmed to show their raised nature (*cf.* Figs. 3, 6, 32).

Figure 31. Inner face of operculum. Occlusor muscles attach to sclerites.

Figure 32. Side view of frontal wall tubular pore and its raised external terminus.

lip). The cardelles form the dividing line between the two areas, *cf.* Figure 28. In side view (Fig. 20), the orifice is not a flat plane. The poster curves outward in lepralioid fashion. There is no difference in size between orifices of the ovicelled and non-ovicelled zooecia.

The zooecial orifice is covered by an operculum shaped to fit (Figs. 20, 31).

It has been the universal and long-time practice for bryozoologists to use the term "chitinous" when describing opercula of those bryozoa which have a more or less horny, yellowish to brown, sometimes unevenly stiffened or reinforced operculum. Its use is based more on the visible physical appearance of the substance (its resemblance to the insect exoskeleton) rather than on its chemical composition. Dr. Libbie Hyman suggests that the term "cuticularized" would be more appropriate since little chemical evidence exists for the presence of chitin in the ectoprocts (bryozoa). So, where I have used the term "chitinized" in the present and in past articles in connection with bryozoa the term "cuticularized" would perhaps have been more suitable.

The *H. carsonae* operculum is lightly cuticularized (Fig. 31). Near its lateral borders, internally, are parenthetical sclerites to which attach delicate occlusor muscle fibers and from which a flange may develop in some older zoids.

The orifice in many old zoids is overgrown or secondarily calcified and may be entirely sealed over or obliterated (Figs. 17, 18). Sometimes the secondary calcification extends to the avicularium and ovicell, so that the avicularium too fuses over and the ovicell opening is blocked by a dome-shaped calcareous lamina (Figs. 6, 23, 24). Peristome and oral spines are absent.

Ovicells: The ovicells were collected long past the breeding season because larvae are absent. The ovicells are either empty or partitioned off by the internal secondary calcification lamina.

Ovicells generally occur in groups at periodic intervals along the stalk. They are not immersed or covered over by the frontal of the next zoid but rest on a cushion formed by it (Figs. 3, 8). They are large, salient, non-porous. Their surface is beaded to tuberculate and faintly ridged. The thin raised rim about the highly arched opening is continuous with the raised mural rims.

Avicularia: An avicularium occurs on some zoids, either ovicelled or non-ovicelled. Its size varies from small to medium, some avicularia being twice as large as others. In position it is constant, always sub-oral, median, slanting obliquely forward-downward, away from the orifice.

A pivot bar or hinge (Figs. 18, 28) for articulation with the mandible separates the avicularial surface into two slightly inclined regions: (a) the back area and (b) the mandibular, beak or rostral area. The rostral area is closed by the mandible. The membrane-covered back area is shorter and broader than the rostral area and in this species is always nearest the orifice, the rostral area being the farthest away, both in the mid line and sub-oral (Fig. 20).

In face view the avicularium is broadly oval, wider than long, perched on a wide mound-like avicularial chamber of variable size. Usually three, occasionally more or fewer, small pores perforate the front of this chamber.

There is a great difference in the degree of development or complexity of the soft structures inside the avicularia of various species but in *H. carsonae* they are well developed.

Levinsen (1909) called the avicularia and their contents heterozooecia and heterozooids; Borg (1926) heterozoids; Silén (1938) heterozoids, avicularial polypides, polymorphic individuals. Earlier references exist to the avicularial contents (Waters, 1888, 1892, 1900) but Waters' 1892 account is very adequate and understandably figured, although Silén (1938) published an extensive and excellent study of avicularia of several species.

The avicularial contents of *Hippadenella carsonae* are similar to those described by Waters (1892, pp. 272–274, and Pl. 19, Figs. 1, 2, 4, 5) for *Lepralia foliacea* Ellis and Sollander. In *H. carsonae* the avicularial chamber contains the avicularial polypide, avicularial glands, abductor mandibuli and adductor mandibuli muscles (Figs. 4, 20, 22, 27). The function of the polypide and glands is unknown. Marcus (1939, Pl. 19, Fig. 46) shows the avicularial contents of seven species and on p. 275 says of avicularial glands: "These glands can neither belong to the nervous, nor to the nutritive, or reproductive system and might perhaps have something to do with the stronger skeleton of the Ascophora . . . the function of the(se) organs still remains unknown; they might be poisonous." The *H. carsonae* avicularial glands are sometimes large, hollow and alveolar, with a large lumen and thin wall. Other times they are partly filled with a homogeneous, hardened secretion. The two bilaterally placed glands are united in a saddle-shaped unit that curves about the two bundles of adductor tendons and follows the contours of the very wide avicularial chamber. The avicularial polypide is a small, dense cellular body pinched in the middle so it seems double. It is attached to the back of the avicularial gland isthmus, in the mid-line. A vestibulum connects the polypide to the rostral area below the mandible tip, although this is difficult to see because of the opacity of the calcareous wall, the infrequency of favorably oriented crushed specimens and the small size of the soft structures involved.

The musculature of the avicularium is well developed. The adductor mandibuli muscles are more anterior, proximal and massive than the abductor mandibuli muscles. The adductor muscles have numerous short, thick, faintly striated muscle fibers. Their endings at origin and insertion differ in appearance. Their origin is over an extensive area on the walls and floor of the avicularial chamber. Their insertion is in two small pits, one at each side of the lucida on the inner mandibular surface. The muscle fibers attach bluntly and broadly at the origin while before the insertion is reached the muscle fibers have given way abruptly to delicate membranous and fine tendinous tissue (Figs. 4, 20). The tendon fibers attach by bead-like enlargements to the insertion site (Figs. 1, 25, 27).

The abductors form a very diffuse, sparsely fibered curtain against the distal end of the avicularial chamber. Their fibers originate on the back avicularial chamber wall and insert on the cuticularized membrane very close to the pivotal bar against which the mandible articulates. They do not seem to be striated and are considerably more slender than the adductor fibers. Marcus (1939, p. 273) states that in species studied by him the avicularial adductors are striated but the abductors smooth. This is true of *H. carsonae* also.

The avicularium is topped by a cuticularized mandible and a back area membrane (Figs. 20, 27). The mandible has a narrowly elliptical lucida flanked on each side by a somewhat semicircular pit into which the adductor tendons attach. A broad cuticularized band reinforces the mandible edges and base (Figs. 1, 22). A

short, delicate, transparent and serrated membrane decorates its front border (Figs. 9, 25), hanging down vertically, so it is almost invisible from the top (Fig. 1).

Distribution and ecology: Hippadenella carsonae turned up in only two dredgings of Jan. 29, 1948, Comdr. D. C. Nutt collector; one fragment from Sta. 101, and a pintful of twigs from Sta. 104. Both stations were from the Ross Sea area, Antarctica, off Cape Royds, Ross Island, from 58 fathoms. Most of the twigs were empty of polypides and relatively clean of extensive extraneous growths or encrustations. When other forms did grow on or in them these were relatively few in number and sparsely distributed over the branches, so apparently the twigs of *H. carsonae* did not present as hospitable a stratum for settling of other forms as does *Phylactellipora lyrulata* or some other species. The following organisms or their products are attached to the dead parts of *H. carsonae* twigs from Sta. 104: brown and green Folliculinids, Foraminifera, brown and white sponges, yellow egg cases (of flatworms?), calcareous tubes of annelids and scraps of various bryozoa. The bryozoa growing in small patches or attached to *H. carsonae* are several species of the Order Cyclostomata and the following of the Order Cheilostomata: *Cellaria moniliorata, Hippothoa bougainvillei, Hippothoa distans, Phylactellipora lyrulata* and a number of other species awaiting fuller identification. The Cyclostomata are especially numerous and seem to favor this species. Some Sta. 104 *H. carsonae* twigs grow on or partly engulf alcyonarian and sponge spicules, Cyclostomata, *Cellaria moniliorata, Cellaria vitrimuralis, Smittina ordinata* and other cheilostomes yet to be identified. In a discussion of *Smittina ordinata* (Rogick, 1956, p. 300) reference was made to *Smittina ordinata* growing on "other Bryozoa at Sta. 104," the other bryozoa in this case being *H. carsonae.*

Hippadenella carsonae specimens are deposited in the Smithsonian Institution, U. S. National Museum, USNM Cat. Nos. 11357 through 11364.

Summary

1. The morphology of *Hippadenella carsonae*, new ectoproct from the Antarctic, is described in detail and measurements made of many of its structures.

2. At the time of its collection, Jan. 29, ovicells were empty of embryos but developing eggs were in the ovaries. Developing embryos were absent from the body cavity also.

3. Living polypides occurred at the tips of some twigs but most of the material was dead or empty of polypides, at the time of collection.

4. The species has an unusually well developed avicularial apparatus consisting of large glands, reduced polypide, abductor and adductor muscles.

5. So-called "oral" or "sub-oral" glands are present. They are near the operculum but have no actual connection with, or proximity to, the true polypide mouth.

6. The function of "oral" glands, avicularial glands and avicularial polypide is unknown in this species, and a matter for speculation in other species.

7. Other peculiarities of this species are the oblique tubular frontal wall channels (frontal pores) and the mode of secondary calcification where trabeculae span the orifice and a domed lamina seals the ovicell.

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EXCYSTATION OF APOSTOME CILIATES IN RELATION TO MOLTING OF THEIR CRUSTACEAN HOSTS

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The dependence of certain aspects of the life cycle of a parasite on certain physiological activities of its host is a common phenomenon. One need only mention, as examples, the synchronicity of reproduction in malaria parasites, which is dependent on the diurnal rhythm of activity of the host (Stauber, 1939), and the appearance of sexual reproduction in the intestinal protozoa of the roach *Cryptocercus*, which is provoked by the molting of the host (Cleveland and Nutting, 1955). In a similar way, some species of apostome ciliates exist as small cysts (phoronts) on the gills of various Crustacea and excyst only at the time when the host molts (Chatton and Lwoff, 1935). The excysted forms (trophonts) then engorge rapidly on the proteinaceous fluid in the shed skin of the host. The much larger organisms so produced form free-living cysts. Within these cysts a series of divisions occurs which results in the formation of a number of small daughter ciliates (tomites). These swim about, apparently without feeding, until they have been drawn into the gill chamber of a suitable crustacean host. Here they encyst on the gills and again remain quiescent until the new host molts. The entire cycle depends on a single meal obtained from the molting fluids of the host, and the molting of the host provides the only stimulus to excystation (Chatton and Lwoff, 1935).

It seemed of interest to attempt to produce excystation *in vitro*.

Materials and Methods

Two host-parasite combinations have been used: (1) the fiddler crab *Uca pugnax* and a probably undescribed species of *Gymnodinioides;* (2) the hermit crab *Pagurus longicarpus* and *Gymnodinioides inkystans*. The fiddler crabs were kept in dishes with a shallow layer of sea water which was changed daily. Isolated individuals were kept in paper cups with a little sea water and a screen cover. The hermit crabs were maintained in running sea water. Isolated crabs of this species were placed in 100-ml. beakers covered with a piece of gauze held on by a rubber band. The beakers were immersed in an aquarium of running sea water. Both species were fed pieces of clam once or twice a week.

Individuals of *Uca pugnax* which were near the molt were generally recognizable by a peculiar pale cast to the carapace and legs. Those in the process of molting were observed to have milky white blood. In order to identify crabs very near the molt, the tip of a leg was cut off to permit a drop of blood to exude. The blood had a clear appearance except just before molting, when it was milky.

Crabs of the species *Pagurus longicarpus* near molting could be recognized by a marked gray color of the carapace and legs, as distinguished from the reddish cast

of individuals which had recently molted. In small groups of isolated "gray" crabs about 50% molted within three days after isolation, whereas none molted in corresponding groups of "red" crabs.

For the *in vitro* experiments, sterile Petri dishes holding two discs of filter paper and one or two depression slides were used. The filter paper was moistened with sterile sea water. In the concavity of each depression slide was placed a droplet of sterile sea water containing, per ml., 500 or 1000 units of penicillin G and 0.5 or 1 mg. of streptomycin. The lower concentrations were used in the experiments with *Uca* and the higher concentrations in the experiments with *Pagurus*. A crab selected to serve as a source of infected gill material was immersed briefly in 70% ethanol, rinsed in sterile sea water, and blotted on sterile filter paper. The legs were cut off close to the body with sterile scissors and the blood allowed to exude into the droplet of sea water with antibiotics. Two such blood-sea water mixtures could be prepared from one crab. In the experiments with *Uca* the blood was diluted by the sea water about 1 : 1, in those with *Pagurus* about 1 : 3. The carapace of the crab was then torn off. At this step, if the animal was close to molting, the old carapace cuticle would readily come loose revealing the soft newly formed skin beneath it. After exposure of the gill chamber, the gills were plucked off and placed in the mixtures of blood and sea water with antibiotics.

The preparations were kept at room temperatures in dim light and observed with a dissecting microscope for the appearance of trophonts. The gills were later placed between slide and coverslip and examined for the presence of phoronts and for possible signs of excystation.

Results

A. *Observations*

1. *Gymnodinioides* sp. of *Uca pugnax*. About 80% of the crabs of this species examined showed a few to many phoronts on their gills. The incidence of trophonts was, however, much lower. Out of a series of 105 recently shed skins only 24 contained trophonts. This might have been the result of very rapid engorgement and early escape of the trophonts from the exuviae, especially since molting usually occurred during the night. On the other hand, it might be that *Uca* is a relatively unfavorable host. The phoronts were frequently surrounded by a cellular reaction, often containing considerable brown pigment. Such a host reaction was never seen in *Pagurus*.

The living trophonts of this ciliate had a more pointed and twisted posterior end than those of *Gymnodinioides inkystans* from *Pagurus*. A silver preparation suggested 10 or 11 rather than 9 ciliary bands. The developmental cycle was typical of the genus *Gymnodinioides* (Chatton and Lwoff, 1935). More detailed morphological study would be needed for the precise identification of this organism, which does not quite fit any of the described species of *Gymnodinioides*.

In gill cuticle material taken from crabs found in the act of molting, small trophonts which had already excysted were present, as well as cysts showing a motile trophont within, and also seemingly unchanged resting phoronts. Cysts containing a motile trophont did not show the conspicuous large refractile granules present in most of the other phoronts. This might indicate a utilization of reserve food granules during the encysted state, a suggestion already made by Miyashita

(1933). The following observation on re-infection, however, is not entirely in accordance with this idea.

One *Uca* isolated in a small dish with a little sea water molted during the night. In the morning engorged trophonts were found swimming about in the dish. The crab was removed to a separate dish. By the following day cysts undergoing tomite formation were observed in the first dish and the crab was returned to it. The next day active tomites were noted in the water of this dish. One day later (three days after the molt) the crab was killed and its gills examined. Eighteen phoronts were found. All of these had few reserve granules and most had a large vacuole near the posterior end. In several this vacuole was seen to collapse and re-form slowly. Had these cysts not been found on a host which was known to have just molted and just been re-infected, they might have been considered "ripe" phoronts ready to excyst.

2. *Gymnodinioides inkystans* of *Pagurus longicarpus*. At least 80% of the molt skins of *Pagurus* examined during three summers contained moderate to large numbers of trophonts which were clearly *G. inkystans*. Phoronts were found on the plicae of the gills of most of the hermit crabs examined.

B. *Experiments in vitro*

1. *Gymnodinioides* sp. of *U. pugnax*. In an initial experiment gills from a crab in the act of molting were placed in a droplet of sea water and in sea water containing a segment of leg integument. Active small trophonts were already present in the gills, but nowhere else, at the time of the preparation. Eight hours later only small trophonts were seen in the preparation with sea water alone, but in the other preparation numerous partially to fully engorged trophonts were swimming about. A few were already encysting. By the second day many active tomites had been formed. Thus, once excystation had occurred, the engorgement and subsequent development *in vitro* were essentially normal.

In all the later experiments, with both host-parasite combinations, the methods detailed in the section on Materials and Methods were used. The general plan was to pair a crab judged to be not near the molt with one considered close to molting. The gills of each were divided into two portions. One portion was placed in a droplet of sea water with antibiotics plus blood of the same crab, and the second portion in a similar droplet with blood of the other crab.

Out of five experiments of this type in which numerous phoronts were present on the gills of both crabs, fully formed trophonts ready to excyst and showing slight movements were found in three. In these three experiments the crab judged to be near molting had milky blood, whereas in the other two experiments the crab considered near molting had clear or faintly turbid blood. In two of the three positive experiments the signs of excystation occurred only on gills of the crab near molting in its own blood. In the third, they occurred on gills of the crab near molting in the blood of the non-molting one. In no case did phoronts from the gills of the non-molting crab show signs of excysting.

2. *Gymnodinioides inkystans* of *P. longicarpus*. Seven experiments of the paired type were done in which phoronts were present on the gills of both the non-molting crab and the crab near molting. The following observations were made.

Exp. 1. Seven hours after placing of the gills in the blood-sea water-antibiotics mixture, four large trophonts were seen in the preparations containing gills from the crab near molting in its own blood, and one small trophont in the preparation of gills from this same crab in the blood of the non-molting crab.

Exp. 2. No trophonts were seen after seven hours, but after one day one large trophont was present in the preparation of gills from the crab near molting in its own blood, and two large trophonts in the preparation of gills from the crab near molting in the blood of the non-molting crab.

When the gills of the non-molting crab in its own blood were then examined under higher magnification, the unexpected observation was made of three phoronts which showed ciliary movement within the cyst (out of a total of 16 seen). Of 20 phoronts seen on the gills of the non-molting crab in blood of the one near molting, none showed movement although three had a large vacuole near the posterior end.

Exp. 3. A large trophont was present after one day in the gills from the crab near molting held with its own blood. None of the other preparations showed any indication of excystation.

Exp. 4. After one day four trophonts had developed from the gills of the crab near molting held with its own blood, and one from the gills of this crab with blood of the non-molting crab.

Exp. 5. Within seven hours six trophonts had developed from the gills of the crab near molting in its own blood, and one trophont from the gills of this crab in blood of the non-molting one. By the next day two additional trophonts had appeared in the latter preparation.

Exp. 6. Six trophonts developed within seven hours from the gills of the crab near molting in blood of the non-molting one. Partial drying had occurred in the preparation containing gills of the crab near molting in its own blood.

Exp. 7. No trophonts or signs of excystation could be found in any of the preparations.

Discussion

Excystation of both species of *Gymnodinioides* can evidently take place *in vitro* from phoronts which have not yet begun to excyst *in vivo*. The host crustacean must, however, be very close to molting time for this to occur, so that the statement of Chatton and Lwoff (1935) that these phoronts excyst only in response to molting of their host still holds. Phoronts from the gills of crabs near molting excysted, on the whole, a little better in the presence of the homologous blood than in the presence of blood from a non-molting crab. Miyashita (1933) noted that certain "ripe" cysts of *G. caridinae* from a fresh-water shrimp excysted within a few minutes after being placed under a coverslip in body fluid of the host, whereas many "unripe" cysts did not. It might be that the successful excystation occurred only with cysts which happened to have been taken from a shrimp near molting.

The results of the experiments described in the present paper indicate that the encysted phoronts of *Gymnodinioides* may require a series of stimuli from the host in order to prepare them for the final stimulus which produces excystation just before the actual molt. Such a course of events would be somewhat analogous to that described by Cleveland and Nutting (1955) for the sexual phenomena of the protozoa of *Cryptocercus*. Protozoa transferred from a roach at one stage of the

molting cycle to another at a different stage invariably died, whereas those transferred to another roach at the same stage continued their development.

Summary

Observations and experiments have been made with the encysted phoronts of *Gymnodinioides inkystans* on the gills of the hermit crab (*Pagurus longicarpus*) and of *Gymnodinioides* sp. on the gills of the fiddler crab (*Uca pugnax*). The phoronts of both species would excyst *in vitro,* in a mixture of crab blood with sea water and antibiotics, and give rise to engorged trophonts, only if the cysts were taken from gills of a crab which was near molting. This excystation occurred somewhat more readily in the presence of blood from the same crab, near the molt, than in the presence of blood from a crab not close to molting. It is concluded that the encysted phoronts probably require a series of stimuli from the host in order to prepare them for the final stimulus which produces excystation just before the actual molt.

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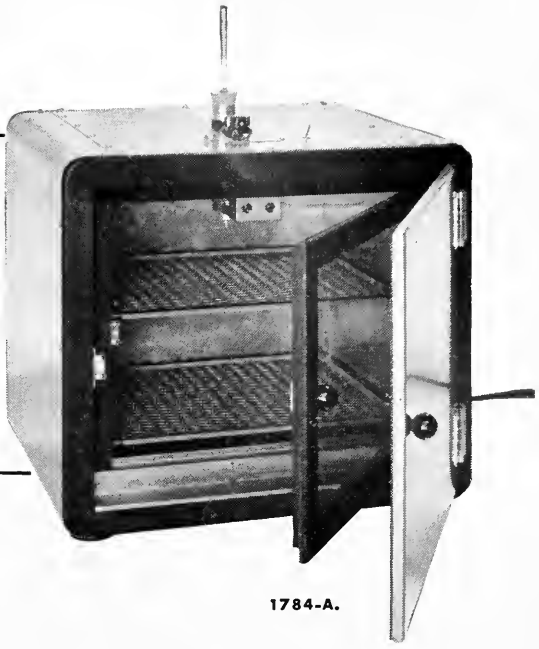

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THE
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DIFFERENCES IN SUSCEPTIBILITY TO WHOLE-BODY GAMMA IRRADIATION IN THE LAYERS OF THE RETINA OF BUFO

BENNET M. ALLEN AND MARION HUBBLE DEVICK

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Differences in susceptibility to irradiation constitute a problem of biological significance heightened by the fact that they occur in comparable tissues in different groups of animals. We have found that in *Bufo* the cells destroyed under the conditions of our experiments include certain small nerve cells of the brain, of the olfactory membrane, and of the retina. This paper deals with the latter because it offers an especially instructive example of these principles.

Materials and Methods

Recently metamorphosed *Bufo boreas halophilus* with trunk lengths of 10 to 12 mm. were firmly held in the zone of maximum irradiation while being exposed to total-body irradiation by a cobalt^{60} source. The average dosage rate was 950 r/minute. Fixation was by Bouin's fluid. Serial paraffin sections were cut at a thickness of one to five microns. A thickness of three microns was the most suitable. Slides were left over-night in commercial H_2O_2 in order to expose the rods and cones by depigmenting the strands from the pigment layer.

Some material was stained with Delafield's hematoxylin and eosin, but by far more useful was Heidenhain's iron-alum hematoxylin, slides being left one-half hour in each of the two solutions. This stain proved especially valuable not only because of its sharpness but because it stained the pyknotic nuclei most intensely.

Results

Gamma irradiation of 10,000 r, 20,000 r, 30,000 r, and 60,000 r caused destruction to the nerve cells of the inner nuclear and ganglionic layers. The few nuclei surviving after a dose of 60,000 r may be largely identified as those of the fibers of Mueller and the amacrine cells. On the other hand, even 60,000 r within the twenty-four hour survival period of the toads caused no destruction to the outer nuclear layer nor to the rods and cones belonging to them. At the lower irradiation levels the ganglionic layer appeared to be somewhat less susceptible than the inner nuclear layer, but with an irradiation of 60,000 r it, too, was completely destroyed (Fig. 2). The occurrence of structures interpreted as chromosomal vesicles in all

the retinal nuclei is considered to be normal because they are the same in experimental and control animals.

Toads irradiated with 10,000 r or 20,000 r and killed at twenty-four hours (Fig. 4 and Fig. 6) showed considerable destruction of the inner nuclear layer but this was almost complete at 30,000 r and 60,000 r. Toads irradiated at 10,000 r

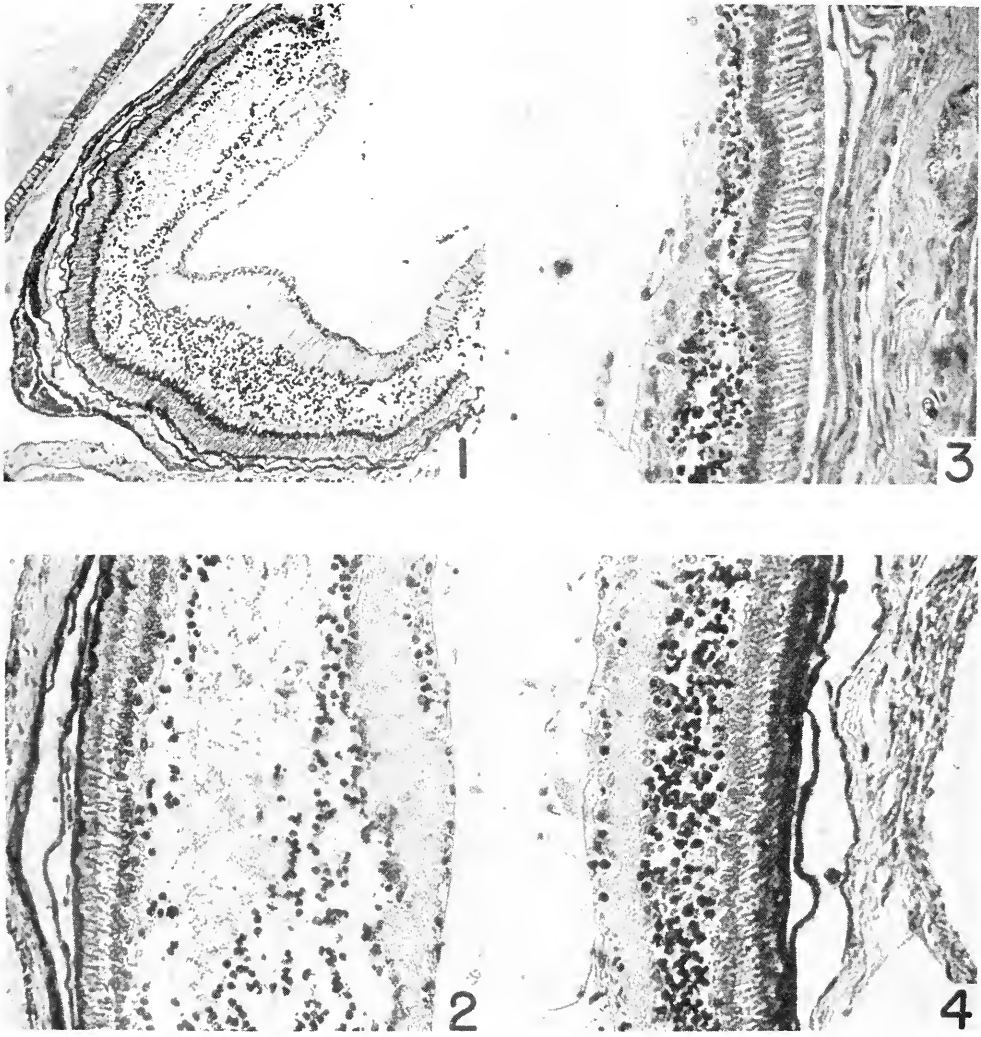

FIGURE 1. The retina at twenty-four hours after being given 60,000 r; × 60.

FIGURE 2. A higher magnification of the area in Figure 1, showing a portion of the more edematous area. × 239.

FIGURE 3. The retina six days after having been given 20,000 r. The thickness of the internal nuclear layer is reduced. The lowest part of the picture is the nearest to the ora serrata. × 329.

FIGURE 4. The retina twenty-four hours after having been given 20,000 r. × 239.

and 20,000 r and killed at an interval of six days after irradiation (Fig. 3 and Fig. 7) showed decided reduction in the thickness of the inner nuclear layer due to resorption of dead nuclei.

With doses of 60,000 r, groups of toads were killed immediately and at short intervals of one hour, two hours, and three hours after irradiation. In general, the effect was a delayed one, pyknosis becoming more and more extensive up to 24 hours. This was observed both after x-ray and gamma irradiation, the results being closely similar. A few cases of pyknosis were seen by the time irradiation was completed. In these early stages pyknosis involved a darkening of the nuclei and only later was there destruction of the cytoplasm. These pyknotic cells occurred throughout the inner nuclear layer of the retina but were most numerous a short distance in from the margin. Not only do the nuclei become deeply pyknotic but decided edema of the retina results. This was localized in the case of lower irradiation dosages, but with 60,000 r the entire retina becomes very heavily pyknotic and edematous (Fig. 1).

Figures 6, 7, 8 and 9 show portions of the retina under different degrees of irradiation. Pyknosis is roughly proportional to the amount of the dose. It is clear that even the highest dose does not affect the outer nuclear layer or the rods and cones, as observed at 24 hours or at six days after irradiation with 20,000 r.

Experiments were performed to show the effect of divided doses on the basis of a 60,000 r total. Irradiation was given on consecutive days and at the following rates: 10,000 r, six times; 20,000 r, three times; and 30,000 r, two times. Destruction of the inner nuclear layer was comparable to that caused by a single dose of 60,000 r. There was a marked reduction in thickness of the inner nuclear layer due to resorption during the course of the experiment (Fig. 8). Divided doses did not cause the amount of edema produced by a single dose.

A very interesting condition is seen at the ora serrata (Fig. 10). The cells of this region show no visible differentiation and there is no sharp line of demarcation between prospective sensory and nerve cell layers, but the nuclei toward the cavity of the eyeball can be followed to the inner nuclear and ganglionic layers, and we consider them to be prospective nerve cell nuclei. It is significant that irradiation renders these highly pyknotic. On the other hand, the nuclei adjacent to the choroid coat, considered to be prospective sensory cells, are not pyknotic. In the region more peripheral to the ora serrata there is likewise no pyknosis in spite of the fact that it is the zone where mitosis had added to the retina, up to a stage shortly before this. It would seem clear that some change has taken place in the prospective nerve cells that renders them especially sensitive to irradiation.

Following the differentiated nerve cell layers central to the ora serrata the distribution of pyknotic nuclei shows that cells of the peripheral portion of the retina are far more readily destroyed than the ones nearer to the center of the retina. At a lower degree of irradiation, 10,000 r and 20,000 r, this is quite evident but at 60,000 r the entire retina is deeply affected.

Discussion

The effects of irradiation upon the retina of amphibians have been chiefly studied in young stages. Brunst (1955) applied x-rays in doses of 1000 r to 8000 r to axolotl larvae from 9 to 65 days of age, the experiment being terminated in 18 to

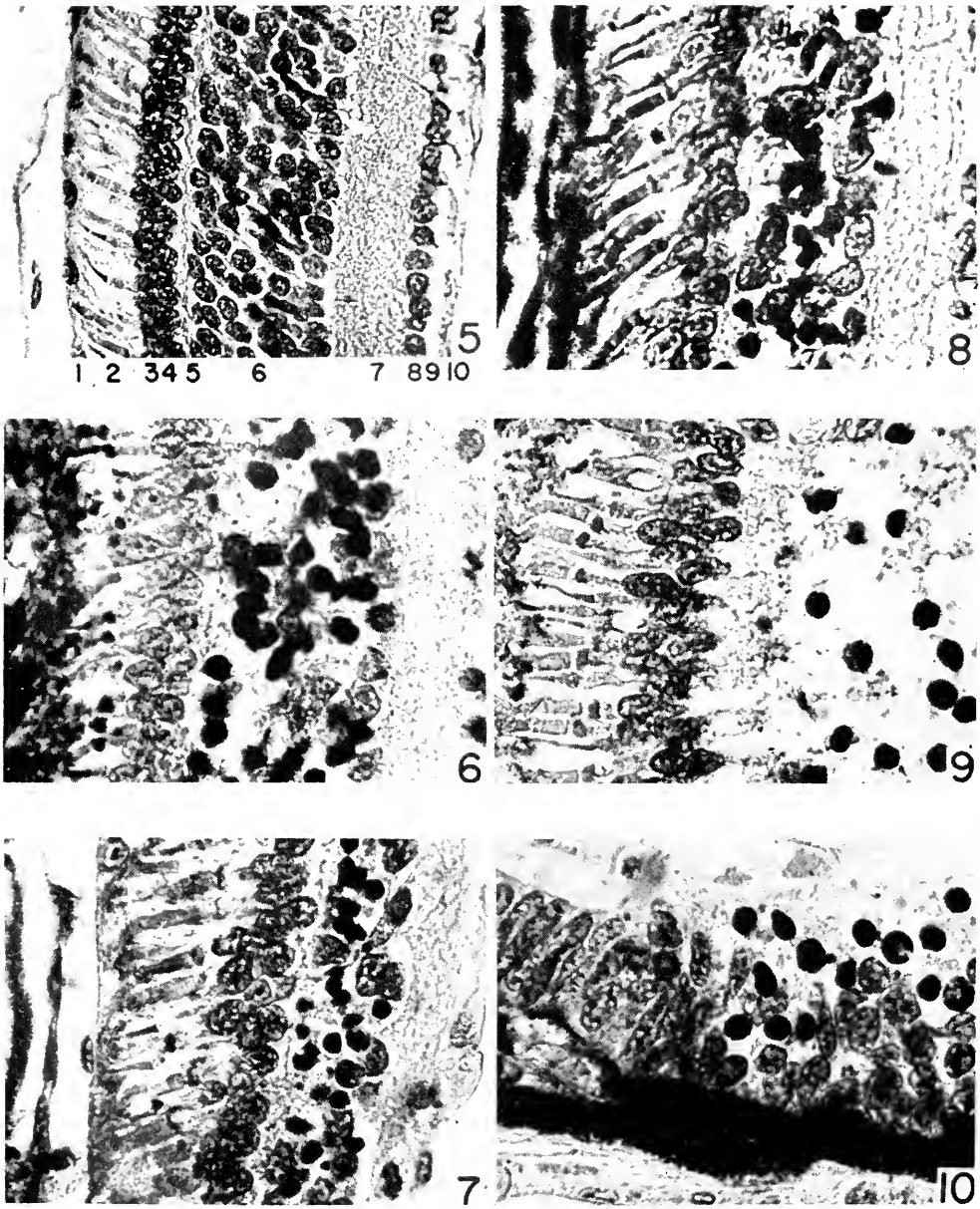

FIGURE 5. Section from the retina of a normal control animal. (1) pigment layer, (2) the rod and cone layer, (3) outer limiting membrance, (4) outer nuclear layer, (5) outer plexiform layer, (6) inner nuclear layer, (7) inner plexiform layer, (8) ganglionic layer, (9) nerve fiber layer, (10) internal limiting membrane. × 1110.

FIGURE 6. The retina twenty-four hours after having been given 20,000 r. × 1402.

FIGURE 7. The retina six days after having been given 20,000 r. Pyknotic nuclei are shrunken and resorbed. The thickness of the inner nuclear layer is reduced as compared to Figure 6. × 1402.

28 days after irradiation. He stated that in all cases the rod and cone layers disappeared first. Eventually the retina degenerated leaving only pigment cells. Brunst (1955) further states (p. 289): "These observations justify the conclusion that the eyes of animals used for this investigation are organs in the process of differentiation and active growth and are therefore, according to the law of Burgonie and Tribondeau, sensitive to roentgen irradiation."

Rugh (1954) finds that in *Ambystoma* larvae 22 mm. in length, x-irradiation of 15,000 r not only causes destruction of mitotic cells, but is equally destructive in regions lying quite apart from them. In a discussion following the reading of this paper, Rugh stated (p. 63), "the rods and cones are separated from the pigment layer but are not individually damaged as are the neuroblast cells. . . . The rods and cones are apparently not relatively sensitive or delicate."

In our own work the rods and cones and the plexiform layers are completely developed (Fig. 5). At the same time this condition has been rather recently attained. The peripheral region of the retina is the youngest part, having been built up as a result of mitotic activity in the region of the ora serrata, as shown by Spear and Glucksman (1941). We have shown that irradiation with 10,000 r and 20,000 r produces very heavy destruction of the inner nuclear and ganglionic layers a short distance central to this region. This leads to the assumption that the susceptibility of these cells is conditioned by their degree of maturity, together with the intensity of the irradiation. In our work we find no evidence that the outer nuclear layer or the rods and cones are affected even by 60,000 r in the 24 hours through which the toads survive. Edema is localized in the case of 10,000 r and 20,000 r irradiation but it is general when 60,000 r is given in a single exposure. It is of secondary importance because it does not appear when 60,000 r is given in divided doses. We have shown that the amount of pyknosis produced by divided doses appears to be roughly equal to that observed when irradiation is given in a single dose. This was shown in *Triturus* by Brunst and Sheremetieva-Brunst (1949).

In sharp contrast to our findings in *Bufo*, the investigators who have irradiated the eyes of mammals have found most extensive injury to the outer nuclear layer, notably to the nuclei of the rods and to the rods themselves. The cones with their nuclei were less affected. This was the finding of Lorenz and Dunn (1950), who exposed newborn mice to 400 r of x-irradiation and killed them at the end of twelve months. Noell *et al.* (1954) gave x-irradiation to the eyes of rabbits, resulting in the destruction of the visual cells while the inner retinal layers were spared. Similar findings were recorded by Brown *et al.* (1955), and Cibis and Brown (1955), who used gamma and x-irradiation upon the monkey, *Macaca rhesus*. With doses of 10,000 r the rods and their nuclei were affected as early as two hours after irradiation. Only when doses exceeded 30,000 r, was there considerable destruction of nerve cells of the inner nuclear layer.

FIGURE 8. This animal was given 10,000 r on 6 consecutive days. The amount of pyknosis is comparable to that produced by 60,000 r in one dose. The thickness of the inner nuclear layer is much reduced by resorption. There is no edema. × 1402.

FIGURE 9. The retina twenty-four hours after having been given 60,000 r. A large amount of edema and many pyknotic nuclei. × 1402.

FIGURE 10. Undifferentiated margin of the retina near the ora serrata, twenty-four hours after 60,000 r was given. The pyknotic nuclei are continuous with the inner nuclear and ganglionic layers. The pigment epithelium is toward the bottom of the picture. × 1402.

Noell (1953, 1955) experimented upon rabbits by injecting sodium iodate, sodium iodacetate, and also by applying oxygen poisoning as well as x-irradiation. All of these were especially injurious to the rods and in a lesser degree to the cones. They all produced quite comparable effects. The nuclei of the outer nuclear layer were largely killed except those belonging to the cones. He found that with the sodium iodate-poisoning, the pigment epithelium was first affected and the injury to the sensory organelles came later, leading to the view that there is a causal relation.

It is clear that in all of the above papers dealing with experiments upon mammals, it was shown that the rods and their cell bodies in the outer nuclear layer were most sensitive to irradiation, while the nerve cells were affected in far less degree. We have shown that just the reverse is true in *Bufo,* within the time limits used. In fact, we find no injury to the rods and cones and their cell bodies in the outer nuclear layer, while on the other hand, the inner nuclear and ganglionic layers show a very high degree of pyknosis. We shall not attempt in this paper to speculate upon the reasons for this difference but the facts are clear enough, and the point is most significant.

Attention is called to the sensitivity of the prospective nerve cells observed at the ora serrata where structural differentiation has not yet taken place. It is evident that in very early stages these prospective nerve cells have already undergone invisible changes that render them vulnerable to irradiation.

Allen (1956) showed that irradiated toads used in this work not only suffered heavy destruction of the nerve cell layers of the retina but also of the nerve cells of the brain.

Summary

1. Gamma irradiation doses of 10,000 r, 20,000 r, 30,000 r and 60,000 r were given to recently metamorphosed *Bufo boreas halophilus.* Toads receiving the two lower dosages were killed at the end of twenty-four hours and at six days. Those receiving the two higher dosages were all killed at twenty-four hours. Cell destruction was found in the inner nuclear and ganglionic layer of the retina but none was found in the outer nuclear layer nor in the rods and cones.

2. Destruction increased as the dosage increased. At doses of 10,000 r and 20,000 r, pyknotic nuclei were most abundant near the marginal portion of the retina, where the cells had been formed more recently than in the central portion. In animals that were given 60,000 r, pyknosis of retinal nerve cells was general and almost complete.

3. Divided daily doses of 10,000 r given six times, 20,000 r three times, and 30,000 r two times produced as much destruction as a single dose of 60,000 r. The amount of edema in these cases was never as great as that which was caused by a single dose of 60,000 r.

4. At the ora serrata where structural differentiation was not yet evident, cells interpreted as prospective nerve cells were destroyed while there was no destruction of prospective sensory cells of the outer nuclear layer.

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LARVAL DEVELOPMENT OF PALAEMONETES PUGIO HOLTHUIS [1, 2]

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Knowledge of the larval development of the marine species of *Palaemonetes* of the eastern United States is limited to Faxon's (1879) account of the development of *P. vulgaris*. This study was based almost wholly on planktonic larvae collected in an area from which two other closely related species, *P. pugio* and *P. intermedius*, have been described since (Holthuis, 1949). Holthuis (1952) feels that even mature adults of these species often have been confused. The larvae, presumably, may be quite similar, although those of the two latter species are unknown.

The problem of distinguishing between decapod species during the larval phase has received relatively little attention. Lebour (1927 through 1943) has found generic, and in some instances specific, taxonomic characters for British decapod larvae, but sometimes only was able to state that larvae of certain species were alike. Gurney (1942) feels that similarity between larvae indicates the relationship between the species. Often larvae of commercially valuable decapods have been studied without consideration of the larvae of related species. Churchill's (1942) account of the zoeae of the blue crab, *Callinectes sapidus*, is thought by Hopkins (1944) to include larvae of another species, possibly *C. ornatus*. Pearson's (1939) description of the development of the white shrimp, *Penaeus setiferus*, has been questioned by Burkenroad (1949) and Heegaard's (1953) descriptions of larvae of the same species are thought by Gunter (editorial comment in Heegaard, 1953) to include larvae of other penaeids.

Among species most studied there has been relatively little agreement on either the form or the number of larval stages. Churchill (1942) found five blue crab zoeae. Hopkins (1943, 1944) found four, but feels that a fifth may exist which has never been found. Sandoz and Rogers (1944) obtained a pre-zoeal blue crab in the laboratory which has not been found in nature. Heegaard (1953) questions the number of white shrimp stages found by Pearson (1939). The number of naupliar stages reported for other species of *Penaeus* varies still more (Hudinaga, 1942; Heldt, 1938). Many of these accounts were based on larvae caught in plankton and have been questioned. The validity of a reconstruction depends upon the ability of the author to recognize species during the larval phase. The difficulty is apparent, especially when another closely related species may exist in the same region.

Intraspecific variation in development among euphausids has been fairly well established (MacDonald, 1927; Fraser, 1936; Boden, 1950, 1951), but variation

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in decapod larvae has been denied (Gurney, 1942). Heegaard (1953), however, feels that, at least among penaeids, variation in the rate of larval development may result in differences in the number and form of larval intermolts for each species. Some doubt may have been cast on this hypothesis by the paucity and questionable identity of the material on which it was based. The pre-zoeal stage of *Callinectes* reported by Sandoz and Rogers (1944) has usually been dismissed as abnormal.

The present paper is first a description of the larval development of *Palaemonetes pugio* Holthuis based on observations of larvae reared in the laboratory. The larvae and the development are compared to that of *Palaemonetes vulgaris* (Say) reared concurrently. Finally, the considerable variation in the number and structure of larval intermolts, found in the development of both species, is described.

The author wishes to express his sincere appreciation to Professor C. G. Bookhout, under whose guidance and direction this work was done and who read and suggested improvements in this manuscript. Thanks are also due to Dr. T. R. Rice, who supplied stocks of all unicellular algae used, and to Dr. Fenner A. Chace, Jr., who provided working space and specimens of *Palaemonetes* from the collection of the U. S. National Museum.

Methods

Mature adult *P. pugio* and *P. vulgaris* are abundant in the vicinity of the Duke University Marine Laboratory at Beaufort, N. C., from late April until mid-October. Egg-bearing females were caught in dip nets and held individually in dishes of sea water in the laboratory until the eggs hatched, after which they were preserved for later identification and reference.

Groups of 10 newly-hatched larvae, all from the same clutch of eggs, were placed in clean four-inch finger bowls of sea water. The water was changed only if evidence of cloudiness appeared. The bowls of larvae were placed near north windows but not in direct sunlight. Because of other experiments which required constant illumination in an adjacent part of the laboratory, the room was never completely dark. No method of controlling the temperature of the building was available during the summer months. Most of the larvae were reared at temperatures which ranged from about 25 to 27° C. and none at lower than 20° C.

The diet of each larva was the same during its lifetime, but a variety of different foods or combinations of foods was offered to various larvae. Some were not fed. Other individuals received daily rations of either a species of unicellular algae, or a combination of species. Algae used included two species of *Nitzschia* and one species of each of the following genera: *Chlamydomonas, Thorocomonas, Nannochloris, Porphyridium* and *Pyramimonas*. Some larvae were fed species of algae combined with zooplankton which had been obtained by net and first killed by immersion in distilled water to prevent the fortuitous inclusion of living larvae similar to those being studied. The diet of some other larvae consisted of freshly killed zooplankton alone. A few larvae were fed on chaetognaths removed from the plankton and some others were fed tiny bits of the visceral mass of the mud snail, *Nassarius obsoletus*. The remaining larvae were fed living *Artemia* nauplii. Food was added to the bowls and uneaten food was removed daily.

Each larva was inspected daily under the low power of a stereoscopic binocular microscope. A record of molting by each larva was kept. Only the presence of

all or a large part of an exuvium or cast exoskeleton was accepted as evidence that a larva had molted. Morphological changes in larvae established which individuals had undergone the molt.

Camera lucida drawings were made of entire living larvae which had been anesthetized with ethyl carbamate and placed on slides beneath supported cover slips. These preparations were then sealed with oil. Larvae lived for as long as two hours under observation. Individual appendages from alcoholic specimens were studied and drawn.

Finally, adult females from which larvae were obtained were identified after comparison with type and other material at the U. S. National Museum.

Results

The number of individuals of each species which survived each of the several molts is given in Table I. Only those larvae which were fed diets containing some animal tissue were able to survive. The number of ecdyses and, conversely, the number of intermolts or so-called larval stages were not constant among individuals of the same species.

In general, the differences were slight and the similarities great between the larvae of *P. pugio* and those of *P. vulgaris*. In some instances, preserved larvae were identical. Among living larvae, however, a difference in the distribution of chromatophores on the ventral surface of the abdomen proved to be an invariable index to species. Larvae of *P. pugio* bear, on the sternites of abdominal somites

Table I

Number of larvae of Palaemonetes pugio and Palaemonetes vulgaris which survived each molt in the laboratory and the number of postlarvae obtained by metamorphosis. Column headings indicate diet of larvae

Molt number	*Palaemonetes pugio*					*Palaemonetes vulgaris*			
	No food	Unicellular algae	Algae plus animal tissue	Animal tissue	*Artemia* nauplii	No food	Unicellular algae	Algae plus animal tissue	*Artemia* nauplii
0	60	280	608	732	100	80	100	667	390
1	42	162	491	464	92	0	1	30	324
2	0	0	143	177	87		0	15	282
3			77	84	87			13	261
4			54	70	85			11	227
5			47	50	82			11	191
6			37	36	82			9	149
7			29	28	69			8	131
8			24	25				8	
9			18	14				8	
10			17						
11			17						
Number postlarvae	0	0	16	6	65	0	0	6	122

2 and 3, pairs of chromatophores. The larvae of *P. vulgaris* bear chromatophores on the sternite of abdominal somite 3, but lack pigment spots on abdominal sternite 2.

Although the individual larvae and the general pattern of development of *P. pugio* are nearly identical to that of *P. vulgaris*, differences between individuals of the same age and molting history and differences in the duration and tempo of larval development were observed in each species. Larvae which passed through the greatest number of molts during development showed the least morphological change after each molt. The structural characteristics of the individual larva were more readily associated with its total length than with its age or the number of molts completed. Although larvae which were of the same age often differed from one another in extent of development completed, those of about the same size were quite similar in structure regardless of the age or previous molting history. All larvae were alike upon hatching. This similarity began to disappear after the second molt. Considerable variation was found after the third molt, but, at the end of larval development, all larvae again resembled one another. The sequence or order of developmental events was the same for all larvae. The number of steps or stages passed through varied.

Differences in the form of larvae were accompanied by variation in the actual rate of development. Larvae fed living *Artemia* nauplii usually metamorphosed at the seventh molt which occurred about two weeks after hatching. Others, however, required from two to four weeks and as many as 13 molts to complete larval development. The variation observed, therefore, was primarily one of tempo.

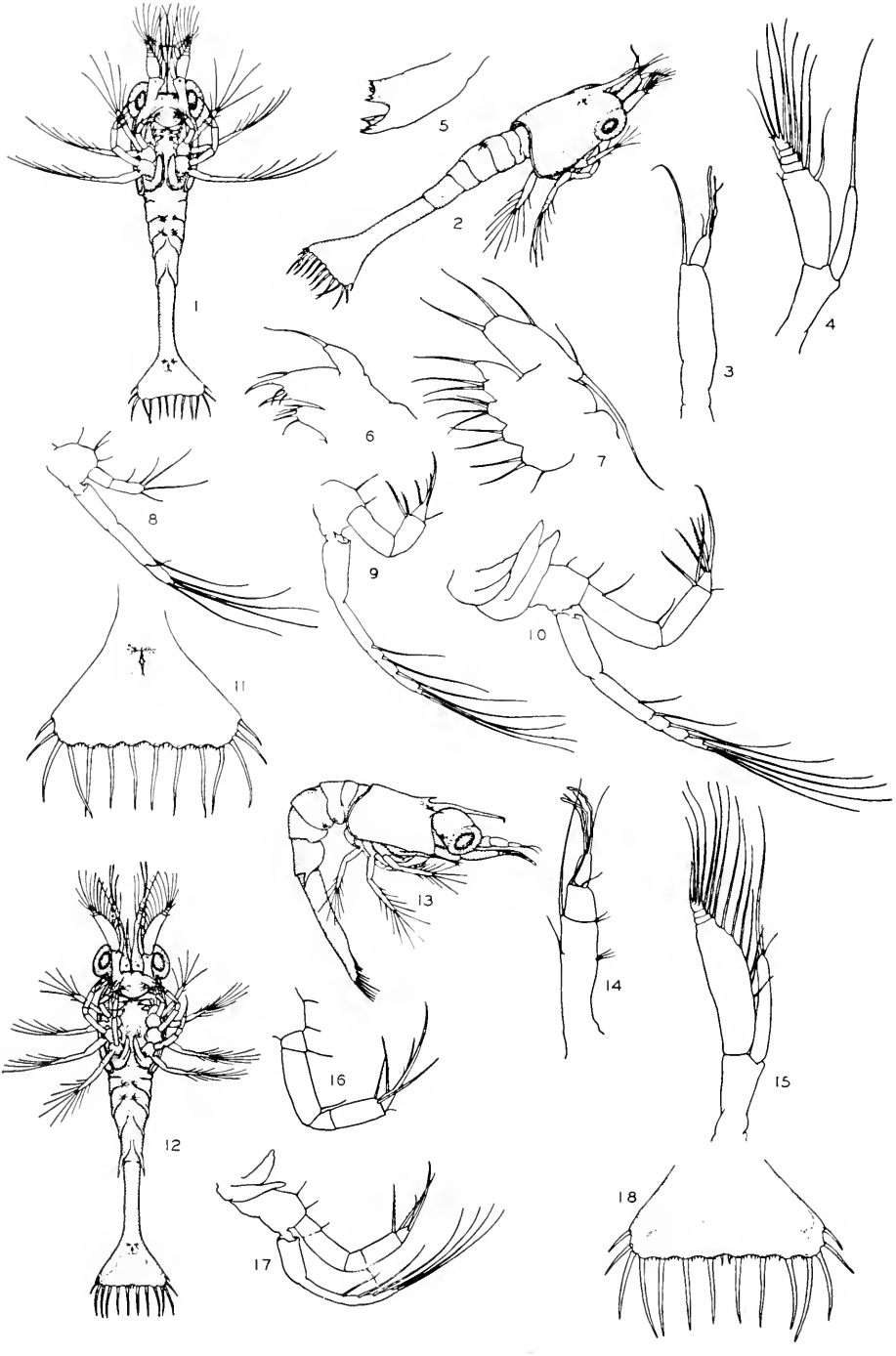
Larvae of Palaemonetes pugio

The eggs carried by adult females were from 0.5×0.6 to 0.6×0.9 mm. in size. They usually hatched within a few days, but always within two weeks or not at all. The prezoeal molt occurs immediately before hatching (Burkenroad, 1947), and the larva emerges from the egg as a zoea.

First zoea (Figs. 1–11): total length about 2.6 mm. Carapace, rostrum and abdomen without spines or teeth. Rostrum curved slightly downward at end. Abdomen of 6 somites, the last of which is not separate from the fan-shaped telson (Fig. 11). Telson with 14 spines. Eyes sessile, contained beneath carapace.

Antennule (Fig. 3) simple; the single basal segment bears terminally a long seta and a short outer flagellum; outer flagellum with three slender and one stout aesthetes and a short seta. Antenna (Fig. 4) biramous; basis unsegmented; flagellum of one segment, shorter than scale with a long terminal seta; scale of a long basal segment, which is convex on inner side, and four short terminal segments, with nine long setae on inner side and three short setae outside near tip.

Mandible (Fig. 5) without palp; incisor process with three teeth at tip; molar process with fine-toothed cutting edge; two movable teeth in angle between molar and incisor processes. First maxilla (Fig. 6) uniramous; coxa with five inwardly directed spines; basis with three spines and two teeth; endopod simple, palp-like, with a terminal seta. Second maxilla plate-like, biramous; protopod three-lobed, armed with three, two and four setae; endopod unsegmented, bears on a lobe near the proximal end two and terminally one setae; exopod a flattened gill bailer with three setae anteriorly, one laterally and one posteriorly.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First maxilliped (Fig. 8) biramous; coxa reduced; basis with four medially directed setae; endopod two-segmented, the distal segment with four terminal and one median setae; exopod longer than endopod with four apical and two sub-apical setae. Second maxilliped (Fig. 9) biramous; coxa reduced; basis with two setae; endopod three-segmented, with two strong spines at junction of ultimate and penultimate segments, ultimate segment with two smaller spines, a seta and a strong terminal claw; exopod longer than endopod with a cylindrical proximal and a flattened paddle-like distal segment which bears four apical and three pairs of sub-apical setae. Third maxilliped (Fig. 10) biramous, larger than second maxilliped, but generally similar to it; endopod with two setae on proximal segment; exopod with four apical and three or four pairs of sub-apical setae.

First and second pereiopods (Fig. 10) rudimentary. Other appendages lacking.

Prominent groups of red and yellow-green chromatophores located dorsally at bases of eyes and at junction of abdominal somites 2 and 3. Paired groups of chromatophores ventrally on basal segment of antenna, on labrum, on thoracic sternites 1 and 8, on abdominal sternites 2 and 3 and on telson just anterior to anus.

This larva corresponds very closely to the first zoea of *P. vulgaris* and to Faxon's (1879) description of the first stage larva of that species. It differs from the larva of *P. vulgaris* chiefly in the presence of a pair of chromatophores on abdominal sternite 2, which are lacking from the latter species. The basal segment of the antennal scale of *P. vulgaris* is less convex on its inner side and the scale is narrower than in *P. pugio*.

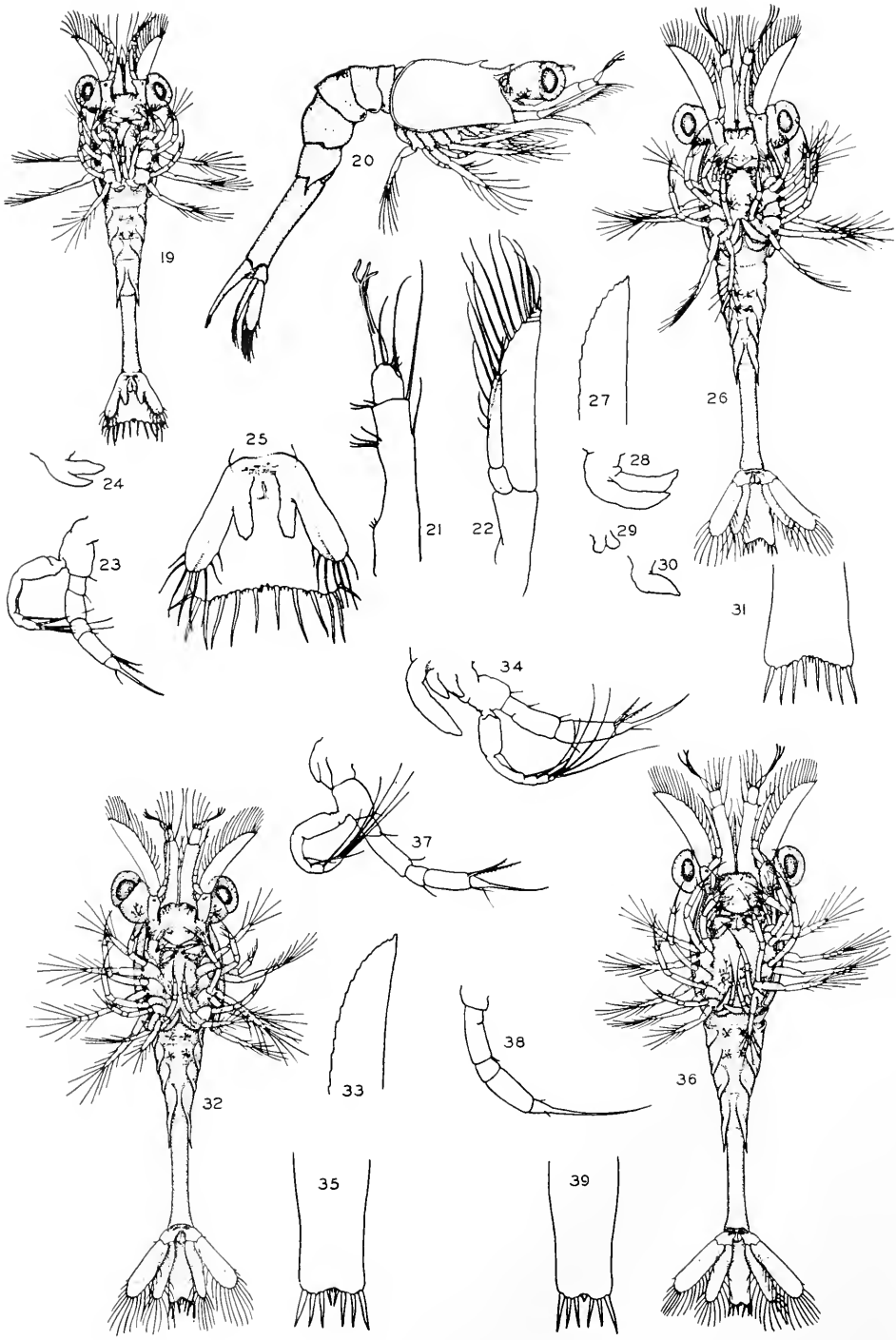
Second zoea (Figs. 12–18): length about 2.8 mm. Differs from first zoea in the following: Carapace with supra-orbital and branchiostegal spines. Rostrum recurved at tip and with one dorsal rostral tooth located on the carapace just behind orbit. Pleurum of fifth abdominal somite terminates as a posteriorly directed tooth. Telson (Fig. 18) with 14 large and two minute spines. The outlines of the uropods often visible within telson. Eyes stalked with chromatophores on postero-ventral side of stalk.

Antennule (Fig. 14) with peduncle segmented, segments marked by long setae on inner side, a long and a short seta on distal end of peduncle; outer flagellum with two slender and two stout aesthetes. Antennal scale (Fig. 15) with three short segments at the distal end and 14 setae; antennal flagellum terminates in a long and two short setae.

First maxilla with four teeth and three spines on basis. Exopod of second maxilla with five anterior setae. Endopod of third maxilliped (Fig. 16) five-segmented.

First pereiopod (Fig. 17) biramous; coxa reduced; basis with two setae; endopod five-segmented, ischium, carpus and dactylus with a seta each, two stout spines at junction of propodus and dactylus, dactylus terminates in a strong claw; exopod as in third maxilliped.

PLATE 1. Larval development of *Palaemonetes pugio*. Entire larvae ×19; appendages ×54. Figures 1–11 of first zoea. Fig. 1: ventral view. Fig. 2: dorsolateral view. Fig. 3: antennule. Fig. 4: antenna. Fig. 5: mandible. Fig. 6: first maxilla. Fig. 7: second maxilla. Fig. 8: first maxilliped. Fig. 9: second maxilliped. Fig. 10: third maxilliped and first and second pereiopods. Fig. 11: telson. Figures 12–18 of second zoea. Fig. 12: ventral view. Fig. 13: lateral view. Fig. 14: antennule. Fig. 15: antenna. Fig. 16: endopod of third maxilliped. Fig. 17: first and second pereiopods. Fig. 18: telson.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The second zoea corresponds to the second zoea of *P. vulgaris* and to Faxon's description of the second stage larva of that species. The second zoea of *P. vulgaris* differs from the corresponding larva of *P. pugio* in lacking chromatophores on abdominal sternite 2. All larvae which had molted once were in the form described.

Third zoea (Figs. 19–25): total length about 3.2 mm. Differs from the previous larva in the following: Sixth abdominal somite separate from telson. Telson (Fig. 25) narrower than before, armed with 12 large and two small spines.

Antennular peduncle (Fig. 21) with two long setae ventro-distally, two long setae on inner side, and, on a protuberance near the proximal end which will be the stylocerite, three short setae; a rounded prominence bearing three or four short setae and located dorsally near the distal end of the peduncle is the antennular lobe; outer flagellum with three stout aesthetes. Antennal scale (Fig. 22) with two short segments at distal end and 15 setae; antennal endopod separated into a short peduncle and an unsegmented flagellum which terminates in two short and two minute setae.

Second pereiopod (Fig. 23) biramous; coxa reduced; basis with at least one seta; endopod five-segmented, first and last segments with setae, two stout spines arise from junction of propodus and dactylus, dactylus terminates in a strong claw; exopod shorter than endopod, similar in structure to other thoracic exopods. Third pereiopod (Fig. 24) biramous, rudimentary.

Uropod (Fig. 25) biramous, unsegmented, with rudimentary inner ramus; outer ramus with 7 or 8 setae.

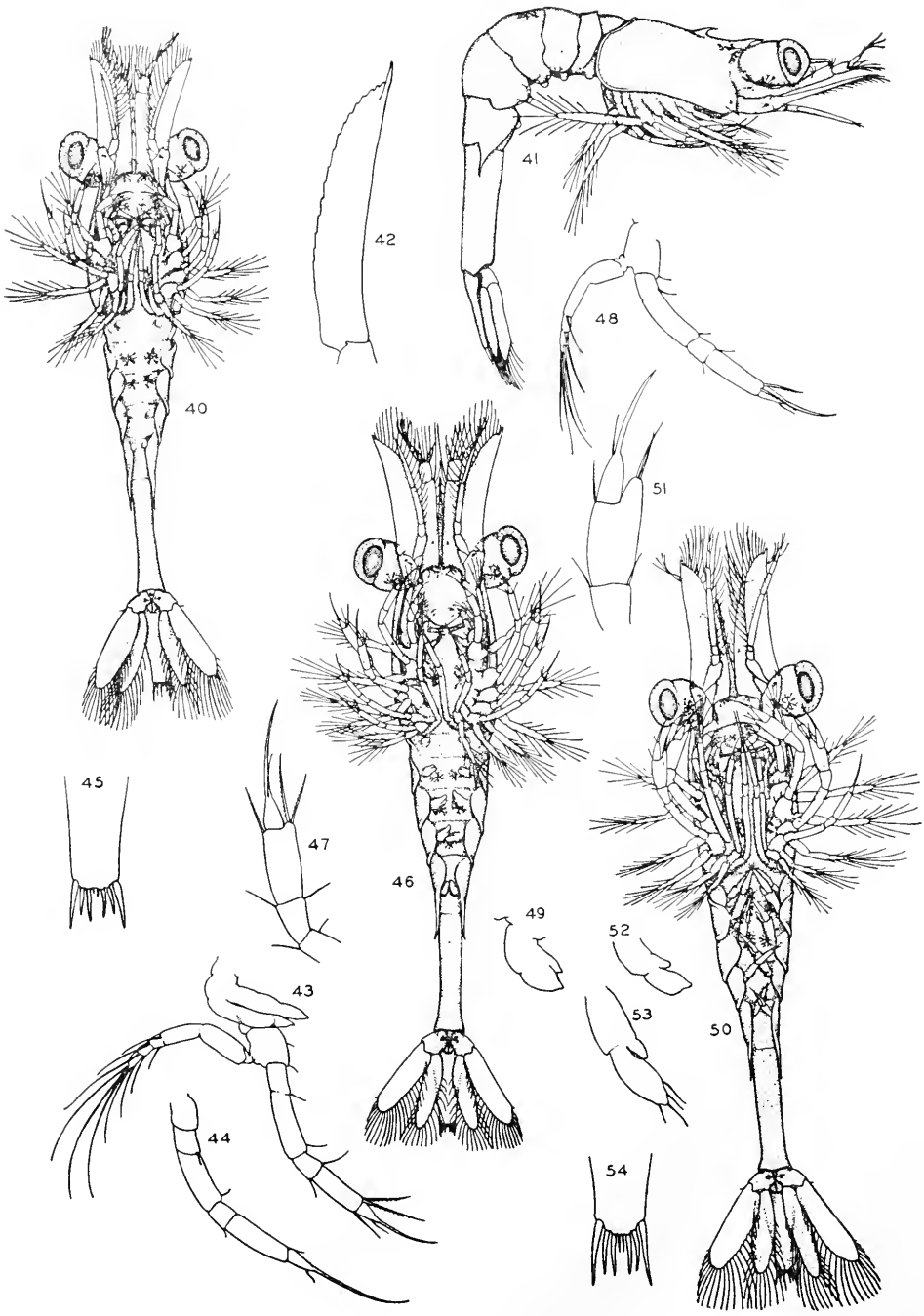
The third zoea corresponds to the third zoea of *P. vulgaris* and to Faxon's third stage. It differs from the third zoea of *P. vulgaris* in the presence of chromatophores on abdominal sternite 2. Variation in the form of larvae which had molted twice was noted in the total length and in the number of rudimentary pereiopods present. Larger larvae had, in addition to the appendages described above, rudiments of third and fourth pereiopods. All larvae which had molted twice were third zoeae.

Fourth zoea (Figs. 26–31): length about 3.5 mm. Differs from the third zoea in the following: Two dorsal rostral teeth on the carapace. Telson (Fig. 31) but little wider posteriorly than anteriorly, armed with eight stout and two small spines.

Antennular peduncle with four long distal setae and a protuberance which is the rudiment of the inner flagellum. Antennal basis separated into two segments by an oblique fissure at the articulation of the peduncle; scale (Fig. 27) not segmented, its disto-lateral tip (spine) projecting slightly, blade with 16 or 17 setae.

Second pereiopod larger than before. Third and fourth pereiopods (Figs. 28 and 29) biramous, rudimentary. Fifth pereiopod (Fig. 30) uniramous, rudimentary. Uropod biramous; basis unsegmented; endopod shorter than exopod bears 8 setae; exopod with 12 setae.

PLATE II. Larval development of *Palaemonetes pugio*. Entire larvae × 19; appendages × 54. Figures 19–25 of third zoea. Fig. 19: ventral view. Fig. 20: lateral view. Fig. 21: antennule. Fig. 22: antenna. Fig. 23: second pereiopod. Fig. 24: third pereiopod. Fig. 25: uropods and telson. Figures 26–31 of fourth zoea. Fig. 26: ventral view. Fig. 27: tip of antennal scale, setae omitted. Fig. 28: third pereiopod. Fig. 29: fourth pereiopod. Fig. 30: fifth pereiopod. Fig. 31: end of telson. Figures 32–35 of fifth zoea. Fig. 32: ventral view. Fig. 33: tip of antennal scale, setae omitted. Fig. 34: third, fourth and fifth pereiopods. Fig. 35: telson. Figures 36–39 of sixth zoea. Fig. 36: ventral view. Fig. 37: third and fourth pereiopods. Fig. 38: fifth pereiopod. Fig. 39: telson.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The fourth zoea of *P. pugio* corresponds to the fourth zoea of *P. vulgaris* but has no equivalent in Faxon's descriptions of the larvae of that species. The fourth zoea of *P. pugio* differs from that of *P. vulgaris* in the distribution of abdominal chromatophores and in the projecting tip of the spine of the antennal scale, which, in *P. vulgaris* larvae, does not extend beyond and free of the blade. Not all larvae which had molted three times correspond to the fourth zoea as described here. Among those larvae which molted the least number of times during development, this form was not represented by any intermolt.

Fifth zoea (Figs. 32–35): total length about 3.5 mm. The fifth zoea differs from the fourth in the following: Telson (Fig. 35) narrower posteriorly than anteriorly, armed with six stout and two slender spines. Antennular peduncle with 5 long setae distally; inner flagellum a separate segment tipped with a short seta. Antennal scale (Fig. 33) with 19 setae, tip projecting; flagellum divided into a short proximal and a longer distal segment.

Mandible with three serrate movable teeth. Basis of first maxilla with five teeth and three setae. Middle lobe of basis of second maxilla with three setae; exopod with six to seven anterior setae.

Third pereiopod (Fig. 34) biramous; coxa reduced; basis with at least one seta; endopod five-segmented, ischium, merus, carpus, and dactylus with setae, two stout spines at junction of propodus and dactylus, dactylus terminates in a claw; exopod shorter than endopod with four apical and two pairs of sub-apical setae. Fifth pereiopod longer than fourth (Fig. 34), both rudiments. Uropodal endopod nearly as long as exopod, with 13 setae; exopod with a short seta on outside near proximal end and a short tooth in dorso-lateral corner, with 16 long setae.

The fifth zoea of *P. pugio* differs from the fifth zoea of *P. vulgaris* as do fourth zoeae. There is no corresponding larval stage in Faxon's account of development of *P. vulgaris,* but a larva of this form was obtained by molt and considered abnormal by Faxon. This form may be skipped in the development of either species.

Sixth zoea (Figs. 36–39): total length about 3.7 mm. Differs from the previous larva in the following: Antennular peduncle with three plumose setae on inner side, angle of stylocerite is acute. Antennal scale with 20 plumose setae. Fifth pereiopod (Fig. 38) uniramous; coxa reduced; short setae on merus, propodus and dactylus, dactylus terminates in a long claw. Uropodal exopod with 16, endopod with 13 or 14 setae.

The form described as the sixth zoea may be present in the development of either *P. pugio* or *P. vulgaris.* The species differ in the ways previously discussed. This form is not represented among Faxon's larval stages, but was seen by him and considered abnormal. This form may be skipped in the development of either *P. pugio* or *P. vulgaris.*

Seventh zoea (Figs. 40–45): total length about 4.4 mm. The seventh zoea

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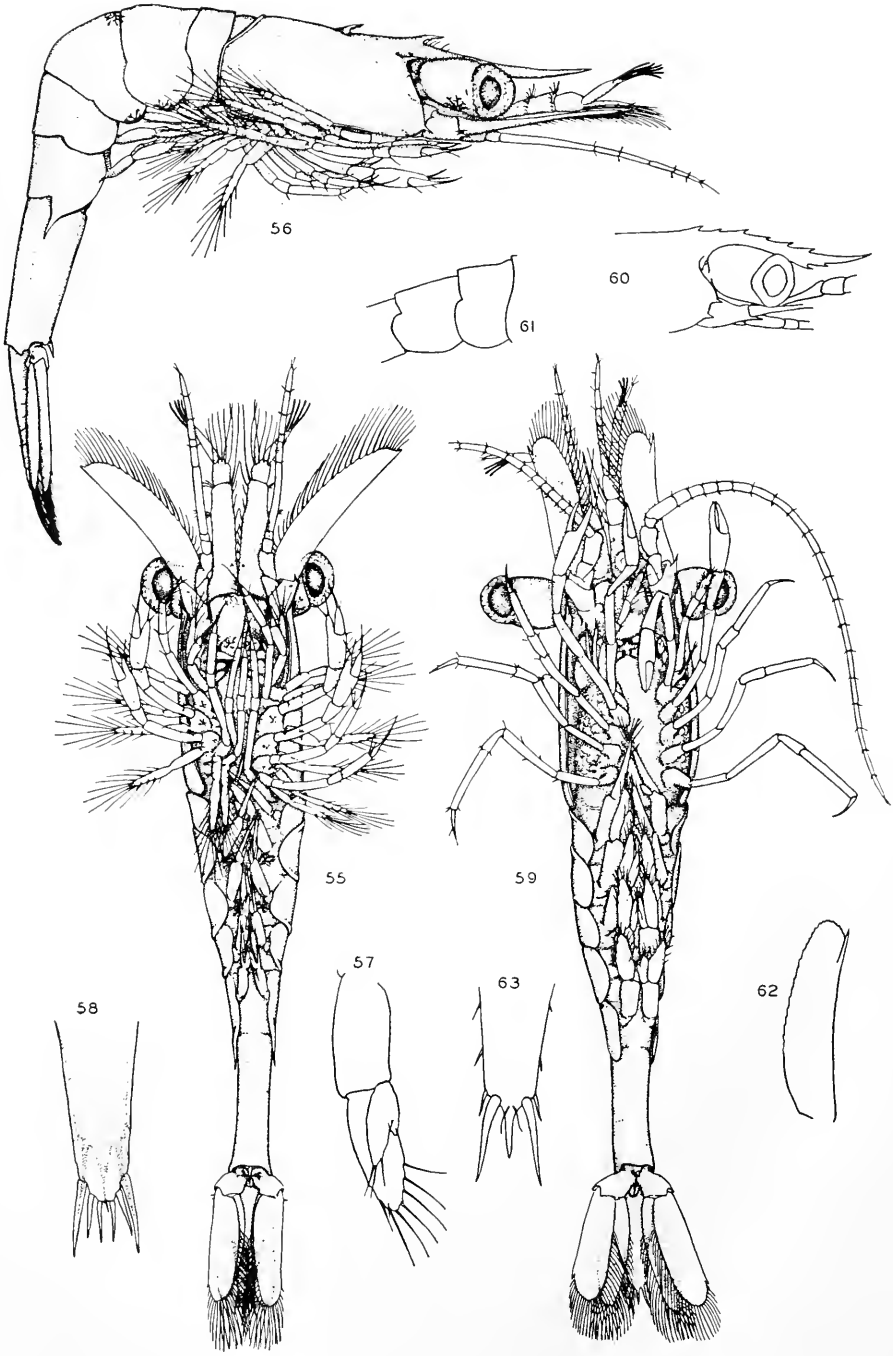
PLATE III. Larval development of *Palaemonetes pugio.* Entire larvae × 19; appendages × 54. Figures 40–45 of seventh zoea. Fig. 40: ventral view. Fig. 41: lateral view. Fig. 42: antennal scale, setae omitted. Fig. 43: third and fourth pereiopods. Fig. 44: fifth pereiopod. Fig. 45: telson. Figures 46–49 of eighth zoea. Fig. 46: ventral view. Fig. 47: first cheliped. Fig. 48: fourth pereiopod. Fig. 49: second pleopod. Figures 50–54 of ninth zoea. Fig. 50: ventral view. Fig. 51: first chela. Fig. 52: first pleopod. Fig. 53: second pleopod. Fig. 54: telson.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differs from the sixth in the following: A minute anal spine present. Antennular peduncle with five setae on inner side; outer antennular flagellum with three stout and one slender aesthetes. Antennal scale (Fig. 42) with 20 setae. Fifth pereiopod with a seta on each segment.

First to fifth pleopods represented by small uniramous buds.

The seventh zoea of *P. pugio* differs from the seventh zoea of *P. vulgaris* as the sixth zoeae differ and in regard to a small tooth on the ventral side of the antennular peduncle of the latter species. This tooth appears later in the development of *P. pugio* and is smaller than in *P. vulgaris*. Considerable variation in the development of the last three pereiopods was found in larvae first having pleopod buds. Among those larvae which, in their development, had skipped the forms described as fourth, fifth and sixth zoeae, pereiopods 3, 4 and 5 were rudiments. Among other larvae pereiopod 5 was still rudimentary at the time of the appearance of pleopod buds. The seventh zoea described here corresponds most closely to Faxon's fourth larval stage of *P. vulgaris*. Larvae of either species may have molted three, four, five or six times when the form described as the seventh zoea is achieved.

Eighth zoea (Figs. 46–49) : total length about 4.9 mm. The eighth zoea differs from the previous larva in the following: The anal spine is stronger. The antennular peduncle bears, about mid-way of the proximal segment on the ventral side, a small tooth, 7 setae on inner side, 6 setae at distal end. Antennal basis with a tooth on ventral side at junction of scale; scale with 23 setae; flagellum of three or four segments.

Propodi of first and second pereiopods (Fig. 47) swollen and protuberant at inner distal corner, forming, with dactylus, the beginning of a chela. Fourth pereiopod (Fig. 48) biramous; coxa reduced, basis with a seta; endopod five-segmented, ischium, merus and dactylus with one seta each, carpus with two setae, junction of propodus and dactylus with three spines, dactylus tipped with a claw. Fifth pereiopod with a short spine arising from the middle of dactylus.

First to fifth pleopods (Fig. 49) small, biramous, rudimentary. Uropodal exopod with 20, endopod with 18 setae.

The eighth zoea of *P. pugio* corresponds to the eighth zoea of *P. vulgaris* from which it differs in the ways previously stated. This form corresponds to Faxon's fifth larval stage of *P. vulgaris*. There was considerable variation in the development of the pleopods, but all had non-setose, biramous pleopod rudiments.

Ninth zoea (Figs. 50–54) : length about 5.1 mm. Differs from the previous larva in the following: Rostrum setose in the angle of the anterior tooth. Outer antennular flagellum with four equal, sub-apical aesthetes and a slender aesthete arising from mid-way of the segment. Antennal flagellum longer than scale, five-segmented; scale with 26 plumose setae. Entire outer edge of exopod of second maxilla setose. First maxilliped with a simple epipod, basal portion enlarged with 8 setae, proximal segment of exopod with two setae.

PLATE IV. Larval development of *Palaemonetes pugio*. Entire larvae and postlarva × 19; larval appendages × 54. Figures 55–58 of tenth (last) zoea. Fig. 55: ventral view. Fig. 56: lateral view. Fig. 57: second pleopod. Fig. 58: telson. Figures 59–63 of postlarva. Fig. 59: ventral view. Fig. 60: lateral view of rostrum and anterior portion of cephalothorax, × 19. Fig. 61: lateral view of fourth and fifth abdominal pleurae, × 19. Fig. 62: antennal scale, setae omitted, × 19. Fig. 63: telson, × 54.

First and second pereiopods chelate (Fig. 51), the fixed finger tipped by the two spines formerly located at junction of propodus and dactylus. First to fifth pereiopods with arthrobranchs.

First to fifth pleopods biramous. First pleopod (Fig. 52) rudimentary. Second (Fig. 53) to fifth pleopods with sparsely setose exopods. Uropodal exopod with 24, endopod with 17 setae.

The ninth zoea of *P. pugio* differs from the ninth zoea of *P. vulgaris* as previously described. The ninth zoea corresponds to Faxon's stage 6, first intermolt. Variation was noted in the extent of development of the inner ramus of the pleopods, the recurvature of the rostral tip, and the extent of development of the chelae.

Tenth zoea (Figs. 55–58) : total length about 6.3 mm. The tenth zoea is the form representing full larval development. It differs from the previous larva in the following: Tip of rostrum curved slightly upward. Telson (Fig. 58) slender posteriorly, armed as before. Inner antennular flagellum with six aesthetes and two short apical setae. Antennal flagellum of 7 segments; scale with 30 setae, its tip projecting or not. Mandible with four or five movable teeth in angle between incisor and molar processes. Basis of first maxilla with 6 teeth. Exopod of second maxilla fringed with 29 setae.

Basis of first maxilliped with 9 setae and a bilobed epipod; proximal segment of exopod with 5 or 7 setae. Pereiopods essentially as before.

First pleopod with rudimentary endopod. Second (Fig. 57) to fifth pleopods biramous with setose exopods and endopods; endopods with small *appendices internae*. Uropodal exopod with 29 and endopod with 26 plumose setae.

The tenth zoea of *P. pugio* differs from the tenth zoea of *P. vulgaris* chiefly in the chromatophores of the ventral abdomen as previously discussed. This form corresponds to Faxon's sixth larval stage, intermolts 2 and 3. Some variation in the extent of development of the *appendices internae* of the pleopod endopods was noted.

Postlarva (Figs. 59–63) : length about 6.2 mm. Rostrum shorter than antennal scale, with six dorsal teeth, the first of which is on the carapace directly over the posterior margin of the orbit, and two ventral teeth, the first of which is directly beneath the last dorsal tooth; tip of rostrum free of teeth (Fig. 60). Carapace with antennal and branchiostegal spines. Posterior margins of abdominal pleurae rounded (Fig. 61). Anal spine present. Telson (Fig. 63) with a tooth extending back from the mid-point posteriorly, two pairs of terminal spines of which the inner pair are longer, a pair of setae mid-ventrally near the distal end, and two pairs of dorso-lateral spines about ½ and ¾ of the way from the proximal end.

Antennular peduncle of three segments; stylocerite less than ½ the length of the basal segment of peduncle; antero-lateral spine of basal segment exceeding anterior margin of segment; inner side of peduncle with 10 setae; basal segment containing a statocyst and a short ventral tooth. Inner antennular flagellum simple, five-segmented. Outer antennular flagellum four-segmented, bearing on the anti-penultimate segment two, and on the penultimate segment three aesthetes, and a tuft of setae on the ultimate segment. Length of antennal scale (Fig. 62) about four times its width, outer margin slightly concave; anterior end of spine projects free of blade and is slightly shorter. Antennal flagellum over half of total length.

Mandible strong, incisor process stouter than in larval mandible; teeth of molar process large, forming a triangular surface, with or without movable teeth in angle.

Basal portion of first maxilla bilobed, each lobe bearing on its inner surface numerous coarse setae; endopod palp-like. Basal portion of second maxilla bilobed, each lobe bearing on its inner surface numerous coarse setae; endopod unsegmented with neither lobes nor setae; exopod setose around edge.

Basal portion of first maxilliped large, bilobed, the lobes with coarse setae directed inwardly; endopod reduced, bears two apical setae; exopod with six setae on proximal segment and four long setae at tip of distal segment; epipod large, bilobed. Second maxilliped with five-segmented endopod, ultimate and penultimate segments wider than long, armed with coarse spines; exopod with two setae; epipod small, bilobed. Third maxilliped with four-segmented endopod, coarsely setose throughout; endopod reduced; epipod tiny, bilobed.

First pereiopod chelate, somewhat stouter and shorter than second pereiopod; exopod a rudiment or lacking. Second pereiopod chelate, cutting edges of chela without serrations or teeth. Carpus shorter than palm; exopod rudimentary if present. Third, fourth and fifth pereiopods not chelate, exopods rudimentary if present. Arthrobranchs at bases of pereiopods.

Endopod of first pleopod rudimentary. Endopods of pleopods 2 to 5 with *appendices internae*. Uropodal exopod sparsely setose along outer edge with a tooth and a movable spine in the disto-lateral corner, numerous setae around the tip and on inner edge; endopod with setae on inner edge and around tip.

The distinctive distribution of chromatophores which characterized the larva is lost in the postlarva. The young prawn appears colorless to the unaided eye, but actually has numerous tiny chromatophores on the cephalothorax and abdomen. The postlarva of *P. pugio* is strikingly similar to the postlarva of *P. vulgaris*. The characters used to separate adults of these species are undeveloped in postlarvae. No attempt is made here to offer characters by which the two species may be distinguished at this stage of development. The postlarva corresponds to Faxon's seventh stage.

The sequence of larval intermolts

The descriptions of zoea larvae given above were based on the structure of individuals reared in the laboratory. Since the number of molts during development is not constant, it is obvious that certain of the described forms may be omitted in the life history of any individual. The relationship between the number of molts and the structure of intermolts observed in the laboratory is given in Table II. Column 4 of Table II shows that, for some larvae, the sequence of successive intermolts was that given in the text descriptions. Columns 5 to 9 show progressive omission of more of the described forms. The data suggest a relationship between diet and the number and form of larval intermolts.

Discussion

Larvae of *Palaemonetes* reared in the laboratory were structurally identical to those from nature described by Faxon (1879). This resemblance extended even to certain larvae considered abnormal by Faxon but shown by rearing to belong to series of intermolts which ultimately metamorphosed to produce normal postlarvae. The distinctive distribution of abdominal chromatophores, which was found to be diagnostic for the species treated in this paper, was not noted by Faxon

TABLE II

The relationship between molting and form of Palaemonetes pugio and Palaemonetes vulgaris larvae fed various diets. Numbers in column 1 refer to text descriptions. Numbers in parentheses refer to described variations. The sequence of forms through which larvae pass in development is given in columns 4 to 9.

Zoea larva No.	Approx. total length (mm.)	Recognition character	Non-living animal plus unicellular algae		Intermolt number Non-living animal		*Artemia* nauplii	
1	2.6	Sessile eyes	1	1	1	1	1	1
2	3.0	Stalked eyes	2	2	2	2	2	2
3	3.2	Telson and uropods	3	3	3			
(3)	3.3	4th and 5th pereiopod rudiments				3	3	3
4	3.5	2 dorsal rostral teeth	4	4	4	4		
5	3.5	3rd pereiopod	5	5				
6	3.7	5th pereiopod	6	6	5			
7	4.4	Pleopod buds	7	7	6	5	4	4
8	4.9	Pleopod rudiments	8		7	6		
(9)	5.0	Chelae		8			5	5
9	5.1	Pleopod exopod	9	9	8	7	6	6
10	6.1	Pleopod endopod	10			8	7	
(10)	6.3	Appendices internae	11	10	9	9	8	7
PL	6.3	Postlarva	12	11	10	10	9	8

who found pigment spots to be variable in position. Minor morphological details, which might serve as indices of species, were not discussed or figured by Faxon. It seems possible that he may have dealt with larvae of more than a single species, although Holthuis (1952) feels that the adults and, presumably, the first stage larvae described by Faxon were *P. vulgaris*.

The normality of larvae reared in the laboratory has been questioned by Gurney (1942) who believes that abnormal stages may be reared under artificial conditions. Gurney further states, however, that "extra stages," through which each individual need not pass in development, occur in nature. Numerous references to these extra stages are available (Faxon, 1879; Gurney and Lebour, 1941; Lebour, 1940).

Fraser (1936) and Boden (1950, 1951) defined stages or norms of variation in euphausid Furcilia larvae on the basis of the frequency of occurrence of all the forms encountered. As a result of these analyses it is possible to state that some of the forms are normal and some abnormal in a purely statistical sense. No treatment of the frequency of occurrence of variation in larvae of a decapod is available. It is not presently possible to state with certainty that any individual decapod larva is either normal or abnormal except on the basis of the course of its development. Defining normal development, however, presents great difficulties. MacDonald (1927) and Fraser found numerical predominance of certain of the euphausid Furcilia stages over others. It was assumed that certain of the stages may be skipped in development. This assumption was confirmed by Fraser in the laboratory. The normality of individual larvae and the course of larval development possibly depends upon extrinsic factors. Sandoz and Rogers (1944) found optimum temperature and salinity conditions for molting of *Callinectes* larvae and that the tempo of molting might be reduced by a sub-optimal diet. In view of the

lack of evidence to the contrary, the variation observed in the structure of larvae and the tempo of development in *Palaemonetes* is considered to be within the limits which may be considered normal for the species.

The apparent discrepancy in the proposed developmental sequence, shown by the unfolding of pereiopods 3 and 5 before the appearance of pleopods in some larvae and after the pleopod buds have been formed in others, may be a function of rate rather than sequence of development. Pereiopod 3 appears after the second molt and is followed by pereiopods 4 and 5. These may appear all at once in the third intermolt among rapidly developing larvae, or their appearance may be in separate intermolts. All the fourth intermolt larvae have five pairs of pereiopods of which the last three are rudimentary. Among rapidly developing larvae the pleopod buds appear in the fourth intermolt, before the last three pereiopods have unfolded. If pleopods do not appear in fourth, fifth or sixth intermolt larvae, pereiopods 3 and 5 will have become functional before the pleopod buds are first seen. Pleopods always follow pereiopods in appearance. The length of time or number of intermolts which intervene may permit some variation in the status of pereiopod unfolding at the time of the pleopod appearance.

The present concept of the crustacean larval stage has contributed to confusion regarding development. Most authors refer to each larval intermolt as a stage. Numbers are assigned to these stages which presume a knowledge of the molting history of the individual. Thus, a larva is assumed, on the basis of structure alone, to have molted a certain number of times and, presumably, to be of a certain age. The basis for this implication is an assumed norm of development on which reasonable doubt may be cast.

Development in arthropods is made to appear discontinuous by the inflexibility of the exoskeleton during the intermolt period. The morphology of the individual larva, which cannot change during the intermolt period, is determined by the extent of development completed at the time of the last molt. Fraser (1936) feels that, within limits, the time of molting may shift slightly backwards and forwards. This fluctuation, superimposed on a continuous process of development, was suggested as the cause of variation in euphausiid larvae. Heegaard (1953) has suggested variation in the rate of larval development in penaeids, but did not discuss molting. No variation in larvae is possible if either a causative or a casual relationship between molting and development exists so that each larval molt occurs at a precise time in development. The presence of larvae of the same molting history which vary widely in size and form argues in favor of the independence of the frequency of molting from the rate of larval development.

Arbitrary stages may be defined in crustacean development but should not be thought of as inflexible, natural steps through which each individual passes. The choice between few or many stages is possible. If, as has frequently been the case, each form found is described as a stage, then many individuals may skip certain stages in development. If few stages are defined, certain individuals may pass through what might be called extra stages during the course of normal development.

The inference that the variation in tempo of larval development is related to diet is inescapable. Trophic conditions may vary in nature during the breeding season of each species. At Beaufort, *Palaemonetes* larvae hatched in late April or May become part of a plankton community which is poor in total number of

organisms. A possible response to this sub-optimal condition might be prolonged larval life with a greater number of larval intermolts. Normal development of a decapod may itself vary according to the season of the year.

Summary

1. *Palaemonetes pugio* Holthuis and *Palaemonetes vulgaris* (Say) were reared in the laboratory from eggs through metamorphosis.

2. The larvae of these species are very nearly identical except for a pair of chromatophores found on abdominal sternite 2 of *P. pugio* but lacking from *P. vulgaris*. The sequence of development is the same for each species.

3. Individual larvae which were of the same age or which had molted the same number of times were not necessarily alike. Larvae of the same size, regardless of age or the number of molts completed, were alike. This discrepancy between age and development seems associated with the diet of the larvae.

4. The molting frequency of the larvae was independent of the rate of development.

5. Descriptions of a series of *P. pugio* larvae which illustrate the sequence of events in larval development are given. Some of these forms may be skipped.

6. The concept of the crustacean larval stage has assumed a constancy of development at variance with the facts observed in rearing experiments. The form of a larva alone may not be regarded as indicative of its age or previous molting history.

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THE RELATIONSHIP BETWEEN DIET AND LARVAL DEVELOPMENT OF PALAEMONETES [1, 2]

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Larval development of crustaceans consists, in its simplest form, of growth and the addition of somites and limbs. The primitive pattern has been obscured among higher crustaceans by the degree of development achieved by the embryo before hatching and by the magnitude of developmental change which may become evident after each larval molt. Gurney (1942) and most authors think of the mode of development for each species as fixed and regard the number and sequence of definitive larval stages as constant.

Variation in the number and form of larval intermolts of *Palaemonetes pugio* Holthuis and *Palaemonetes vulgaris* (Say) reared in the laboratory has been described by Broad (1957). The present paper is a consideration of the relationships between diet and survival, molting frequency, rate of larval development and the number and form of intermolts during development of these species.

The author is indebted to Professor C. G. Bookhout, under whose guidance this work was done. He also wishes to express his thanks to Dr. T. R. Rice, who kindly furnished stocks of all species of unicellular marine algae used.

Methods

Larvae dealt with were hatched in the laboratory from eggs carried by adult females and were reared through metamorphosis. Culture methods have already been discussed (Broad, 1957) and need not be repeated in detail.

Each larva was fed the same diet throughout its life, but several different foods were offered to different individuals. The diets differed from one another generally and specifically. There were five general diet categories: no food; unicellular marine algae; algae and non-living animal matter; non-living animal tissue alone; and living *Artemia* nauplii. Specific differences in diet were between the several combinations of the available foods in each general category.

Larvae which were not fed nevertheless had available whatever food might be obtained from the raw sea water in which they were reared. The form of the mouthparts of these larvae makes it extremely unlikely that particles not visible to the unaided eye could be utilized as food.

Some individuals were fed species or combinations of species of unicellular marine algae. These species were maintained in unialgal culture in the laboratory.

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Clumps of cells which settle in older cultures were used as food. The algae available were from T. R. Rice's stocks of *Nitzschia closterium* and *Nitzschia* sp., (Bacillareae), *Chlamydomonas* sp.; *Thorocomonas* sp.; *Nannochloris* sp. (Chlorophyta); *Porphyridium* sp. (Rhodophyta) and *Pyramimonas* sp. (Chrysophyta).

Other larvae were fed non-living animal tisues alone or in combination with one or more of the algal species. Animal matter used was obtained largely from the plankton. Zooplankton, collected by net, was killed by immersion in distilled water to prevent the fortuitous inclusion of living larvae which might later be confused with individuals being reared. The general zooplankton (and possibly a few contaminating phytoplankton cells) was fed to some larvae. Others were fed on chaetognaths removed from the killed plankton. A few individuals were fed macerated gonad of the mud snail, *Nassarius obsoletus*.

The larvae usually hatched at night. The day on which free-swimming individuals were found was called the first day of larval life. Larvae and the bowls in which they were held were inspected daily. Only the presence of an exuvium was accepted as evidence of molting. Molts were considered to have occurred the day on which the cast exoskeleton was found. Uneaten food was removed and fresh food added at the time of daily inspection.

Results

Palaemonetes larvae were found to ingest almost any particulate matter with which they came in contact. There was no evidence of chemoreception or of selection of any type of food. Cannibalism was infrequently observed. In feeding, the zoea larvae grasp and hold objects with the maxillipeds while the maxillae and mandibles function as jaws. There was no indication of ability to obtain food by filtering.

Larvae fed diets which differed in general composition showed different rates of survival and development and molted at different frequencies, but those fed diets which differed only in specific composition did not. All the larvae are grouped for treatment of data into five general categories according to the diet received.

Diet and survival of larvae

Figures 1 and 2 show the per cent of mortality observed at each molt among *P. pugio* and *P. vulgaris* larvae fed various diets. Most of the deaths occurred at the time of molting, but some individuals were lost during the intermolt phase. These are included among the larvae which died at the time of the next molt. The per cent of larval mortality at molt N is that fraction of the total number of larvae which completed molt N-1 but did not survive molt N, and includes larvae lost during intermolt N. Loss of some intermolt larvae was due to removal of specimens for preservation or study, and the apparent mortality accordingly is biased upward when the total number of larvae involved is small or in the later molts.

Larvae which were not fed did not survive nor did those which were fed only phytoplankton cells. Sixty *P. pugio* larvae were starved. Forty-two of these survived one molt and two individuals molted twice, but none lived longer than 10 days. Among 80 *P. vulgaris* larvae which were not fed, none molted and all died within 5 days. Feeding any of a variety of unicellular marine algae did not seem

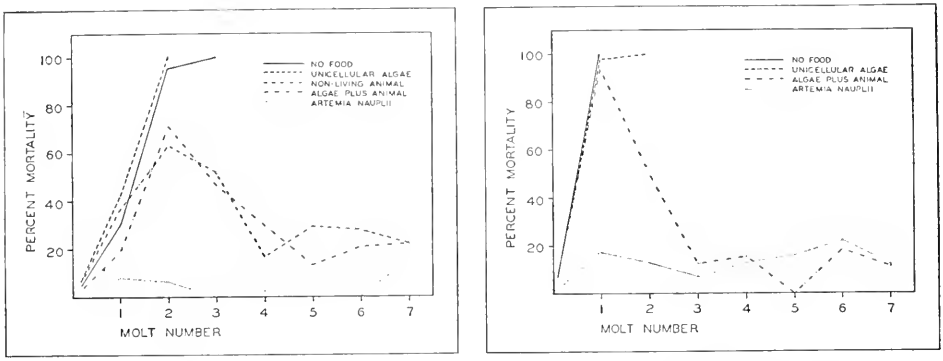

FIGURE 1. Mortality at each of the first seven molts among *Palaemonetes pugio* larvae fed various diets. Per cent mortality at each molt is based on the number of larvae which survived the last molt.

FIGURE 2. Mortality at each of the first seven molts among *Palaemonetes vulgaris* larvae fed various diets. Per cents computed as for *P. pugio* (Fig. 1).

to improve survival or molting in either 280 *P. pugio* or 100 *P. vulgaris* larvae. The survival of algae-fed larvae shown in Figures 1 and 2 closely approximates that of starved larvae.

Larvae that were fed foods of animal origin, either living or non-living, alone or in combination with algae, were able to survive through metamorphosis in the laboratory. The mortality of 608 *P. pugio* larvae that were fed diets of non-living animal matter, mostly zooplankton, combined with phytoplankton cells is shown by the dash-and-two-dots line in Figure 1. Sixteen of these individuals metamorphosed. The dash-and-dot line in Figure 1 shows the mortality of 732 *P. pugio* larvae that were fed non-living animal matter alone. In general the two curves are alike. Both reflect, in peaks at the second molt, the ability of this species to survive a single molt without food. Only 6 individuals metamorphosed on a diet of freshly killed zooplankton or other non-living animal matter. By far the best survival was shown by larvae fed living *Artemia* nauplii. The mortality of 100 of these is shown by the dotted line in Figure 1. Sixty-five individuals survived metamorphosis. Another 40 *P. pugio* larvae, for which it was not possible to determine daily the state of development of each individual, were fed living *Artemia* nauplii. Mortality at each molt of these individuals is not shown, but 33 metamorphosed.

The mortality of 667 *P. vulgaris* larvae that received a diet of non-living animal matter combined with algal cells is shown by the dash-and-two-dots line in Figure 2. The initially high mortality shown by starved larvae is also evident for these larvae, and contrasts with the early independence of available food shown by *P. pugio*. Six postlarvae survived metamorphosis. The dotted line in Figure 2 shows the mortality observed among 390 *P. vulgaris* larvae fed living *Artemia* nauplii. One hundred twenty-two of these metamorphosed in the laboratory.

Diet and the frequency of molting

Molting of *P. pugio* larvae is shown in Figure 3. The differences in molting frequencies between larvae which survived and most of those which did not on

each diet are insignificant, although those individuals which did not maintain a regular molting schedule did not survive. In general, the range of days during which specific molts occurred among larvae which lived is more restricted for the earlier than for the later molts. This most likely results from some variation in the molting frequencies of larvae fed similarly.

Since, except for the first two molts, there is little or no overlap in the means and standard deviations, or sometimes even the ranges, of corresponding molts by larvae fed different diets, the frequencies suggested by the diagram seem to be statistically separable. Average molting frequencies may be computed, although these become more or less meaningless since the interval between hatching and the first molt and that between the last larval molt and the molt of metamorphosis must be included. The interval between hatching and the first molt is usually somewhat longer than that between subsequent molts. The molt of metamorphosis, although not exactly comparable to other molts, is also included in the computations of average molting frequencies. Among larvae which survived metamorphosis on a diet of mixed plant and animal matter, the frequency of molting varied from one molt every 3.15 to 4.0 days with an average frequency of one molt every 3.70 days. Those larvae which survived on a diet of non-living animal matter alone molted once every 2.36 to 2.67 days with an average frequency of one molt every 2.51 days. Molting among larvae which survived metamorphosis on a diet of living *Artemia*

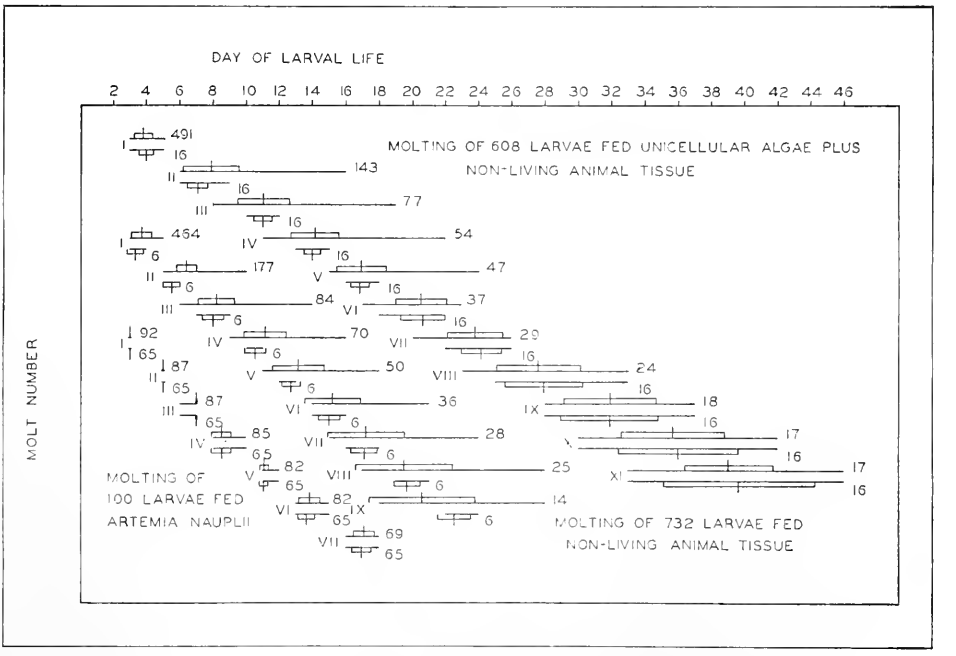

FIGURE 3. Frequency of the several molts among *Palaemonetes pugio* larvae fed three different diets. Number of each molt is given by Roman numbers. Upper line of each pair shows days during which molt occurred. Lower line gives days on which those larvae which completed metamorphosis molted. Means shown by vertical lines. Box on each side of mean is one standard deviation. Arabic numbers show sample size.

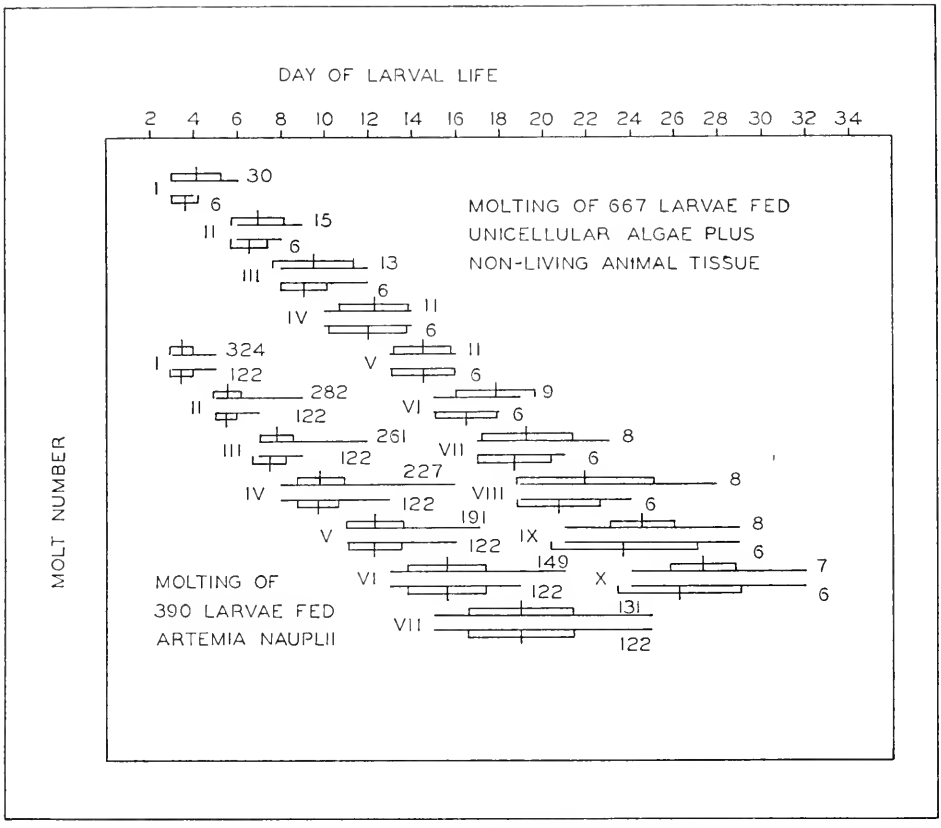

FIGURE 4. Frequency of each molt among *Palaemonetes vulgaris* larvae fed two different diets. See legend of Figure 4 for explanation.

nauplii was observed to occur with a frequency of once every 2.30 to 2.50 days with an average of one molt every 2.43 days.

The molting of *P. vulgaris* larvae, shown in Figure 4, shows little statistical separateness between molting frequencies of larvae fed different diets. Because of the initially high mortality of these larvae the samples are even smaller than those for *P. pugio*. Fourth and fifth molts seem to be distinct between the two groups of larvae, but, possibly due to a slowing-down of the molting frequency as the time of metamorphosis approaches, the later molts are not. Among those larvae which survived on a diet of mixed animal and plant matter, the molting rate was one ecdysis every 2.4 to 3.1 days with an average of a molt every 2.62 days. The average molting frequency for larvae which metamorphosed on a diet of living *Artemia* nauplii was one molt every 2.67 days, but the molting of these varied from one molt every 2.1 to 3.6 days.

Diet and the rate of larval development

An approximation of the rate of larval development may be obtained from the number of days between hatching and metamorphosis. This, however, is not

completely satisfactory since metamorphosis need not always occur at what appears from morphological criteria to be the end of larval development. Thus, Faxon (1879) found "last stage" *P. vulgaris* larvae which molted in the laboratory gave rise to other "last stage" larvae morphologically indistinguishable from their previous form. The larvae of both *P. pugio* and *P. vulgaris* reared in the present study sometimes passed through two or more identical intermolts at the end of development and before metamorphosis. At this time, depending upon other conditions not presently understood, the larva may metamorphose, as most of them do, or possibly may remain larval for some time.

P. pugio larvae fed a diet of mixed animal and plant matter metamorphosed from 29 to 49 days after hatching. Most of the larvae metamorphosed at the eleventh or twelfth molt, but a few metamorphosed as early as the ninth or tenth molt, or as late as the thirteenth molt. Larvae of this species fed a diet of non-living animal matter alone metamorphosed from the nineteenth to the twenty-eighth day after hatching and at the eighth, ninth, tenth or eleventh molt. Those individuals fed a diet of living *Artemia* nauplii metamorphosed from 17 to 21 days after hatching, usually at the seventh, but sometimes at the eighth molt.

P. vulgaris larvae fed a diet of mixed animal and plant matter metamorphosed from 24 to 34 days after hatching. The molt of metamorphosis was most often the tenth, but two individuals metamorphosed at the eleventh and thirteenth molts. Those larvae fed a diet of living *Artemia* nauplii metamorphosed from 14 to 30 days after hatching, usually at the seventh molt. Metamorphosis was also noted at the sixth, eighth, ninth and tenth molts.

A second approximation of the rate of development might be obtained by comparing the structure of larvae to age and molting history. In spite of the variation noted in duration and tempo, the sequence of development was the same for all larvae and for both species. Except for differences in color patterns which are specific, all newly-hatched *P. pugio* and *P. vulgaris* larvae were essentially alike. For the present purposes this first zoea larva may be characterized by the presence of all head appendages, three pairs of functional maxillipeds, two rudimentary pereiopods, sessile eyes, a fan-shaped telson and a lack of spines. After a single molt all larvae acquire stalked eyes, spines on the carapace and abdomen, and functional first pereiopods. Until after the second molt variation in form is almost non-existent, but, from the second molt to the end of larval development, there may be wide variation in the form of larvae of the same age or molting history. A table which summarizes the relationship between intermolt number and form of larvae fed variously has previously been presented, and the morphology of the several developmental steps or stages has been discussed in detail (Broad, 1957). For the present purpose it suffices to reiterate that the number of intermolts in development of either species dealt with may vary, but that the sequence of events in development does not. Approximate ages of intermolts discussed below may be obtained from Figures 3 and 4.

Following the second molt, third intermolt, or third zoea larvae show variation in the number of pereiopod rudiments. Those individuals fed *Artemia* had rudiments of pereiopods 3, 4 and 5 while those fed other diets lacked pereiopods 4 and 5. Great variation was evident among fourth intermolt larvae. Those individuals fed *Artemia* bore rudiments of the pleopods but none of the others did. Among larvae fed non-living animal foods alone, pleopod buds first appeared in the fifth or

sixth intermolt and not until the seventh intermolt in larvae fed diets which combined algae with animal matter. The intervening intermolts, 4, 5 and 6, differed from one another in the development of pereiopods.

Fifth intermolt larvae which had been fed *Artemia* had pleopod rudiments and chelae. The pleopod buds of fifth or sixth larval intermolts fed non-living animal tissue alone became rudiments after a single molt, but chelae did not appear until after two molts. Some of the eighth larvae fed algae and animal matter bore chelae, but others had none.

The pleopods of all sixth intermolt larvae which had been fed *Artemia* bore setose exopods. These were first evident in seventh or eighth intermolts which had received non-living animal food and in ninth zoea larvae which had been fed algae plus non-living animal foods.

The final larval form has been characterized by both setose endopods and *appendices internae* on pleopods. Most larvae fed *Artemia* nauplii achieved this final form in the seventh intermolt, although some individuals required two steps after the sixth zoea for *appendices internae* to appear. Metamorphosis occurred at the seventh or eighth molt. *Appendices internae* made their appearance in ninth intermolt larvae fed non-living animal matter, and metamorphosis occurred at the tenth molt. Among larvae fed algae and animal matter, the final larval form was reached in the tenth, eleventh, or even sometimes the twelfth intermolt. Metamorphosis usually occurred at the following molt.

Discussion

Since survival of larvae fed algal diets alone did not differ from that of zoeae which were not fed, it would seem that the algal species available had no value as food for either *P. pugio* or *P. vulgaris* larvae. In order to survive, the larvae must find some particulate food, probably animal in nature.

A physiological distinction between *P. pugio* and *P. vulgaris* is possible in the ability of the former species to molt once and survive up to ten days without food while the latter neither molts nor lives longer than five days without food. Speculation regarding the survival value which might be associated with this relatively greater independence of trophic conditions, though interesting, is futile in view of the presently limited knowledge of the geographic and ecological distribution of the species of *Palaemonetes*. The ability of either species to adjust development to external conditions and the rather indefinite time of metamorphosis are both of positive survival value.

It is possible that the varying rates of survival, molting and development noted among larvae reared in the laboratory may be due to differences in the total quantity rather than in the quality of food available. Two factors limited the amount of non-living animal tissue available to larvae fed this diet. In order to retard fouling of the water in the bowls, the total quantity of food added daily had to be kept within limits. Since food was always left, the quantity was at first thought to be sufficient. Non-motile food, however, sank to the bottom of the bowls. The larvae swam near the surface. It has already been stated that contact between zoea and food seemed to be the result of chance encounter rather than active search on the part of the larva. The low probability of encounter between larvae swimming near the surface and food lying on the bottom might account for the uneaten food left each day.

If clumps of algal cells were added to the diet, a third limiting factor is also added. Larvae fed algae actually ingested the material offered. The red, green or brown color of the cells could be seen in the gut and feces of the larvae. Since the algal species used have been shown to be of no nutritive value, it seems possible that, where algae plus another food is offered, the ingestion of the nutritively inert material may restrict the intake of other foods which can be digested and utilized.

Ne restrictions were placed on the total number of *Artemia* nauplii fed. The nauplii swam near the surface with the zoea larvae. The probability of encounter between larva and food was greatest when the diet consisted of living animals.

It has been suggested that intraspecific variation in crustacean larvae may arise from extrinsic causes (Broad, 1957). Sandoz and Rogers (1944) found that poorly nourished *Callinectes* zoeae molted only after a relatively long period and were smaller than other larvae which had received more food. Templeman (1936a, 1936b) found reducing the amount of food given *Homarus* larvae lengthened the intermolt period and sometimes resulted in the production of an "extra" larval intermolt. The present data suggest that the amount of food available to *Palaemonetes* during its larval life may affect both the rate of development and the frequency of ecdysis. The independence of these variables is suggested by the differences in the magnitude of response to sub-optimal feeding. Although the longest regular interval between molts was only 1.9 times the least, the duration of larval life was extended 3.5 times over the most rapid successful development.

Heegaard (1953) has suggested that different developmental rates among decapod larvae may be caused by "external as well as internal" factors. The amount of available food may be an external factor which affects both the rate of development and, independently, the frequency of molting. Variation in the form of larvae under these conditions might be restricted only by the morphological limitations of species. If norms of developmental stages exist among decapods, as Fraser (1936) has found among Euphausids, constancy of environment might be considered of prime importance in their establishment. Extra or abnormal larval stages found in nature or in the laboratory as well as stages skipped in development may reflect an adaptibility to environmental variation during development.

Summary

1. Larvae of *Palaemonetes pugio* Holthuis and *Palaemonetes vulgaris* (Say) reared in the laboratory showed differences in survival, frequency of molting and rate of development which may be associated with the amount of food available.

2. Larvae were unable to survive if fed diets of either single species or combinations of species of several unicellular marine algae or if not fed. Starved *P. pugio* larvae were able to survive one molt without food but starved *P. vulgaris* larvae died without molting.

3. Larvae of both species lived through metamorphosis if fed a diet which included animal tissue. The best survival was obtained by feeding living *Artemia* nauplii.

4. The frequency of molting, the duration of larval life and the number of larval intermolts in the development of *P. pugio* and *P. vulgaris* vary according to the quantity of food available. The frequency of molting and the rate of development are suppressed by a reduction in intake of food.

5. Variation in molting frequency independent of the rate of development makes possible variation in the form and number of larval intermolts.

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X-RAY EXPERIMENTS WITH MOLGULA MANHATTENSIS: ADULT SENSITIVITY AND INDUCED ZYGOTIC LETHALITY

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Sessile organisms which pump large quantities of sea water through their bodies suggest themselves as ideal material in which to study biological effects of radioactive contaminants. Because of their relationship to the chordates, ascidians have particular attraction. Furthermore, many are functional hermaphrodites providing both sperm and eggs for simultaneous irradiation. *Molgula manhattensis* has the additional advantage of self-fertility so that crosses using gametes from the same individual can be made, as well as outcrosses.

In the absence of radiobiological information on Molgula, the present x-ray experiments were performed rather than employing isotopes with their more difficult dosimetry. Isotope experiments may be performed in the future after a more complete knowledge of what may be expected from external radiations is at hand.

Materials and Methods

Preliminary experiments were performed during the summer of 1955 with the assistance of Robert L. Sullivan. Specimens of Molgula were collected for us by personnel of the M. B. L. Supply Department. Irradiation was delivered by the M. B. L. generator in "A" position at 6000 r per minute (187 KV; 28 ma; filtration $\equiv$ 0.2 mm. Cu). To achieve massive doses without overheating, the animals were immersed in sea water within an inner chamber, and crushed ice was packed into the space between it and the outer wall of the plastic container. After irradiation, animals were kept in individual glass jars (50 $\times$ 80 mm.) in running sea water.

Failure to contract in response to prodding with a blunt instrument was the criterion for death. An additional check on presumptive death was obtained by holding such animals until post-mortem flaccidity and degeneration became obvious. The results indicated that adults would be adequately resistant to whole-body irradiation which would allow the full range of dosages required for a genetic experiment on induced dominant lethality.

In 1956 animals of adult size were obtained both through the M. B. L. Supply Department and by personal collection. The latter procedure seemed necessary when it was discovered that masses of Molgula held in the Eel Pond "live-cars" and in laboratory aquaria did not consistently exhibit gonads with mature gametes. The adequacy of the supply of eggs and sperm can be determined by examination with a low power microscope, usually after peeling off the test, always after removal of a portion of the mantle.

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Adults were irradiated in position "B" at 2500 r per minute, again using the Woods Hole machine, the Coolidge tubes this time not as close to the animals.

Eggs were obtained by suction applied to a capillary pipette inserted into the lumen of an ovary. They were expelled into a stender dish of sea water. Immediately after withdrawing eggs from both ovaries, sperm suspensions were prepared by macerating a portion of each testis in a separate dish of sea water (3–5 cc.).

Eggs from each ovary were divided into three groups: (1) a control as a check against accidental fertilization during removal, (2) a sample which was fertilized with sperm from the adjacent testis, and (3) a sample which was cross-fertilized with sperm from another Molgula. Sixty-millimeter flat stender dishes, containing one inch of sea water, were used. They were placed on the sea table with their bases in running water. Fifteen hours later, tadpole development was scored using a stereoscopic dissecting-type (48 ×) microscope. Immediately after completion of these observations, the degree of cleavage for 100 objects was scored using a compound microscope (16 mm. objective, 10 × ocular).

During July and the first half of August, pair-mating experiments were set up daily to obtain simultaneous data (a) for eggs from an irradiated animal fertilized by sperm from an untreated one, (b) for the reciprocal cross of treated sperm to untreated eggs, (c) from selfing the irradiated animal, and (d) selfing the non-irradiated member of the pair. The goal was to obtain data from at least five pairs of crosses for each of five chosen doses.

T. H. Morgan's 1942 paper led us to expect a technical difference between the two ovaries in difficulty of removing unfertilized eggs. Therefore we kept separate controls and made separate crosses for each side of each animal. However, upon analysis of results, no significant differences between sides were demonstrable and the data for the two sides of each animal serve merely as replications.

From August 13 through August 18 the daily plan of experiment was modified. On each day, five or more adult animals were simultaneously exposed to one of the five chosen doses. The gametes obtained from five treated animals were mixed to provide outcross data when both sperm and eggs were treated. At least five samples (averaging 800 objects/sample) were scored for each mass fertilization. On August 15 a mass outcross control was obtained.

From August 20 through August 24, five animals were treated simultaneously at each of the five doses and selfed to obtain more information about the variability between material from different treated animals.

From August 27 to September 1, miscellaneous experiments were set up: crosses of three different animals, each given a different dose, to a single untreated animal, and selfing crosses in which several doses were investigated each day.

Results

Lethality of adults

An exploratory experiment indicated that the critical dose of x-ray for the adult organism lay between 36,000 r and 60,000 r. This involved observations on 72 sea-squirts, 8 of which were controls, along with 8 samples of 8 animals given doses graded between 1000 r and 120,000 r.

Two subsequent experiments of 40 each were set up with controls and samples irradiated at the following doses: 36,000 r, 42,000 r, 48,000 r, 54,000 r and

60,000 r. In both the control and 36,000 r groups, individual animals were still alive more than a month after the date of irradiation. Also, although most of the animals given 42,000 r died during the first week, one lived almost a month. At higher doses no animals survived the fourth day, with the average time of death 2.4 days after treatment. This places the lethal dose between 42 and 48 kiloroentgens. This is considerably less than the radiation required to kill adult insects (Sullivan and Grosch, 1953), brine shrimp (Grosch and Erdman, 1955) and vegetative microorganisms (Bacq and Alexander, 1955). On the other hand, such amounts of radiation are many times that required to kill mammals.

Induced zygotic lethality

A summary of the relative proportion of tadpoles obtained among the ova and zygotes studied is given in Figure 1. Consistently fewer swimming tadpoles emerged than developed to tadpole morphology. Furthermore, curves for data from irradiated eggs tend to lie below those representing sperm. This trend becomes statistically significant at higher doses and it should be re-emphasized that the data come from paired matings.

With self-fertilization, involving sperm and eggs from irradiated sea-squirts, the developmental yield is strikingly decreased (solid line, "Both Treated"). Along with the curve for selfing data, results from mass outcross experiments are shown by the broken line in Figure 1. With one exception, points obtained by calculating the mean are nearly identical whether selfing or outcrossing has been the procedure, provided both sperm and eggs are from irradiated animals. The standard errors omitted from the outcross curve in order not to further complicate Figure 1 are less than 2%.

An additional group of data obtained from selfing five animals irradiated simultaneously at each dose also gives a curve similar to the solid line at the lower doses and identical with it at the three higher doses. Therefore it is not shown here.

Since it has been corroborated by three separate sets of experiments, the curve above 5,000 r is a good representation of expectation when both sperm and eggs come from gonads irradiated *in situ*. Furthermore, this "Both-Treated" curve is predictable on the basis of one of the laws of probability: when two events are independent, the probability that both will occur simultaneously is the product of their separate probabilities. Thus, multiplying survival when sperm are x-rayed by survival when eggs are x-rayed we obtain the following:

$$\begin{aligned}
.33 \times .075 &= .0248 = 2.5\% \text{ for } 20{,}000 \text{ r} \\
.34 \times .185 &= .0629 = 6.3\% \text{ for } 15{,}000 \text{ r} \\
.45 \times .43 &= .1935 = 19.4\% \text{ for } 10{,}000 \text{ r} \\
.545 \times .51 &= .2779 = 27.8\% \text{ for } 5{,}000 \text{ r}
\end{aligned}$$

These values calculated from Figure 1 data for the five higher doses are all within the range of one standard error from the "Both-Treated" curve shown in Figure 1. The theoretical value for 1000 r, 24.6% (.53 × .465), is lower than the "selfing" value obtained in pair experiments but falls within the range found in outcross experiments when both sperm and eggs are from treated animals.

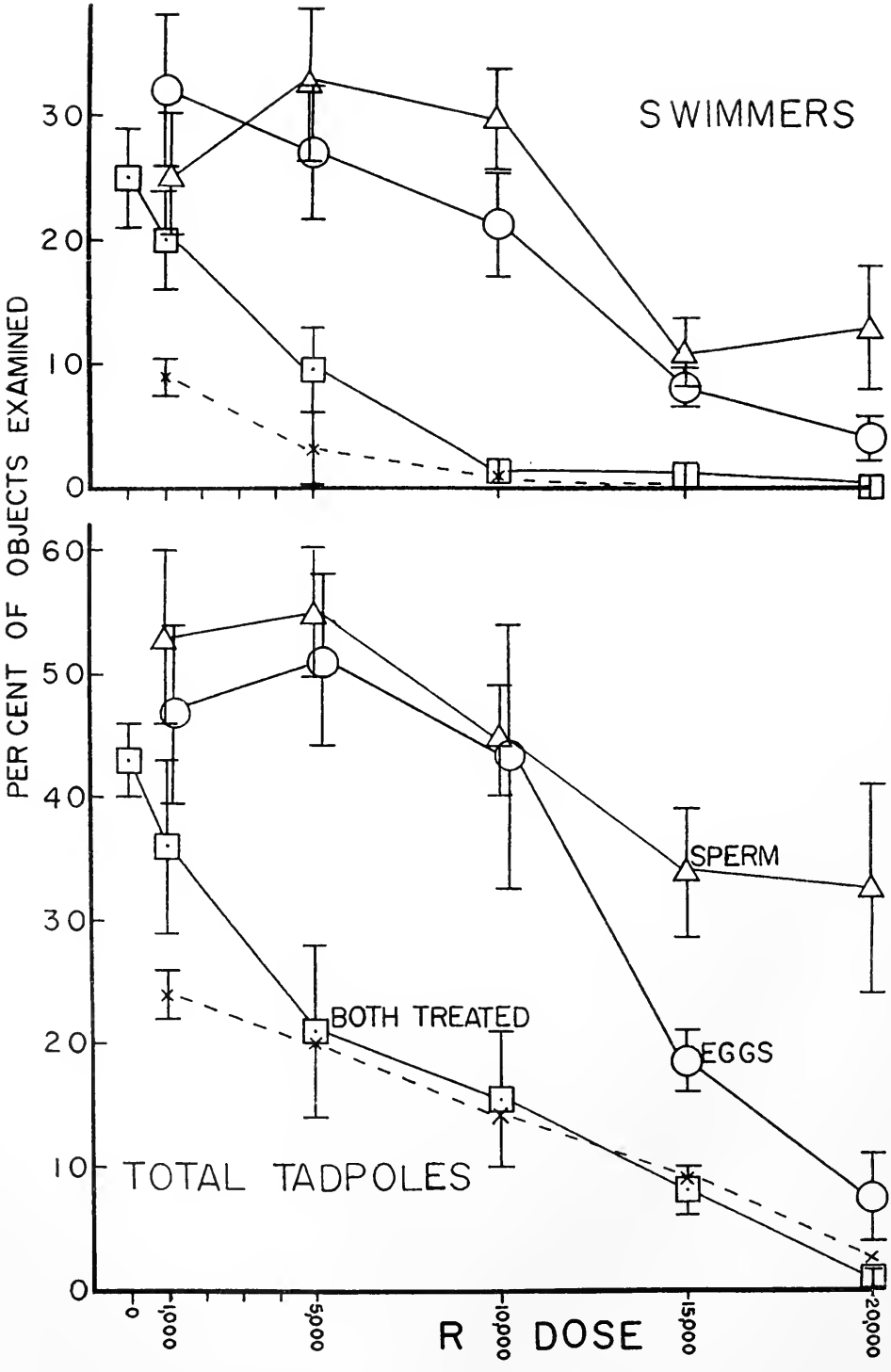
SWIMMERS
PER CENT OF OBJECTS EXAMINED
30
20
10
0
60
50
40
30
20
10
0
SPERM
BOTH TREATED
EGGS
TOTAL TADPOLES
0
1000
5000
10000
15000
20000
R DOSE

TABLE I

Development in the residuum of eggs and zygotes, tadpoles having been counted. The scoring was done on 100 objects in each case. Values are given in per cent, representing failure to develop to tadpole morphology (means and standard errors)

X-ray dose in r	Both gametes treated			Sperm treated	Eggs treated
	Mass outcross	Self-cross 5 simultaneously	Self-cross pair experiments		
0	99.5 ± 0.5		45.8 ± 11.9		
1,000	84.3 ± 3.3	76.5 ± 0.5	54.2 ± 10.8	74.6 ± 6.3	63.8 ± 7.9
5,000	90.5 ± 2.0	72.2 ± 7.4	35.4 ± 12.9	55.4 ± 3.4	57.4 ± 9.8
10,000	89.9 ± 4.7	93.6 ± 2.9	59.2 ± 12.2	65.2 ± 5.6	64.8 ± 12.6
15,000	91.2 ± 1.2	79.8 ± 12.7	70.5 ± 8.4	65.9 ± 5.3	75.0 ± 6.8
20,000	86.1 ± 1.6		36.2 ± 10.2	76.0 ± 5.0	50.4 ± 11.7

No attempt was made to obtain a quantitative record of morphological aberration. However, as an indication of developmental difficulty, thickened, bent and misshapen tails were typical for tadpoles in experiments above 10,000 r.

Cleavage data

In percentages, Table I presents a summary of results when objects other than tadpoles were scored. These are the averages from five or more experiments, in each of which 100 objects were carefully examined. Because of the time required and technical difficulty it is not feasible to examine all of the residuum. In order to understand Table I it must be realized that when few tadpoles develop from a group of eggs there are many undeveloped forms, and vice versa. Accordingly, since the amount of residuum varies, similar percentages with different doses may actually reflect differences. A conversion of the tabulated percentage values into numerical values, such as those plotted in Figure 2, helps to visualize the situation. The average total of eggs per stender dish, 800, is used as a common basis for presentation. The pertinent percentage of tadpoles (see Fig. 1) is subtracted. The applicable cleavage percentage (Table I) of the remainder gives the relative number of embryos plotted in Figure 2.

All embryos considered in Table I and Figure 2 appeared to be in the late gastrula or neurula stages, although at the two highest doses, structure was very disorganized. Presumably if embryos were able to begin development they could continue to such stages before facing an insurmountable developmental crisis. Extremely few embryos were found halted in an early cleavage stage. During the whole summer, when nearly 150,000 examinations were made, only 18 early cleavage types were seen in selfing experiments, and 17 in outcrosses—exceptional individuals making up only 0.02%.

As might be expected, the number of gastrulae and neurulae increased as the number of tadpoles decreased at higher doses. Although this general trend is clear, unidentified sources of variability complicate the picture in selfing and pair-

FIGURE 1. The relative proportions of tadpoles developing from gametes obtained from adult specimens of Molgula after irradiation. Results are contrasted when either or both types of gametes come from x-rayed parents.

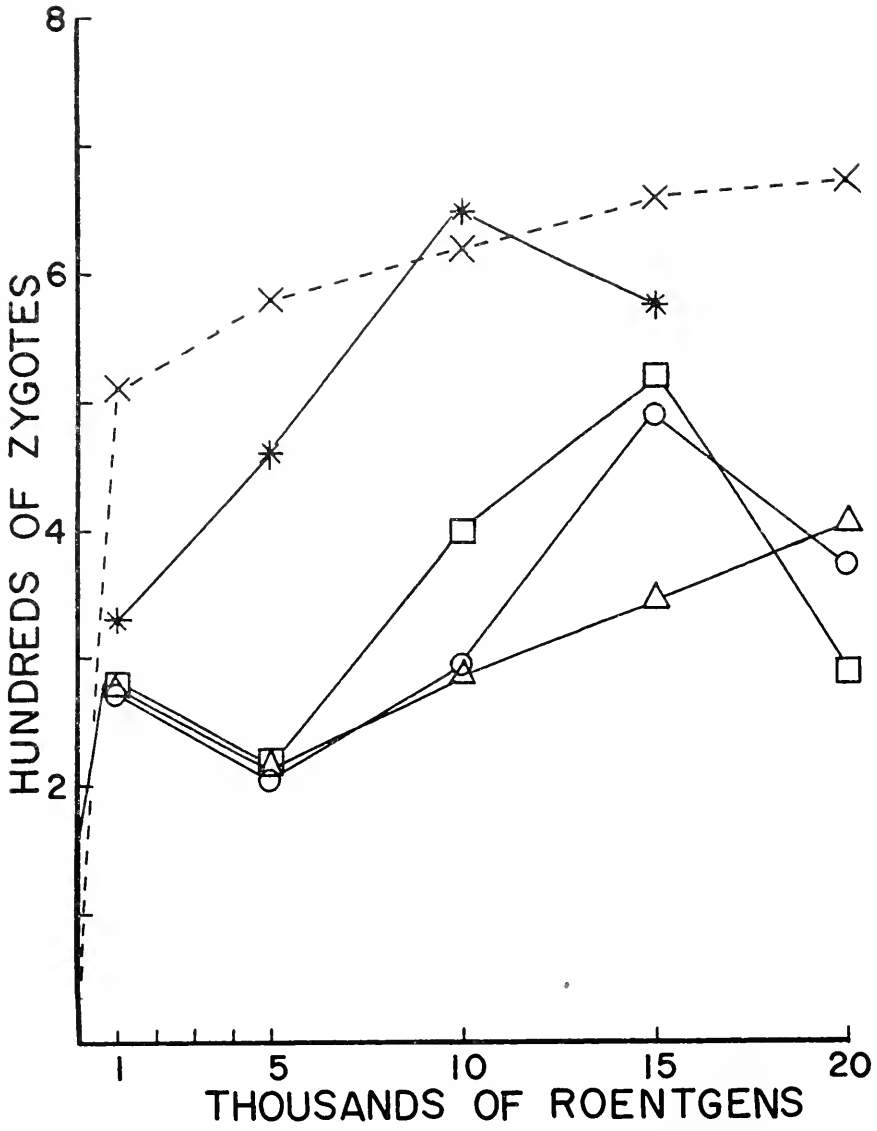

FIGURE 2. Post-cleavage zygotes which failed to develop to the tadpole stage. Results have been put on a common basis by calculations from the average total of eggs per sample, 800. Triangles indicate that the sperm were from treated animals; circles, eggs. Squares represent selfed eggs and sperm from treated animals in pair experiments; asterisks, data from 5 irradiated animals selfed on the same day. X's indicate outcross data, both parents x-rayed. Squares, circles and triangles represent results from pair experiments.

mating experiments. When plotted as in Figure 2, zigzag lines are obtained. Furthermore, in the latter experiments, results when both gametes came from treated parents are not greatly different from those obtained when only one of the

types of gametes has been exposed to radiation. Indeed, even after 5000 r, all three pair mating values were nearly identical. Therefore some general aspect of fertilization common to the experiments should be sought. The higher average values obtained after simultaneously selfing five animals given the identical dose of radiation are consistent with such a view.

Discussion

Relative radiosensitivity of sperm and eggs

In Molgula, relative radiosensitivity of eggs instead of sperm is an interesting parallel to Rugh's (1953) similar findings with the clam Spisula, although his crosses involved gametes which were irradiated after extraction from the parent. Such results are not expected, either on the basis of Henshaw's type of delayed cell division or that of chromosomal-gene effects. Sperm have been found more sensitive on either count.

Mavor and De Forest (1924), who irradiated Arbacia gametes, found that samples scored two days later showed development ranging from gastrula stages to well-formed plutei. The retardation in development was greatest in those larvae developed from x-rayed sperm and increased with dose. Subsequently, Henshaw (1940) explained this phenomenon by a demonstration that radiation-delayed first cleavage is reflected in later development. Furthermore, the apparent difference in sensitivity was due to partial recovery occurring in the eggs prior to fertilization.

Judging from insect experiments, dominant lethals are more readily induced in sperm. In fact, P. W. Whiting (1938) found that at dosages of about 10,000 r, practically every Habrobracon sperm contained at least one dominant lethal. Certain of his experiments more closely resemble the conditions of the present experiment than any other investigations we have been able to find in the literature. In these experiments, female wasps mated before treatment contained both sperm and eggs when irradiated. Comparisons with data from females mated after irradiation, and with parthenogenetic data, indicated dominant lethals to be more readily induced in sperm than even recessive lethals in the eggs. In a definitive Habrobracon paper, Heidenthal (1945) constructed the dominant lethal mutation curve for doses up to the asymptote and demonstrated that those secured for Drosophila (Sonnenblick, 1940; Demerec and Fano, 1944) were quite similar. An even steeper curve has been reported for Mellitobia (Kerschner, 1946). Muller and the Valencias (1949) have since presented Drosophila data which indicate that presumptive deficiencies are far less abundant if eggs are irradiated.

Nucleic acid or its cycle is implicated in both the Arbacia cleavage delay experiments and the dominant lethal experiments with insects. Although usually discussed separately, it seems possible that both types of damage may be reflected in the results obtained by scoring development at a specified time. However, it has been shown that neither phenomenon completely explains the present results. Perhaps a third and somewhat different aspect of cell division—other than chromosomal—is involved. The material may need to be considered from the standpoint of the general physiologist who studies the stimulus for initiating the process of cell division (Heilbrunn, 1955; Heilbrunn and Wilson, 1955; Rieser, 1955).

Especially provocative is an earlier Arbacia paper (Heilbrunn and Young, 1935), which shows that irradiation in the presence of ovarian tissue produced a

considerably greater division delay than irradiation of eggs in sea water alone or in concentrated suspension. At least, sperm inactivation can be ruled out. This requires doses in excess of 100,000 r in marine forms as well as insects (Maxwell, 1938; Henshaw, 1940; Rugh, 1953).

Self-sterility

Another aspect of Molgula investigations which may turn out to be a problem in physiology rather than genetics is fertility-sterility. A range from perfect self-fertility to absolute self-sterility has attracted geneticists to ascidians from the early days of genetics research (Morgan, 1904). However, although T. H. Morgan himself devoted considerable attention to the problem, including experiments with Molgula (1942), neither the genetic basis nor the physiological mechanisms have been completely elucidated.

Observations during the present experiments revise the Molgula picture. Self-incompatibility is not as extensive as previously believed, provided (1) that organisms with undeveloped or senile (degenerate?) gonads are not used, and (2) that no strong chemical cleaning solution or detergent is employed in cleaning glassware. In all our experiments in which these criteria were met, Molgula adults were self-fertile. The influence of a chemical agent was demonstrated dramatically one day when Alcanox had been used on the glassware. In spite of repeated water washings, as is the standard procedure in analytical chemistry, and over-night drying, no development occurred in selfing and only about 5% development in outcrosses. Ordinarily in outcrosses by far the great majority of eggs are fertilized and cleave. Although no details are available, at least one of Morgan's Molgula experiments bears a resemblance to this exceptional one of ours. He recorded a case in which no eggs selfed and only two out of a large number of eggs cleaved when cross-fertilized. Perhaps hot water is the only safe cleaning agent, although the junior author feels that dilute HCl rinses do much to offset the Alcanox type of hazard.

Summary

1. The lethal dose of x-rays for adult specimens of Molgula is placed in the neighborhood of 45,000 r (delivered at a rate of 6000 r/minute).

2. Radiation damage to gametes from irradiated adults can be measured in terms of tadpoles, unhatched or swimming. Eggs proved more sensitive than sperm. Curves when both gametes come from irradiated parents were similar no matter how obtained, pair matings or group matings, selfed or outcrossed. In the latter curves, 10,000 r is about the limit for swimmers, and 20,000 r for tadpole development (rate, 2500 r/minute).

3. The cleavage score for 100 objects residual to developed tadpoles did not provide a regular, clear-cut picture of radiation damage. It is suspected that uninvestigated features of fertilization physiology cloud the issue.

4. Self-incompatibility in Molgula is not as extensive as previously believed. The condition of the gonads must be considered and chemical cleaning solutions should be avoided.

5. It is concluded that the physiology of spindle formation rather than that of nucleination or chromosomal continuity may be a most important aspect of results like the present.

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ESTROGENS IN MARINE INVERTEBRATES

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Material with estrogenic activity demonstrable by bioassay in rodents has been found in marine invertebrate tissues. Steidle (1930), using a mouse bioassay, found traces of estrogens in a sea urchin, *Echinus miliaris,* three molluscs, *Aplysia, Octopus* and *Eledone,* as well as in certain worms and arthropods. Similarly Schwerdtfeger (1932) found estrogens in a sea anemone, *Actinia aquina,* but none in the mollusc *Chiton marginatus.* Donahue (1940) reported small amounts of estrogenically active material to be present in extracts of the Bermuda urchin, *Lytechinus variegatus,* the reef urchin, *Echinometria,* a holothurian, *Stichopus mobii,* and a lobster, *Palinurus argus.* More recently, this same author (Donahue, 1948) made extracts of the shed eggs of another lobster, *Homarus americanus,* purified them by solvent partition, and made estrogen analyses by a fluorometric method. In this way he found 100 international units of estrogen per 100 gm. eggs.

Gordon and Villee (1956) have described an enzymatic assay for estrogens, which is as sensitive as the fluorometric methods now available. Their assay depends upon the fact that human placenta contains a DPN(diphosphopyridine nucleotide)-linked isocitric dehydrogenase which catalyzes the reaction.

$$\text{isocitrate} + \text{DPN} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{DPNH} + CO_2$$

and which is specifically activated by certain natural estrogens. In a limited range the degree of enzyme activation is a function of the amount of hormone present. Since they measured the appearance of reduced DPN spectrophotometrically, it was essential that the material being assayed give a clear solution, and for that reason they found it impossible to analyze blood and tissue extracts. The progress of the reaction can also be measured chemically, by analyzing the reaction mixture after incubation for total keto-acids produced, and in this way relatively impure tissue extracts can be assayed. Such assays done on the ovaries of a number of marine invertebrates are reported here.

Methods

Invertebrate tissues were extracted for assay by the procedure used by Folch *et al.* (1954) for preparing total lipide extracts. The tissue was removed from the animal, blotted gently with filter paper, weighed accurately and homogenized in a Waring blendor containing 20 ml. 2:1 chloroform:methanol per gram of tissue. The homogenate was filtered and the residue discarded. The filtrate was washed

[1] Lalor Fellow, Marine Biological Laboratory, 1956.

once with 0.2 volume of distilled water and once with 0.2 volume of 0.01% aqueous calcium chloride which had previously been equilibrated with 2:1 chloroform:methanol. The extract was evaporated to dryness at room temperature under a gentle stream of clean air. The residue was taken up in ethanol for analysis and if, after thorough mixing, the solids did not go completely into solution, they were allowed to settle and the clear alcoholic extract was used for assay.

A 20% w/v homogenate of term human placenta was made in ice-cold 0.25 *M* sucrose, and centrifuged for 10 minutes at 600 G to remove cell nuclei and debris. The supernatant was then centrifuged at 57,000 G for 60 minutes to remove cellular particulate matter (Villee, 1955). The supernatant from the latter centrifugation contains the soluble matter of the cell, including the DPN-linked isocitric dehydrogenase. The enzyme-catalyzed reaction was carried out in 20-ml. beakers in a Dubnoff incubator at 37°, and was allowed to proceed for one hour. Each reaction vessel contained one ml. of the placental enzyme preparation; one ml. buffer containing 30 micromoles K^+, 10 micromoles Mg^{++}, 20 micromoles phosphate, and 20 micromoles Cl^-, adjusted to pH 7.3; 0.9 ml. water containing 0.75 micromole DPN and 3 micromoles cis-aconitate; and 0.1 ml. ethanol in which the standard or unknown solution was dissolved. The reaction was started by adding the DPN. Crystalline estradiol-17β was used as a standard. Analyses of the reaction mixture for total keto-acid production were made by a modification of the method of Friedemann and Haugen (1943). The amount of keto-acid produced in vessels containing all components except estradiol was used as a blank correction for the standard, and that produced in vessels containing all components except DPN was used as a blank correction for the unknowns. Total nitrogen analyses of the enzyme preparation were made by a micro-Kjeldahl procedure. The keto-acid analysis results were calculated in micromoles keto-acid produced per milligram nitrogen per hour. Duplicate reaction vessels were incubated and analyzed for each of the standards and unknowns in each assay.

Results

Separate standards were assayed with each set of unknowns, and the average results from 12 sets of standards are plotted in Figure 1. The least squares curve for these points is also shown. There is clearly a linear relationship between the logarithm of the amount of estradiol added and the amount of keto-acid produced over the range from 0.05 to 0.25 microgram estradiol per vessel. The index of precision, (λ), for this curve is 0.3 (Bliss, 1944). Recoveries of estradiol added to tissue before extraction averaged 99%.

The amount of estrogen in each unknown was calculated from a simultaneously determined standard curve for the same placental enzyme preparation. Each tissue extract was assayed with at least three different enzyme preparations from three different placentas. Of the tissues extracted, only the ovaries of *Mactra* (*Spisula*) *solidissima* contained significant amounts of estrogenic material, 1.1 $\pm$ 0.4 (mean $\pm$ standard error) milligrams estradiol equivalents per kilogram of fresh tissue. The other tissues, including the ovaries of *Asterias forbesi*, *Arbacia punctulata*, *Strongylocentrotus droebachiensis*, *Loligo pealei*, *Busycon canaliculatum*, *Carcinides maenas*, *Homarus americanus*, and *Limulus polyphemus*, the whole tissue of *Microciona prolifera* and the liver of *Homarus americanus* all contained less than 50 micrograms estradiol equivalents per kilogram of fresh tissue.

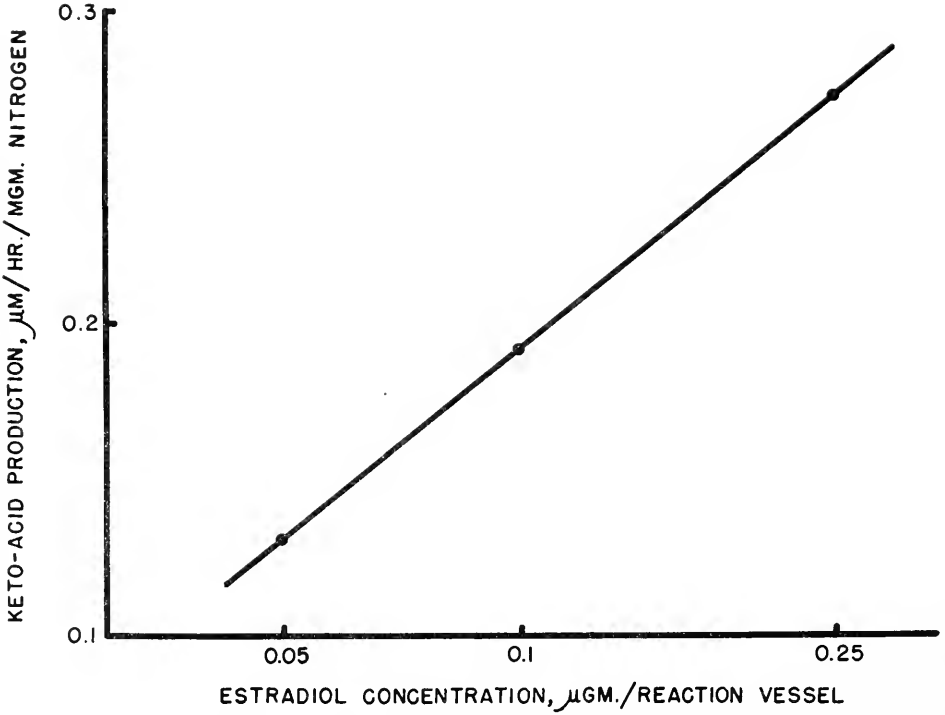

FIGURE 1. Keto-acid production by placental isocitric dehydrogenase as a function of estradiol-17β concentration.

DISCUSSION

The results were calculated in terms of estradiol equivalents, since that compound was used as a standard. It has been shown (Villee and Gordon, 1956) that estrone, equilenin and equilin are as effective as estradiol in stimulating the enzyme, while a variety of other steroids and synthetic compounds do not affect the enzyme unless present in very large quantities. It is thus likely that the material present in *Mactra* ovary is one of these four compounds.

The minimum amount of estrogen that can be detected in this assay is about 0.01 microgram of estradiol, and the tissue concentration that can be accurately determined depends, of course, on the amount of tissue extracted. With samples of tissue of about 10 grams, the analytical blanks are not unduly large and this magnitude of sample was used in these experiments. In all the tissues examined except *Mactra* ovary, the results of the assays indicated the presence of small amounts of active material, confirming the experience of earlier workers. The amounts are probably much smaller than the upper limit of 50 micrograms per kilogram which the present results make certain, but it would be necessary to extract larger quantities of tissue to make accurate estimates of the true amount present.

The precision of which this assay method is capable depends on the same factors as any other bioassay, the index of precision of the standard curve and the number

of replicates which are made. The former is satisfactory and the effort expended in making many replicates by this method is less than with many other forms of bioassay. Even with the small number of assays that were done in the present work on the *Mactra* ovary extracts, one can be quite confident that this tissue contains a very large amount of estrogenic material, presumably estradiol or a closely related compound. One milligram per kilogram is ten times as much as the maximum previously reported (lobster eggs) for any species. Estradiol added *in vitro* has no effect on the metabolism of *Mactra* ovary (Hagerman, 1956) and apparently no physiologic effects of estrogens on molluscs have been described. The material may be present as an evolutionary freak, similar to the occurrence of uric acid as an excretory product in the Dalmatian coach hound, or may have some important physiologic function in this mollusc. Further speculation should await the isolation and complete chemical characterization of the material present in *Mactra*.

Summary

1. An enzymatic method of assay for estrogens suitable for use with crude tissue extracts is described.

2. Of a variety of marine invertebrates examined, only the ovaries of the mollusc, *Mactra* (*Spisula*) *solidissima*, contained appreciable amounts of estrogenic material.

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THE EFFECTS OF X-IRRADIATION ON THE FERTILIZED EGGS OF THE ANNELID, CHAETOPTERUS[1]

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The study of ionizing radiations and their effects on living systems has been of interest to biologists for many years. An important manifestation of such effects is evident in the mitosis of cells or tissues exposed to radiation, and excellent experimental material for the analysis of cytological and developmental consequences of such exposure is available in the eggs of a number of marine invertebrate animals. One of the more favorable forms is the polychaete annelid, *Chaetopterus pergamentaceus*. The eggs of this form, although not transparent, are characterized by moderate amounts of yolk, so that it has been possible to make whole-mount preparations of the irradiated and control eggs which show pertinent cytological detail clearly. Furthermore, the normal development of Chaetopterus has been studied by Mead (1898) and Lillie (1906), and there are available time-tables of development for given temperatures, so that irradiation can be begun at a known stage of mitosis, and subsequent deviations from the normal schedule of events studied in considerable detail.

Materials and Methods

Ripe Chaetopterus males and females were collected by the M. B. L. Supply Department, and maintained in the laboratory in separate large fingerbowls supplied with running sea water. Eggs were obtained by clipping off two or three posterior parapodia from a single female; these parapodia were transferred to pieces of cheesecloth wet with filtered sea water, through which the eggs passed into 100 cc. of filtered sea water contained in each of two (control and experimental) plastic boxes, $3\frac{1}{4}'' \times 2\frac{3}{4}'' \times 1\frac{1}{4}''$ in size and provided with lids. The eggs were allowed to stand undisturbed for 15 minutes; during that period, the first maturation division proceeds to the metaphase (Lillie, 1902). Sperm were obtained by placing one parapodium from a male in 100 cc. of filtered sea water, 15 minutes before they were to be used for insemination.

Immediately after insemination, the lids were placed on both boxes of egg suspension, and exactly thirty minutes after insemination, the experimental eggs were irradiated by the Laboratory x-ray technician, Mr. Alan P. Brockway. Samples from both control and experimental groups were examined at intervals with a binocular dissecting microscope, to ascertain the progress of cleavage and later development. After irradiation, the contents of control and experimental boxes were transferred to two small fingerbowls, each containing an additional 100 cc. of

[1] Work done under A. E. C. Contract AT-(40-1)-1085, and under a grant from the National Institutes of Health, RG-3907.

filtered sea water; both fingerbowls were then kept on the sea water table, with running sea water flowing around them. The room air temperature varied from 21 to 25° C. Control and experimental cultures were examined for the final time 19 to 22 hours after insemination, when the controls were vigorously swimming trochophores.

The x-ray generator used operates simultaneously two Coolidge tubes at 25 ma and 182 KVP, with an inherent filtration of 0.2 mm. copper. The plastic box (from which the lid was first removed) containing the experimental eggs was thus "cross-fired" between two x-ray beams. Since the eggs were distributed in an even layer on the bottom of the box, a fairly uniform field of irradiation was possible. The x-rays were delivered at two different rates. For total dosages below 3570 r, the machine was calibrated at 510 r per minute with the two tubes 48 cm. apart, the bottom of the Lucite box being approximately equidistant between the two tubes. For dosages above 3570 r, the x-rays were delivered at a rate of 2160 r per minute, with the tubes 16 cm. from one another. There was no appreciable rise in temperature during any of the irradiation treatments, and artificial cooling measures were therefore not used. The duration of treatment varied from one-half minute to eight minutes, and the dosages used were 255 r to 17,280 r

Cytological preparations were made of samples from the control and experimental cultures, at times calculated to result in fixation of the eggs at the metaphase or anaphase of the first three cleavages. A modified squash whole-mount technique was devised, which yielded excellent preparations in a minimum of time. The method involved fixation of the eggs in Kahle's fluid [2] (water, 30 parts; 95% alcohol, 16 parts; formalin, 8 parts; glacial acetic acid, 1 part) on cover-slips. A single drop of concentrated egg suspension was placed on one clean No. 1 square cover-slip, and a drop of fixative on a second cover-slip; the second cover-slip (with the fixative) was inverted and dropped face-to-face onto the first cover-slip. No pressure was applied, and if drops of the correct size were used there was no distortion of the eggs. After about five minutes, the two cover-slips (still face-to-face) were transferred as a unit to a small staining jar containing fresh fixative, where they were kept for 15 to 30 minutes. During this period, the cover-slips were carefully separated from one another with watchmaker's forceps; approximately one-half the eggs adhered to each of the two cover-slips, and very few were lost during the process of separation. After separation, the cover-slips were placed in porcelain racks, Chen Type A, provided with wire handles, and subsequent steps were carried out by transferring the racks to tall Stender dishes containing the necessary reagents. Hydration of the eggs through 70% alcohol, 50% alcohol and distilled water was followed by staining for four to six minutes in a solution of Harris' acid haematoxylin, diluted 1:4 or 1:5 with distilled water and filtered before each use. No counterstain was used. After staining, the eggs were "blued" in several changes of tap water alkalinized with 1% sodium bicarbonate solution, and dehydrated in three changes of triethyl phosphate,[2] in carbol-xylol and in two changes of xylol; they were mounted in damar. The preparations were studied at magnifications of 150 ×, 300 × and 660 ×, using a Spencer compound binocular microscope with compensating oculars. Approximately 440 slides were prepared and studied.

[3] We are indebted to Dr. Anna R. Whiting for suggesting the use of these reagents.

Results

The results are summarized in Table I.

It is apparent that relatively low doses of x-rays (255 to 1020 r) had no immediately obvious effects on fertilized Chaetopterus eggs. There was a very slight retardation (3–5 minutes) of the first three cleavages in the experimental eggs after 765 r, as compared with the control eggs, but no other visible gross or cytological effects. However, when the control and experimental cultures were examined 19 to 22 hours after insemination, the trochophores were very abnormal in cultures which had received 500 and 765 r, respectively. They were, for the most part, very disorganized masses, with marked ciliary defects; they moved feebly, if at all. Almost all the larvae were dead in the groups which had received 1020 r, and disintegration of the undifferentiated cytoplasm had occurred.

In most of the experiments where the eggs were treated with 1275 r and higher dosages, there was at least a slight retardation (5–10 minutes) of the first three cleavages, and the majority of the embryos were dead 19 hours after insemination. The few surviving trochophores were very abnormal, with ciliary defects, cytoplasmic blebs, etc.

The occurrence of cleavage retardation reported here is in accordance with the results obtained by other workers. Thus, Packard (1918) observed delays in the division of Chaetopterus eggs irradiated with gamma rays from radium. Cook (1939) demonstrated a two- to five-hour delay in the first cleavage of Ascaris eggs x-irradiated at the one-cell stage. Henshaw (1940a, 1940b) and Henshaw and Cohen (1940) described marked cleavage retardation in Arbacia eggs, after x-irradiation of eggs, or sperm, or both gametes. Carlson (1938) described a cessation of mitosis in grasshopper neuroblasts treated with varying doses of x-rays (100–1000 r). The recovery time for return of mitosis (anaphases being used as the criterion) varied from three hours after 100 r to 22 hours after 1000 r. There must be a considerable difference in the radio-sensitivity of Chaetopterus eggs, as opposed to grasshopper neuroblasts, since complete inhibition or even pronounced delay of mitosis in our experiments required much higher dosages.

X-ray dosages below 8640 r did not result in any obvious cytological damage to eggs fixed at the times of the first three cleavages; at and above that dose level, however, there were often multipolar spindles, chromosome bridges at anaphase, and fragmentation, particularly in eggs fixed at the time of the third cleavage. These were very similar to the aberrations described by Costello, Henley and Kent (1952) in P^{32}-treated fertilized Chaetopterus eggs. The chromosomes in the Chaetopterus egg are small, and the detection of minute cytological abnormalities is not always possible.

Eggs treated with 15,120 r presented a striking cytological picture when they were fixed at the time of the second and third cleavages. At least some degree of karyokinesis had apparently taken place, but it was not accompanied by cytokinesis, so that there were often several interphase nuclei present in one undivided or incompletely divided mass of cytoplasm. The nuclei were elongated and somewhat pear-shaped, with the narrow "stem" of one of a pair of nuclei directed toward the narrow "stem" of what was apparently its sister nucleus. One had the impression that an abnormal mitotic division with, apparently, a thick chromatin bridge, had been arrested during its course. This phenomenon of nuclear division without

TABLE I—*X-irradiation of fertilized Chaetopterus pergamentaceus eggs*

X-ray dose*	Gross abnormalities noted at stage of:				Cytological abnormalities noted in eggs fixed at:		
	1st cleavage	2nd cleavage	3rd cleavage	Trochophores	1st cleavage	2nd cleavage	3rd cleavage
255 r	None	None	None	Very slight: a few dead	None	None	None
510 r	None	None	None	Abnormal: ciliary defects, cytoplasmic blebs	None	None	None
765 r	Slight retardation**	Slight retardation	Slight retardation	Abnormal: ciliary defects, cytoplasmic blebs	None	None	None
1020 r	None	None	None	Mostly dead; those living very abnormal	None	None	None
1275 r	Retardation	Retardation	Retardation	Mostly dead; those living very abnormal	None	None	None
1530 r	Slight retardation	Slight retardation	Slight retardation	Dead	None	None	None
2550 r	Slight retardation	Slight retardation	Slight retardation	Mostly dead; those living very abnormal	None	None	None
3570 r	None	Slight retardation	Slight retardation	Dead	None	None	None
8640 r	Retardation	Retardation	Abnormal 2- and 3-cell stages	Dead	None	None	Multipolar spindles; chromosome fragments and bridges
12,960 r	Retardation	Retardation	Retardation	Dead	None	None	Multipolar spindles; chromosome fragments and bridges
15,120 r	Retardation	Retardation	Retardation	Mostly dead; those living very abnormal	None	Nuclear division without division of cytoplasm. Many very elongated interphase nuclei	Multiple interphase nuclei in undivided or partially divided cytoplasm. Many elongated nuclei
17,280 r	Retardation	Inhibition (most eggs not cleaved)	Inhibition (most eggs 2- and 3-cells)	Dead	None	Undivided eggs	Two interphase nuclei present in each of the 2 cells in most cases. Some 3-cell stages with 1 interphase nucleus per cell; AB cell is one which appears to have divided

* X-ray data: Inherent filtration 0.2 mm. Cu; 25 ma; 182 KVP. Two tubes: 48 cm. apart, 510 r per minute, for doses up to 3570 r; 16 cm. apart, 2160 r per minute, for doses above 3570 r; irradiated eggs equidistant between tubes in all cases.

** Cleavage retardation times were ascertained only approximately; in all cases they varied from 4 to 15 minutes, except in those groups in which cleavage was completely inhibited.

cytoplasmic division, or with incomplete cytoplasmic division, is perhaps the most striking effect noted after treatment of these eggs with high dosages of x-rays.

X-irradiation with 17,280 r resulted in marked effects (both gross and cytological) on the first three cleavages. The division to two cells was very abnormal and noticeably retarded (5–7 minutes), and the two subsequent divisions were almost completely inhibited. There were some two- and three-cell stages present in the samples fixed at the times of second and third cleavage in the controls; the stages fixed 75 minutes after insemination often had two interphase nuclei in each of two cells, again indicating a suppression of cytokinesis at an earlier stage. The three-cell stages (which are not normally found in the cleavage of Chaetopterus, although the polar lobe may simulate a third cell) usually had only one interphase nucleus in each of the three cells. It appeared that cytoplasmic division had taken place only in the smaller AB blastomere at the second cleavage, the larger CD blastomere remaining undivided. The possible significance of this observation will be discussed below.

Discussion

Sensitivity of prophase chromosomes to irradiation

The design of our experiments was such that treatment was begun at prophase of the first cleavage, a time which appears, according to the findings of many observers utilizing a variety of materials, to be very susceptible to radiation. This fact, coupled with the circumstance that division in Chaetopterus eggs is predictable and synchronous (within certain limits imposed by temperature and other environmental factors), makes the material favorable for studies of radiation effects.

One of the early reports on the susceptibility of prophase chromatin to irradiation came from Strangeways and Hopwood (1926), who irradiated chick tissue cultures with x-rays and found the most sensitive period to be at the time immediately before the onset of visible prophase. Sax (1938, 1943) reported the greatest frequency of x-ray-induced chromosome aberrations in Tradescantia microspores which were at the meiotic or mitotic prophase at the time of treatment. Luther (1938) x-irradiated frog eggs, and concluded that the maximum susceptibility was during prophase, the minimum susceptibility at metaphase. The findings of Marshak (1939) are somewhat at variance with the general observation that prophase is the stage of greatest sensitivity; x-irradiated onion seedlings, pretreated with dilute solutions of ammonia, were examined cytologically and the results interpreted to indicate that the chromosomes were most sensitive at the resting stage. This was held to be the case regardless of whether or not ammonia pre-treatment was used.

Henshaw and Cohen (1940) x-irradiated fertilized Arbacia eggs, and found that when cleavage retardation was the criterion, the time of greatest susceptibility was during the period after the male and female pronuclei had come together, and during early prophase. The late prophase stage, immediately before breakdown of the nuclear membrane, was found by Carlson (1941) to be the most sensitive period for grasshopper neuroblast cells, and Swanson (1942) made similar observations on Tradescantia pollen tube chromosomes.

More recently, Bloom, Zirkle and Uretz (1955) utilized microbeams of protons and ultraviolet light for irradiation of individual chromosomes and parts of chromo-

somes, in cultures of Triturus heart tissue. They found that a given dose at the prophase stage produced "sticky" chromosomes and chromosome fragmentation; the same radiation to metaphase chromosomes resulted, again, in sticky chromosomes but very few akinetic chromosomes or fragments were observed. Their findings are of great interest, not only because of the highly localized character of the radiation used, but also because they were able to follow the fate of a single irradiated chromosome in considerable and exact detail by the use of phase optics and time-lapse motion pictures.

When Chaetopterus eggs are x-irradiated beginning 30 minutes after insemination, both polar bodies have usually been given off (except in a few of the experiments performed early in the breeding season, when low temperatures slowed down all features of development of the egg). At 21° C., according to Heilbrunn and Wilson (1948), the male and female pronuclei have approached one another and are fusing, and by 40 minutes after insemination, the fusion nucleus is at the prophase of the first cleavage. This time-table of events proceeds at a considerably faster rate when the temperature is increased, and our data indicate that for most of the experiments reported here, the eggs were at the early prophase stage when irradiation was begun.

Possible effects of irradiation on early development of the eggs

In the ovary of the Chaetopterus female, a definite polarization of the egg is evident, the future animal pole being free in the lumen of the ovarian tubule, and the future vegetal pole being attached to the wall of the tubule (Lillie, 1906). Thus, the subsequent position of the polar bodies is "set" very early in development. The cleavage spindle is formed by the separation of the two sperm centrosomes (Mead, 1898), in a position in the egg which is determined by the position of the male pronucleus. This, in turn, is determined by the polarity of the egg (the sperm nearly always entering in the vegetal hemisphere), so that there is an orderly chain of events, each one of which is predicated on the original polarity of the egg.

Lillie (1906) described the separation of large basophilic granules from the chromosomes of the first cleavage in Chaetopterus. The subsequent division of the egg segregates all these granules into the CD blastomere, none being left in the AB cell. X-irradiation with 17,280 r at prophase resulted, as noted above, in a failure of the CD blastomere to divide at the second or at the third cleavage, so that there appears to have been a drastic effect on the distribution of these granules, as well as on other cytoplasmic movements.

Indeed, irradiation of eggs at the prophase of first cleavage could very well have other far-reaching effects, quite apart from those on the chromosomes. The influence of x-irradiation on viscosity changes during mitosis of the Arbacia egg has been described by Wilson (1950). In unirradiated eggs, there is a marked increase in viscosity which reaches a value three or four times that of the unfertilized eggs at 15 minutes after insemination; this increased viscosity persists for a few minutes, and then drops to almost the level of viscosity of unfertilized eggs shortly before the first cleavage. Approximately the same magnitude of increase was observed in eggs irradiated and then inseminated, but it remained high for a period two to three times that of the control eggs, eventually decreasing shortly before

cleavage. If viscosity changes occur in the fertilized Chaetopterus egg irradiated after insemination, they would be expected to have profound effects on the complex patterns of oöplasmic segregation characteristic of this form. Lillie (1906) demonstrated that there was an exact correlation between the distribution of ectoplasmic spherules (which, before the breakdown of the germinal vesicle, are distributed over the animal two-thirds of the egg, and which come to overflow the vegetal hemisphere after germinal vesicle breakdown) and the distribution of cilia in the trochophore larvae. The cilia apparently do not develop directly from the spherules, however. The fact that the later stages of development in our experimental cultures were almost invariably marked by abnormalities of cilia distribution (if, indeed, the embryos survived to the stage of cilia formation at all) indicates that the irradiation directly or indirectly affected some part of the cilia-producing mechanism.

The phenomenon of polar lobe formation, which is characteristic of the eggs of certain annelids (including Chaetopterus) and molluscs, is a striking indication of the profound changes which occur in the cytoplasm of these eggs within the first few hours after fertilization. Although we observed no visible evidence of malformation of the polar lobe in the irradiated eggs, it is quite possible that there were inconspicuous disturbances in the apportionment of cytoplasmic materials to this structure. It is of interest in this connection to point out that even after 17,280 r, polar lobe formation immediately before the first cleavage was morphologically normal, although delayed. Formation of the second polar lobe after this dosage appears to have been completely suppressed.

In any event, the deleterious effects of x-ray treatment on later development are, *per se,* an indication that irradiation interfered with early processes of differentiation.

The possible role of cytoplasmic effects in radiation damage has been considered in the recent paper by Ord and Danielli (1956), in which they transferred x-irradiated *Amoeba proteus* nuclei to non-irradiated cytoplasm of the same form. The resulting organisms had a somewhat lower percentage of survival than did intact irradiated amoebae. When non-irradiated nuclei were transfered to irradiated cytoplasm, 18–48 hours after irradiation, the animals survived despite the fact that they had received very high doses of x-rays; they were able to form new clones, although the time of first division was somewhat delayed. If the transfers of normal nuclei to irradiated cytoplasm were done sooner than 18 hours after treatment, however, the normal nuclei were apparently lethally damaged by the irradiated cytoplasm in most cases. Ord and Danielli point out that in this study, nuclear damage appears to be a *direct* result of x-ray damage to the nuclei, and an *indirect* result of contact with damaged cytoplasm. They suggest that perhaps the importance of nuclear damage from radiation, as opposed to cytoplasmic damage, may have been somewhat over-emphasized.

Chromosome aberrations

There have been many reports in the literature of chromosome aberrations which occur after irradiation from various sources. An early one was the paper by Packard (1918); he irradiated unfertilized Chaetopterus eggs with gamma rays from a radium source and observed multipolar spindles at the first cleavage, after

the treated eggs had been inseminated. Packard interpreted these multipolar spindles as being due to polyspermy, which he felt was facilitated in some way by the irradiation. Alberti and Politzer (1924a, 1924b) described and figured a variety of cytological abnormalities in the corneal epithelium of larval salamanders which had been x-irradiated; among these anomalies were chromatin bridges at anaphase, telophase and interphase, akinetic fragments and chromosomes, accessory nuclei, multiple akinetic chromosomes, and multipolar spindles. Similar effects were described by Strangeways and Hopwood (1926) in x-irradiated chick tissue cultures, and by Sonnenblick (1940) in the embryos obtained after mating x-irradiated male and female Drosophila adults. Laznitzki (1943) also x-irradiated chick tissue cultures, and described a number of cytological abnormalities resulting from treatment; however, this author emphasizes the fact that no multipolar spindles were observed in irradiated material, which is in contrast to the findings of other investigators. Zirkle and Bloom (1953), utilizing proton bombardment of parts of amphibian heart cells in tissue culture, reported chromosome bridges, inhibition of cytokinesis, and unequal distribution of the daughter chromosomes. Chromosome fragmentation after irradiation has been described by Carlson (1938) for grasshopper neuroblast cells, by Fabergé (1940) for Tradescantia pollen grains, by Bishop (1942) for grasshopper spermatocytes, by Whiting and Murphy (1956) for Habrobracon oöcytes, and by Lesley and Lesley (1956) in plants from treated tomato seeds. We have observed many of the aberrations described above in our material, especially after the higher doses of x-rays.

Henshaw (1940d) studied the multipolar spindles which occurred in fertilized Arbacia eggs after x-irradiation; it is interesting that in contrast to our findings (where multipolar spindles clearly attributable to irradiation were observed no sooner than the third cleavage), he described such effects at the first cleavage. This difference may be due to the fact that Henshaw used considerably higher doses (31,200 r) than were employed in the present study. In the same paper (1940d), he found no evidence of nuclear division without accompanying cytoplasmic division at the first cleavage in his treated eggs. We found karyokinesis without cytokinesis only at the second and third cleavages, which apparently were not studied by Henshaw.

Recently, Bloom, Zirkle and Uretz (1955) irradiated parts of chromosomes of amphibian heart cells in tissue culture, using microbeams of protons or ultraviolet. Irradiation of the kinetochore region resulted in "drifting" of the chromosome until anaphase when it was incorporated as a lobe on the daughter nucleus, or became a small accessory nucleus. Chromosome "stickiness" and fragmentation were also reported. Chromosomes treated with beams of protons or ultraviolet outside the kinetochore region showed no such effects. Bombardment of extra-chromosomal areas of the cells (cytoplasm and the ends of spindles) with relatively large numbers of protons produced no effects at the site of irradiation. Heterochromatic ultraviolet irradiation of the same regions, however, resulted in a disappearance of the spindle and derangement of the characteristic metaphase configuration; a "false anaphase" followed, in which chromosomes, rather than chromatids, moved apart.

It is of interest that many of the chromosome aberrations typical of radiation damage are produced also by a variety of other agents including, for example, low temperature (Callan, 1942; Böök, 1945; Henley, 1950; Henley and Costello, 1949);

high temperature (Briggs, 1947); colchicine (Callan, 1942); and ribonuclease (Kaufmann, MacDonald and Bernstein, 1955).

Androgenesis after irradiation

There has been considerable discussion in the literature as to the possibility of androgenesis occurring in fertilized irradiated eggs, as a consequence of irreparable damage to the egg chromatin, so that development proceeds with only the haploid, paternal complement of chromosomes. Packard (1918) reported that in Chaetopterus eggs which had received heavy doses of gamma radiation before insemination, the female pronucleus remained in a polar position, often attached to the polar body material by a fine cytoplasmic strand in which chromatin threads can be distinguished. Cleavage took place in such eggs in an orderly fashion, however, and the haploid number of chromosomes was present. Whiting (1948, 1955) found haploid androgenetic males in Habrobracon. They developed only from eggs x-rayed in first meiotic metaphase, thereby resembling Packard's results in Chaetopterus.

Henshaw (1940d), on the other hand, reported that both pronuclei participated in the development of irradiated Arbacia eggs, even after heavy doses of x-rays. However, Arbacia eggs are fully mature, with the female pronucleus present, when in the fertilizable condition, whereas Chaetopterus eggs are at the metaphase of the first maturation division.

Whiting (1955) studied Feulgen preparations of Chaetopterus eggs irradiated (during metaphase I) with 60,000 r and fertilized 3½ minutes later with untreated sperm. The majority underwent continued cleavage, and of these 26% appeared to have sperm chromosomes, only, and were therefore androgenetic. Whenever chromatin abnormalities appeared in cleaving cells, there were more than nine chromosomes (the haploid number) present, and in all cells in which there were nine chromosomes, only, no aberrations were visible. Whiting therefore found no evidence of an injurious effect of irradiated cytoplasm upon untreated chromosomes. In the experiments reported in the present paper, apposition of the pronuclei occurred normally in irradiated eggs, and the diploid number of chromosomes appeared to be present. Since the eggs had been inseminated before irradiation, the occurrence of normal fertilization implies that no damage was suffered by the sperm, either directly or as a consequence of its passage through irradiated egg cytoplasm.

The effects of irradiation on later development

Lea (1955) points out that the death of a cell as a result of irradiation usually does not occur immediately but at, or following, the next division of the cell; an immediate lethal effect requires much larger doses of radiation than a delayed lethal effect. The results obtained in the present study confirm this general statement, and are in accordance with the findings of other investigators. Cook (1939), for example, x-irradiated Ascaris eggs at the one-cell stage and obtained highly abnormal embryos, consisting of unorganized masses of cells. Sonnenblick (1940) reported the occurrence of undifferentiated non-viable masses of cells among the progeny of adult fruit flies which had been treated with x-rays. Henshaw (1940c) found that x-irradiation of Arbacia eggs with 14,400–28,800 r resulted in the

appearance of a wide gradation of effects at the pluteus stage, ranging from shortening of the skeletal arms to the formation of a disorganized mass of cytoplasm which subsequently disintegrated. Giese (1946) treated Chaetopterus sperm with 8000–16,000 ergs/mm² of ultraviolet; when such sperm were used to inseminate normal eggs, death ensued, at a stage which is not specified.

Even after x-ray doses as low as 255 r in our experiments, there was some mortality in Chaetopterus trochophores examined approximately 22 hours after insemination. Above that dose level, the larvae were very abnormal or, more commonly, dead. This delayed action was especially striking after some of the lower doses of x-rays, where no particular gross or cytological effects (except for slight cleavage retardation) were discernible at earlier stages after treatment.

Summary

1. Fertilized eggs of Chaetopterus were x-irradiated, beginning 30 minutes after insemination; doses from 255 r to 17,280 r were used, and the duration of treatment was one-half minute to eight minutes. Observations were made of both living and fixed eggs, at various intervals after irradiation, and of living trochophore larvae 19–22 hours after irradiation.

2. The principal effects of relatively low doses (255–1020 r) were found to be a slight retardation of cleavage (3–5 minutes), and the production of abnormal trochophore larvae which were characterized by severe ciliary defects, cytoplasmic blebs, and very feeble movements (following doses of 255 to 765 r). Doses of 1020 r and above resulted in death of most of the larvae by the trochophore stage. The majority of the eggs irradiated with 1275 r and above showed at least a slight retardation of the first three cleavages.

3. Among the cytological abnormalities observed (especially after the higher doses) were multipolar spindles, chromosome fragmentation, and karyokinesis without cytokinesis.

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THE TAXONOMY OF UNARMORED DINOPHYCEAE OF SHALLOW EMBAYMENTS ON CAPE COD, MASSACHUSETTS [1]

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Several papers give brief accounts of unarmored Dinophyceae found along the eastern coast of the United States. Calkins (1902) described from Woods Hole three European species, with one as a new variety. Herdman (1924a) listed five European, sand-living species from Woods Hole. Lackey (1936) listed thirteen species, all European, in his account of Woods Hole protozoa. Martin (1929) described thirteen species, four of which were new, from Barnegat Bay. It would be expected that further study would show many more extensions of range from the east to the west side of the Atlantic. One wonders, though, whether new species would be few, as suggested by these figures, or on the contrary would be many, since studies hitherto have not been very detailed. Further, the Barnegat Bay list suggests that the shallow, estuarine type of habitat has as many species as coastal waters, since Martin's number is matched only by that of Lackey.

The following study covers twenty-six species, of which twelve are completely

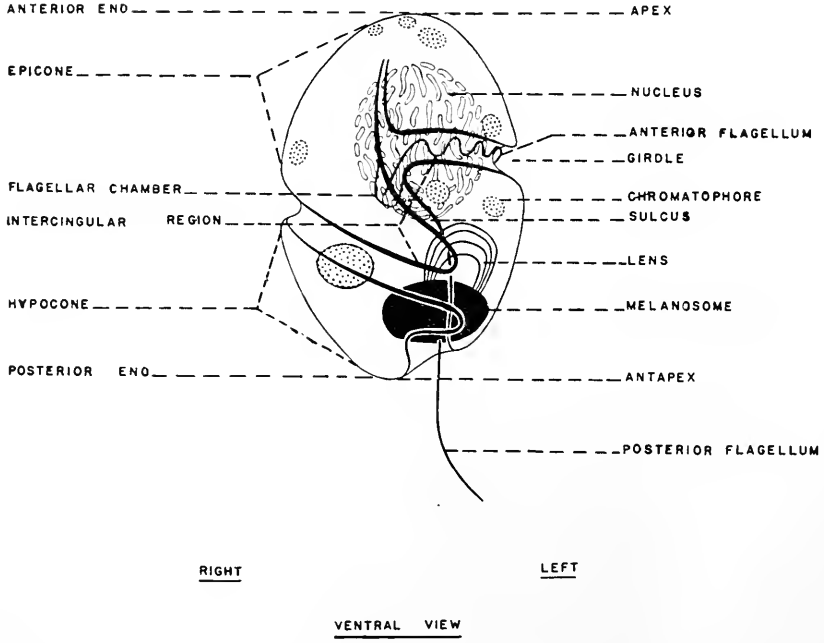

FIGURE 1. Structure of a dinophycean.

[1] Contribution No. 838 from the Woods Hole Oceanographic Institution.

new and nine show extensions of range from Europe. The collections which furnished the great majority of the material were from Great Pond, Falmouth Harbor, Salt Pond, and Uncatena Island Pond, all very shoal embayments on the southern shore of Cape Cod. The expectations, consequently, seem to be fulfilled.

Water samples were concentrated by centrifuging and the Dinophyceae were studied alive in water mounts. At magnifications of × 440 and × 980 drawings were made of specimens which had ceased to swim but showed no deformity due to approaching disintegration. Outline, girdle, and sulcus were drawn with camera lucida; the rest of the structures were drawn free-hand.

For those unacquainted with the morphology of unarmored Dinophyceae and its special nomenclature, Figure 1 illustrates the typical structure of such a dinophycean.

The species studied fall into eight genera, which may be characterized as in the following key:

Key to Genera

Girdle and sulcus rudimentary ..... *Oxyrrhis*
Girdle and sulcus well-developed:
  Solitary:
    Without ocellus and nematocysts:
      Girdle not markedly displaced:
        a) Girdle in anterior third of body ..... *Amphidinium*
        b) Girdle in central portion of body ..... *Gymnodinium*
        c) Girdle in posterior third of body ..... *Massartia*
      Girdle markedly displaced ..... *Gyrodinium*
    With ocellus but without nematocysts ..... *Warnowia*
    With ocellus and nematocysts ..... *Nematodinium*
  Colonial ..... *Polykrikos*

OXYRRHIS Dujardin

Oxyrrhis marina Dujardin

Oxyrrhis marina Dujardin in Kofoid and Swezy, 1921, p. 117, text fig. R, 3.
Oxyrrhis marina Dujardin in Lebour, 1925, p. 19, pl. 1, figs. 6a–6e.

Woods Hole area: Uncatena Island, Salt Pond; March, August, October. New Jersey; White Sea; England; brackish estuary near Nieuport, Belgium; Marseilles harbor; Genoa harbor.

This species is distinguished by the posterior position of its flagella, by the broad excavation of the posterior sulcus, divided midway by a tentacle-like lobe, and by the partial encirclement of the girdle. Other characters are its elongate ellipsoidal form and absence of chromatophores.

AMPHIDINIUM Claparède and Lachmann

Key to Species

Chromatophores present:
  Chromatophore single ..... *A. carteri*
  Chromatophores many ..... *A. wislouchi*
Chromatophores absent:
  Body stout, with rounded ends ..... *A. crassum*
  Body very slender, with pointed ends ..... *A. sphenoides*

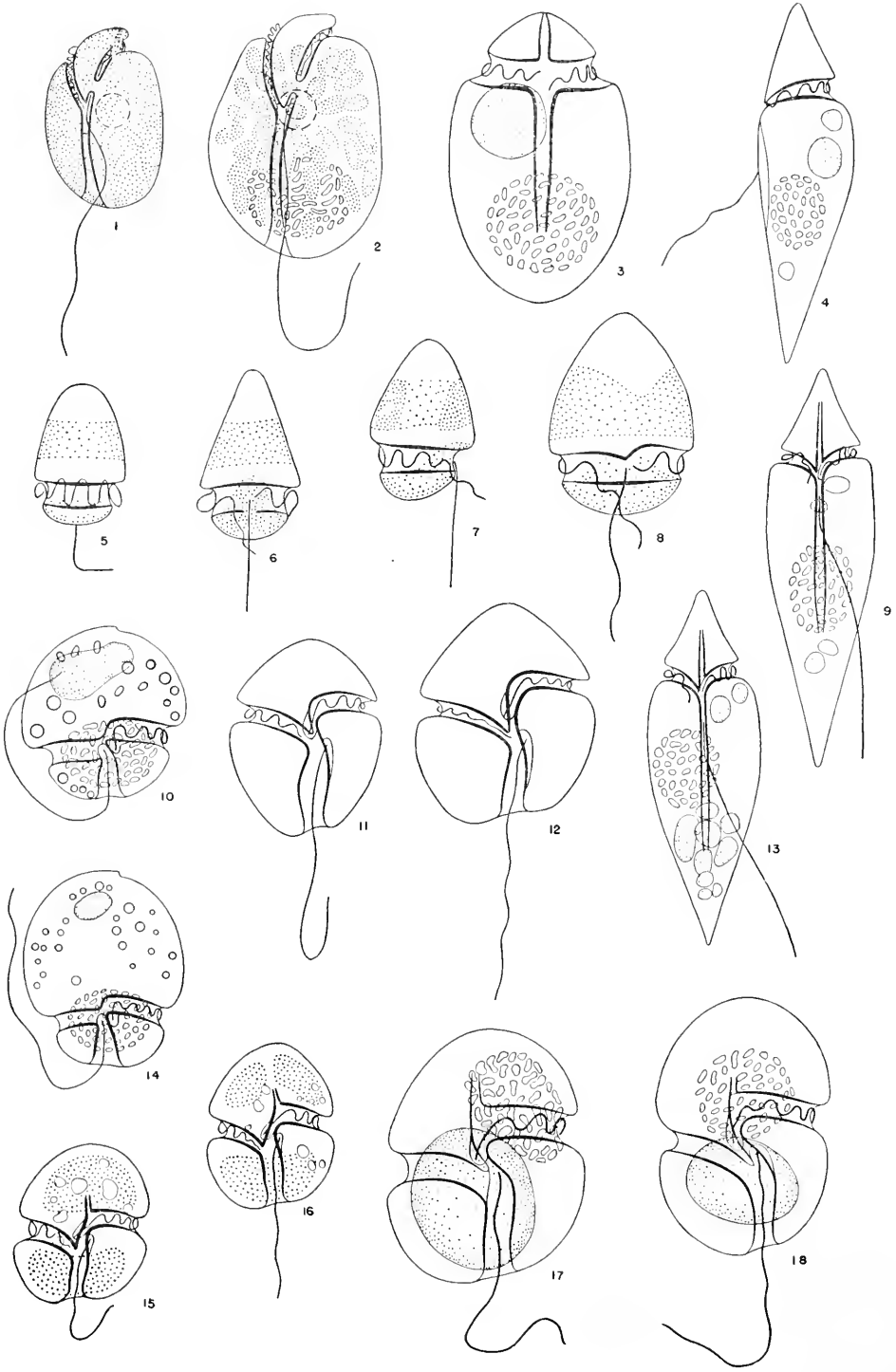

PLATE 1

Amphidinium **carteri** nom. nov.

(Plate 1, Figure 1)

Amphidinium klebsi Carter, 1937, p. 58, pl. 8, figs. 12, 13, 14, 15. (*non* Kofoid & Swezy, 1921).

Body dorsi-ventrally flattened, oval in outline in ventral view, elongate-elliptical in lateral view. Length 12–15 μ, width 8–9 μ. Epicone small, asymmetric, crescent-shaped in ventral view, somewhat flattened at apex; in dorsal view beak-like, rising near right margin and projecting toward left margin. Hypocone truncate-elliptical in ventral or dorsal view, asymmetric, with its right margin convex and left straight or very slightly convex, the antapex broadly rounded. Left limb of girdle starting some distance above posterior end of epicone, running in an arching course anteriorly and laterally, then extending transversely around dorsal surface of epicone, finally leading posteriorly near right margin to round posterior end of epicone, failing, however, to meet the end of the left limb. Sulcus nearer right margin, extending from posterior end of epicone in curving course to antapex. Anterior flagellum inserted at the end of the left limb of girdle; posterior flagellum inserted just below the anterior, separated from it by a "bridge" that separates girdle ends, extending 1.5 body lengths.

Chromatophore single, covering whole inner surface, often perforate, golden brown. Nucleus at posterior end of hypocone, containing short chromatin corpuscles. Pyrenoid present, in center of hypocone. Assimilate granules present or absent.

Woods Hole area: Uncatena Island; October, July. Isle of Wight, England, in brackish pool.

The species described here as *Amphidinium carteri* is identical with Carter's *A. klebsi*. It is considered an independent species since it is much smaller than the forms described as *A. klebsi* by Kofoid and Swezy (1921), Herdman (1924a), and Lebour (1925), and since it has only a single chromatophore.

Amphidinium **wislouchi** n. sp.

(Plate 1, Figure 2)

Amphidinium sp. Wislouch, 1924, p. 121, pl. 3, fig. 11.

Body dorsi-ventrally flattened, oval in outline in ventral view. Length 20–25 μ, width 14–16.5 μ. Epicone small, asymmetric, crescent-shaped in ventral view,

PLATE 1

FIGURE 1. *Amphidinium carteri* nom. nov.
FIGURE 2. *Amphidinium wislouchi* n. sp.
FIGURE 3. *Amphidinium crassum* Lohmann
FIGURES 4, 9, 13. *Amphidinium sphenoides* Wulff
FIGURES 5, 6, 7, 8. *Massartia rotundata* (Lohmann) Schiller
FIGURES 10, 14. *Massartia asymmetrica* (Massart) Schiller
FIGURES 11, 12. *Gyrodinium metum* n. sp.
FIGURES 15, 16. *Gyrodinium estuariale* n. sp.
FIGURES 17, 18. *Gyrodinium glaebum* n. sp.

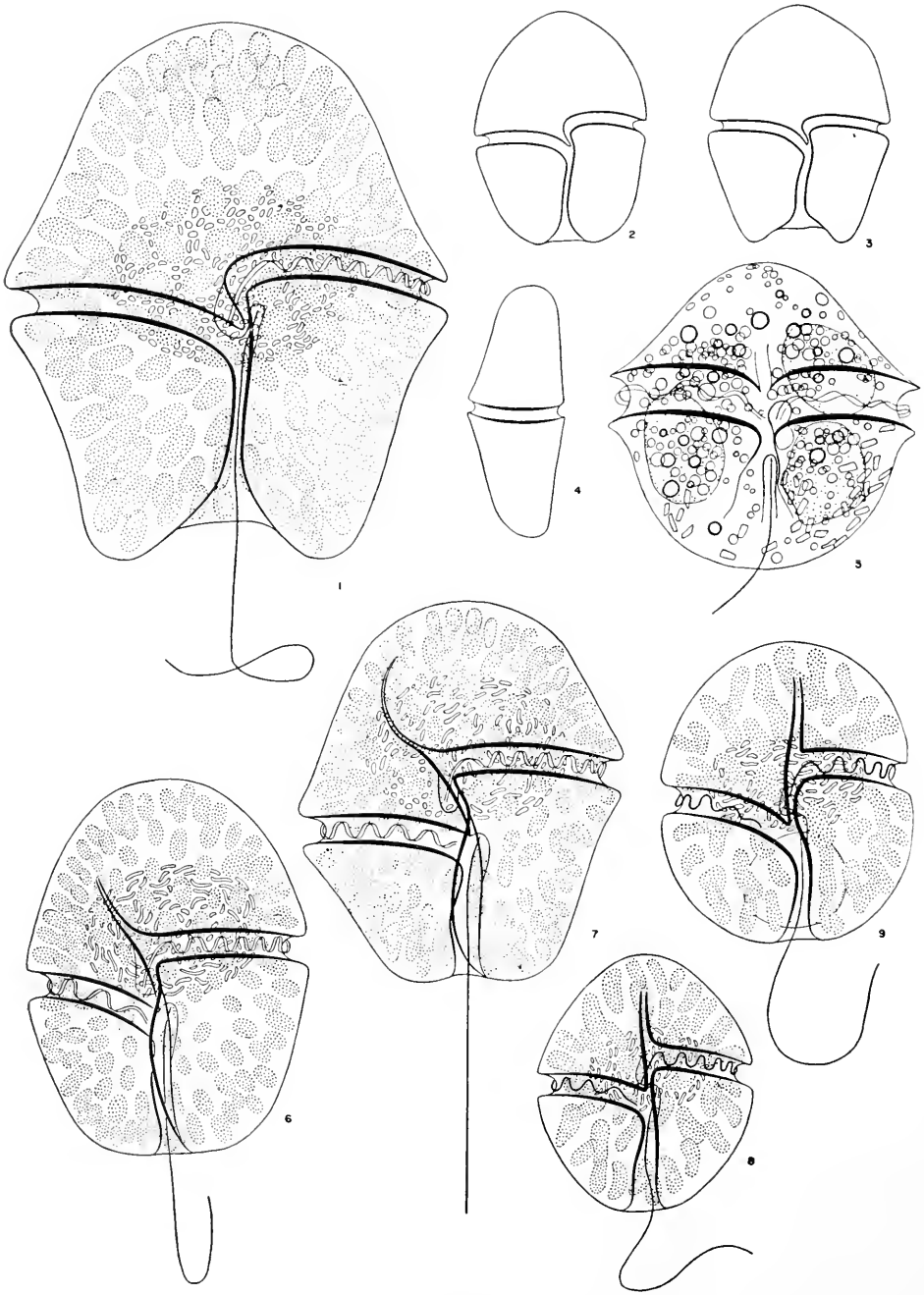

PLATE 2

somewhat flattened at apex; in dorsal view beak-like, rising near right margin and projecting toward left margin. Hypocone truncate-elliptical in ventral or dorsal view, asymmetric, with its right margin convex and left almost straight, the antapex broadly rounded. Left limb of girdle starting some distance above posterior end of epicone, running in an arching course anteriorly and laterally, then extending transversely around dorsal surface of epicone, finally leading posteriorly near right margin to round posterior end of epicone, failing, however, to meet the end of the left limb. Sulcus nearer right margin, extending from posterior end of epicone in curving course to antapex. Anterior flagellum inserted at the end of the left limb of girdle; posterior flagellum inserted just below the anterior, separated from it by a "bridge" that separates girdle ends, extending 1.5 body lengths.

Chromatophores many, elliptical, often arranged in a somewhat radiating manner, with pyrenoid as center.

Woods Hole area: Uncatena Island, Great Pond; October, March. Poland.

This species is about the same as Wislouch's *Amphidinium* sp. and is quite similar to *A. carteri* except for having many chromatophores.

Amphidinium crassum Lohmann

(Plate 1, Figure 3)

Amphidinium crassum Lohmann, 1908, p. 261, pl. 17, fig. 16.
Amphidinium crassum Lohmann in Lebour, 1917, p. 188, fig. 2; 1925, p. 31, pl. 3, figs. 2a–2c.

Body elongate elliptical, circular in cross-section. Length 23–30 μ, width 11–17 μ. Epicone very small, 0.20–0.25 the body length, broadly conical with a pointed apex; hypocone with its sides parallel in the anterior half, rounding into a broad antapex. Girdle not displaced, wide, shallow, its posterior margin wider than the anterior. Sulcus very narrow and shallow, straight, reaching from the apex to 0.66 of the length of hypocone. Flagellar chambers not seen. Anterior flagellum completely encircling body, posterior flagellum not seen.

Chromatophores absent. Nucleus close to antapex, spherical, containing short chromatin corpuscles. Brown ingested bodies and assimilate bodies common.

Woods Hole area: Great Pond, Falmouth Harbor; April, May. Baltic Sea off Kiel; English Channel; Plymouth Sound; Adriatic Sea.

Lohmann (1908) described two similar amphidiniums, *A. crassum* and *A. longum*, the second more slender and with a smaller, more pointed epicone than the first. Lebour (1917, 1925) described as *A. crassum* a type intermediate between these two in proportion of length to width, but with the "fuller" epicone of Lohmann's *A. crassum*. Lebour's type is identical with that described here.

PLATE 2

FIGURES 1, 2, 3, 4. *Gymnodinium nelsoni* Martin
FIGURE 5. *Gymnodinium lazulum* n. sp.
FIGURES 6, 7. *Gyrodinium resplendens* n. sp.
FIGURES 8, 9. *Gyrodinium aureolum* n. sp.

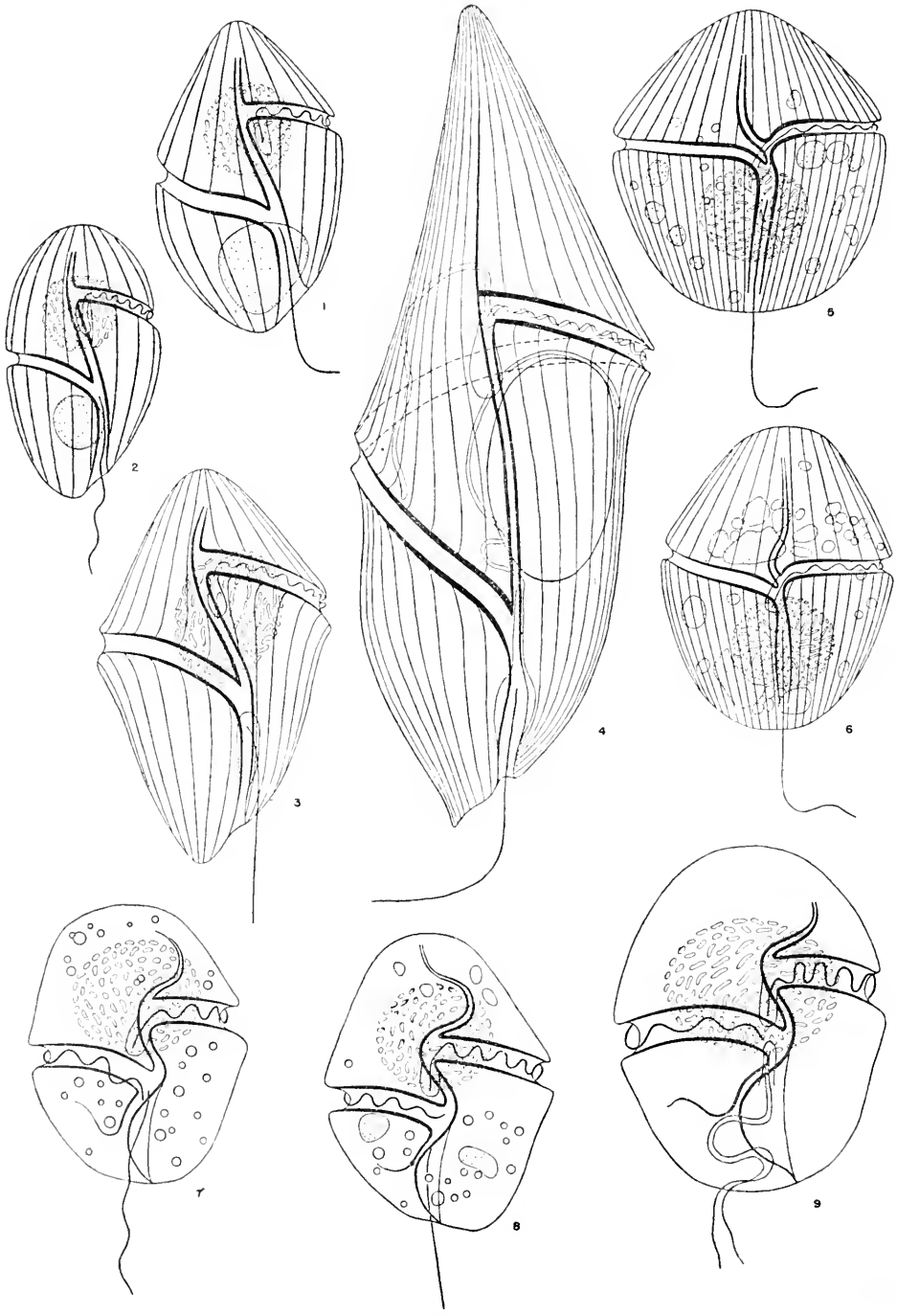

PLATE 3

Amphidinium sphenoides Wulff

(Plate 1, Figures 4, 9, 13)

Amphidinium sphenoides Wulff, 1916, p. 105, pl. 1, figs. 9a–9b.

Body cylindrical, of slender proportions, tapered posteriorly to a sharp point, tapered anteriorly more abruptly to an equally sharp point. Length 35–42 μ, width 12–13 μ. Epicone 0.20–0.25 the total length, symmetrically diamond-shaped or triangular, respectively, in ventral or dorsal view, but asymmetrically triangular in side view, with the dorsal margin more sloping than the ventral; outline contours concave. Hypocone similar but more elongate, triangular in dorsal and ventral view and more sloping along the dorsal than the ventral margin. Girdle deep, broad dorsally but narrowed ventrally, so that in side view the margins appear to converge. Sulcus narrow, running from near the apex to 0.66 the length of the hypocone. Anterior flagellar chamber a mere deepening of the left girdle end (posterior chamber not seen). Anterior flagellum encircling body; posterior flagellum somewhat shorter than the body.

No chromatophores. Nucleus midway the length of hypocone. Assimilate spherules of various sizes present or absent.

Woods Hole area: Great Pond; January. Barents Sea.

GYMNODINIUM Stein (emended by Kofoid and Swezy)

Key to Species

Without striations:
- Chromatophores present ..... *G. nelsoni*
- Chromatophores absent:
  - Body globular ..... *G. lazulum*
  - Body laterally flattened ..... *G. stellatum*

With striations ..... *G. striatissimum*

Gymnodinium nelsoni Martin

(Plate 2, Figures 1, 2, 3, 4)

Gymnodinium nelsoni Martin, 1929, p. 14, pl. 3, figs. 25–26.

Body broadly fusoid, with truncate antapex, very much flattened dorsi-ventrally. Length 50–70 μ, width 38–53 μ. Epicone in ventral view sub-hemispherical to somewhat angled, its sides then straight or concave, and its apex broadly pointed. Hypocone trapezoidal, its sides convex, straight, or concave; its apex wide, emarginate to broadly indented. In lateral view dorsal contour somewhat convex, ventral contour somewhat concave; end-on view with a similar dorsal convexity and ventral concavity. Girdle narrow, deep, displaced one-two girdle widths. Sulcus not present on epicone, narrow and sigmoid in intercingular region, straight and

Plate 3

Figures 1, 2, 3. *Gyrodinium dominans* n. sp.
Figure 4. *Gyrodinium spirale* (Bergh) Kofoid and Swezy
Figures 5, 6. *Gymnodinium striatissimum* n. sp.
Figures 7, 8, 9. *Gyrodinium undulans* n. sp.

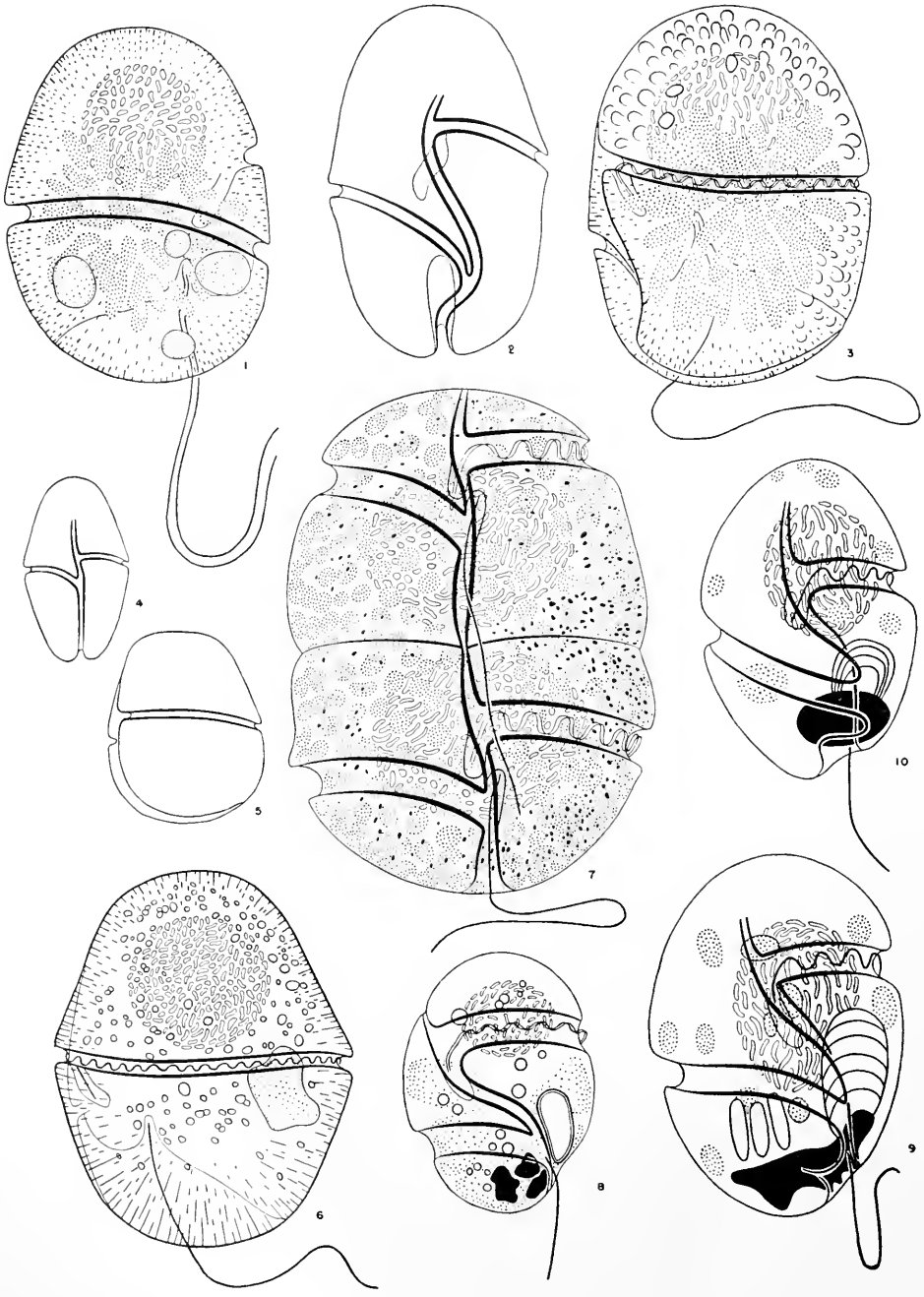

PLATE 4

somewhat wider on hypocone, widening abruptly and showing a large excavation at antapex. Anterior and posterior flagellar chambers overlapping. Anterior flagellum not completely encircling cell; posterior flagellum equal to the body length.

Chromatophores many, rich brown, elliptical, radiating from center of cell. Nucleus central, wider than long, with numberless, elongate chromatin corpuscles. Cells usually free from assimilate and ingested bodies.

Woods Hole area: Great Pond; September. Barnegat Bay, United States east coast.

G. nelsoni is distinguished from its close relative, *G. splendens* (Lebour, 1925), by having short, elliptical chromatophores instead of elongate slender ones.

Gymnodinium **lazulum** n. sp.

(Plate 2, Figure 5)

Body globular, circular in cross-section, epicone and hypocone subequal. Length 28–30 μ, width 30–32 μ. Epicone conical bell-shaped, with marked concavity of outline near girdle; hypocone hemispherical bell-shaped, with concavity of contours less extensive but closer to girdle than in epicone. Girdle wide, deep, without displacement. Sulcus short, narrow, and faintly defined on epicone; on hypocone abruptly widening, more so on the right than the left, its margins fading out about halfway to antapex. Girdle ends dipping in markedly to the bottom of the sulcus, deeply excavating sulcus in girdle region. Anterior flagellum inserted at opening of a pore; pore extending as small pustule from left girdle end posteriorly. Posterior flagellum inserted in trough-like depression of posterior sulcal floor. Anterior flagellum a delicate band completely encircling body; posterior flagellum short, 0.5 body length.

Chromatophores absent. Nucleus not visible. Cytoplasm clear, quite transparent, smoke blue in color. Colored spherules, grading from copper-red through orange and brown to lemon-yellow, abundant, scattered throughout cytoplasm, often clustered near girdle. Angular, crystal-like, slate-gray bodies scattered at random through hypocone. Large, brown ingested bodies common.

Woods Hole area: Great Pond; December, May.

This species was the only one studied with a clear, blue, instead of a granular, gray, cytoplasm. The array of hues of the numberless small spherules, as well as the blue of the cytoplasm, are matched by similar structures in *G. violescens* (Kofoid and Swezy, 1921).

Gymnodinium **stellatum** n. sp.

(Plate 4, Figures 4, 5, 6)

Body laterally flattened, elliptical in side view with sub-truncate anterior end. Length 25–47 μ, thickness 22–39 μ, width 17–25 μ. Epicone in dorsi-ventral view taller than wide, with slightly sloping sides; in lateral view as wide as tall, trape-

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PLATE 4

FIGURES 1, 2, 3. *Gyrodinium uncatenum* n. sp.
FIGURES 4, 5, 6. *Gymnodinium stellatum* n. sp.
FIGURE 7. *Polykrikos hartmanni* Zimmermann
FIGURE 8. *Warnowia parva* (Lohmann) Lindemann
FIGURES 9, 10. *Nematodinium armatum* (Dogiel) Lebour

zoidal, more sloping dorsally than ventrally. Hypocone similar to epicone in shape in dorsi-ventral view, with antapical notch dorsally; in lateral view sub-hemispherical, wider than tall. Girdle narrow, displaced 0.16–0.20 body length. Sulcus narrow, straight except for slight leftward divergence in girdle region, forming a deep hypoconal excavation with anterior limit marked laterally by a vague line running diagonally from posterior dorsal corner toward girdle region on ventral surface. Anterior flagellar chamber a long finger-like pocket extending posteriorly and somewhat dorsally; posterior flagellar chamber a narrow prolongation of hypoconal excavation, reaching almost to anterior chamber. Anterior flagellum encircling less than half the circumference, wide, band-shaped; posterior flagellum body length, sometimes double.

Chromatophores absent. Nucleus within epicone, containing elongate chromatin corpuscles. Peripheral cytoplasm with distinct radial structure. Assimilate occasionally abundant.

Woods Hole area: Salt Pond; October, December, January.

This species is very much like *Gyrodinium uncatenum* in this paper, differing principally in lacking chromatophores, in having a less displaced girdle, and a straighter sulcus. Among hitherto described species, it is similar only to *Gymnodinium bifurcatum* (Kofoid and Swezy, 1921) in its lateral flattening and deep hypoconal excavation.

Gymnodinium striatissimum n. sp.

(Plate 3, Figures 5, 6)

Body globular to elliptical, not flattened, its hypocone slightly larger than epicone. Length 29–43 μ, width 23–31 μ. Epicone conical, pointed, or somewhat truncate. Hypocone similar but more truncate, varying from tapered type with moderately sloping sides and rounded antapex to type with slightly sloping sides and broad antapex. Surface with striations, fewer on epicone (15) than on hypocone (25). Girdle narrow, rather deep, slightly displaced, with distinctive posterior flexure to end of left limb. Sulcus shallow, narrow on epicone, somewhat wider on hypocone, from near apex almost to antapex, distinguished by sharp leftward bend just anterior to girdle. Anterior flagellar chamber prolonged posteriorly. Anterior flagellum completely encircling body; posterior flagellum one body length, sometimes double.

No chromatophores. Nucleus posterior, wholly within hypocone, with elongate chromatin corpuscles. Assimilate bodies of various sizes often abundant.

Woods Hole area: Great Pond; May.

Gymnodinium striatissimum is similar in different number of striations on epicone and hypocone to the much larger *G. multistriatum, G. rubrum,* and *G. translucens* (Kofoid and Swezy, 1921).

MASSARTIA Conrad

Key to Species

Without striations:
- Chromatophores present ..... *M. rotundata*
- Chromatophores absent ..... *M. asymmetrica*

With striations ..... *M. glauca*

Massartia rotundata (Lohmann) Schiller

(Plate 1, Figures 5, 6, 7, 8)

Amphidinium rotundatum Lohmann, 1908, p. 261, pl. 17, fig. 9.
Amphidinium rotundatum Lohmann in Wulff, 1916, p. 103, pl. 2, fig. 11.
Amphidinium rotundatum Lohmann in Van Goor, 1925, p. 285, fig. 4.
Gymnodinium minutum Lebour, 1925, p. 45, pl. 5, fig. 4.
Massartia rotundata (Lohmann) Schiller in Conrad, 1939, p. 11, figs. 17–22.

Body top-shaped, circular in cross-section. Length 8–17 μ, width 6–12 μ. Epicone 2.5 times as long as hypocone, conical, with straight to gently convex sides, its apex pointed or somewhat rounded. Hypocone half as long as wide, broadly rounded, in lateral view asymmetric so that the dorsal portion is larger than the ventral one. Girdle very wide, very slightly displaced, its anterior margin overhanging and of greater diameter than the posterior margin. Sulcus not discernible. Flagellar chambers absent. Anterior flagellum up to twice the girdle circumference in length; posterior flagellum equal in length to the cell body.

Chromatophores two, yellow-brown; one band-shaped, partially encircling periphery of epicone; the other filling bottom of hypocone, extending on ventral surface to epicone. Nucleus not seen. Assimilate bodies present or absent. Pellicle occasionally present.

Woods Hole area: Great Pond, Falmouth Harbor; January to April, August, September. Barents Sea; White Sea; Baltic off Kiel; brackish estuaries near Nieuport (Belgium) and along the coast of Holland; Plymouth Sound; Adriatic Sea.

Several different populations were studied. One had straight sides to the epicone and a pointed apex. Another had convex sides to the epicone and a pointed apex. A third was rather variable, rotund in appearance, often almost colorless, with apex rounded or pointed. In a similar way this distinctive species shows considerable variation as it receives treatment from various investigators. Lohmann describes it with relatively very small hypocone, whereas Wulff describes it with relatively very much larger one. In contrast to these, which have pointed apices, Conrad's has a rounded apex. Van Goor shows a very slender form. Lohmann's and Wulff's figures show a lobed epiconal chromatophore, whereas Lebour's shows a band-shaped chromatophore.

Massartia asymmetrica (Massart) Schiller

(Plate 1, Figures 10, 14)

Gymnodinium asymmetricum Massart, 1920, p. 132, figs. 22A–D.
Massartia asymmetrica (Massart) Schiller in Carter, 1937, p. 59, pl. 8, figs. 17–18.

Body globular, oval in outline, compressed dorsi-ventrally. Length 14–22 μ, width 13–20 μ. Epicone 0.66 body length, hemispherical, with small apical notch. Hypocone small, 0.33 body length, twice as wide as long, broadly rounded, often with slight oblique flattening in antapical region. Girdle very wide, shallow, displaced one girdle width, its anterior margin wider than posterior. Sulcus extending from girdle to antapex, widening abruptly during its course. Anterior

flagellum incompletely encircling body; posterior flagellum 1.0–1.5 body lengths.

Chromatophores absent. Nucleus almost wholly within hypocone, wider than long, containing relatively few, short, unoriented chromatin corpuscles. Large, brown ingested body and smaller assimilate bodies frequent.

Woods Hole area: Great Pond; October, January, February. Isle of Wight, England; estuary near Nieuport, Belgium.

Massartia asymmetrica is similar to *M. vorticella* (Stein) Schiller and *M. stigmaticum* (Lindemann) Schiller, which, however, contain stigmas, and to *M. glandula* Herdman, which differs, however, in its larger size (20–35 μ long) and helmet-shaped instead of hemispherical epicone. Massart's and Carter's figures of *M. asymmetrica* show somewhat greater displacement of girdle than in the specimens described here.

Massartia glauca (Lebour) Schiller

Spirodinium glaucum Lebour, 1917, p. 196, fig. 13.
Gyrodinium glaucum (Lebour) Kofoid and Swezy, 1921, p. 308, pl. 9, fig. 94, text fig. DD, 16.
Gyrodinium glaucum (Lebour) Kofoid and Swezy in Lebour, 1925, p. 54, pl. 7, fig. 4, text fig. 15.

Woods Hole area: Great Pond; October, May. Plymouth Sound; Adriatic Sea; La Jolla, California.

The specimens studied agreed closely in shape with those of Lebour and were not like Kofoid and Swezy's somewhat different form. They were, however, considerably smaller than Lebour's, 28–32 μ instead of 40–56 μ in length. This form is different from other species of *Massartia* in its striations; it is distinguished by its slender form, the slight twist to the apex, and its absence of chromatophores.

GYRODINIUM Kofoid and Swezy

Key to Species

Without striations:
- Chromatophores present:
  - Chromatophores 2 to 4 ..... *G. estuariale*
  - Chromatophores many:
    - Body dorsi-ventrally flattened:
      - Sulcus not deflected on epicone ..... *G. aureolum*
      - Sulcus deflected to right on epicone ..... *G. resplendens*
    - Body laterally flattened ..... *G. uncatenum*
- Chromatophores absent:
  - Sulcus sigmoid:
    - Epicone rounded-conical ..... *G. metum*
    - Epicone hemispherical ..... *G. glaebum*
  - Sulcus bisigmoid ..... *G. undulans*

With striations:
- Length 18–43 μ ..... *G. dominans*
- Length 66–96 μ ..... *G. spirale*

Gyrodinium **estuariale** n. sp.

(Plate 1, Figures 15, 16)

Body ellipsoid; apex often somewhat pointed compared to broadly rounded antapex; often with slight asymmetry, the right side more convex than the left; slightly flattened dorsi-ventrally; with equal epicone and hypocone. Length 11–16 μ, width 9–12 μ. Epicone broadly conical to sub-hemispherical, the right margin often a bit more sloping than the left; hypocone hemispherical to somewhat trapezoidal, with rounded to flattened, oblique, sometimes indented antapex. Girdle deep, moderately wide, displaced 0.25–0.33 body length, strongly posteriorly bending in right limb. Sulcus slight on epicone, markedly deflected to right in intercingular area, widening, and proceeding straight to antapex on hypocone. Anterior flagellar chamber an elongate pocket diverging to right and running beneath posterior flagellar chamber which is a leftward underhollowing of sulcus between girdle ends. Anterior flagellum encircling body completely; posterior flagellum body length.

Chromatophores yellow-brown, one or two in epicone, one or two in hypocone, distinctively inset from periphery. Nucleus not seen. Assimilate bodies usually sparse.

Woods Hole area: Great Pond, Salt Pond, Uncatena Island; July, August, October to January.

Gyrodinium estuariale is very similar to *Gymnodinium vitiligo* and *Gymnodinium veneficum* (Ballantine, 1956), differing in greater displacement of girdle ends, in wider, deeper girdle and sulcus, and in an oblique, instead of symmetrically rounded, antapex. In the intercingular region the sulcus is deflected to the right (passing from anterior to posterior end) in these species, contrary to most gyrodinia. *Gyrodinium estuariale* is similar to *Gymnodinium marylandicum* (Thompson, 1947); but the latter's sulcus follows the longitudinal axis or is deflected slightly to the left.

Gyrodinium **aureolum** n. sp.

(Plate 2, Figures 8, 9)

Body essentially globular, its dorsi-ventral outline either somewhat ellipsoidal or somewhat fusiform, slightly dorsi-ventrally flattened, with subequal epicone and hypocone. Length 27–34 μ, width 17–32 μ. Epicone hemispherical to broadly conical, sometimes slightly truncate. Hypocone similar, but usually distinctly truncate, with antapex faintly indented at times. Girdle wide, moderately deep, displaced 0.20 body length. Sulcus reaching from just behind apex all the way to antapex, with slight, left deflection in girdle region, rather narrow on epicone, wide on hypocone. Anterior flagellar chamber a posteriorly pointed, finger-shaped cavity; posterior flagellar chamber an underhollowing of left sulcal margin opposite right girdle limb. Anterior flagellum completely encircling body; posterior flagellum very long, up to two body lengths.

Numerous yellow-brown chromatophores present, elliptical in shape, usually arranged in a somewhat radiating manner. Nucleus spherical or wider than long, with elongate chromatin corpuscles.

Woods Hole area: Great Pond, Uncatena Island, Falmouth Harbor; December to April.

This species is rather similar to *Gyrodinium aureum* (Conrad, 1926). It is, however, different in its less elongate chromatophores, wider grooves, greater width for the same length, more conical outline of epicone and hypocone, and somewhat less displacement of girdle ends.

Gyrodinium resplendens n. sp.

(Plate 2, Figures 6, 7)

Body broadly fusoid, with truncate apex and antapex, moderately flattened dorsi-ventrally. Length 36–62 μ, width 32–48 μ. Epicone and hypocone similar, equal, trapezoidal in outline, their sides convex, straight, or concave; the apex somewhat rounded, the antapex with sulcal indentation. Girdle deep, moderately wide, displaced 0.20–0.25 body length. Sulcus extending onto epicone as very narrow superficial groove, diverging to right; in intercingular region narrow, vertical or left deflected; on hypocone running straight to antapex, superficially narrow but broad beneath projecting lappet of left margin. Anterior and posterior flagellar chambers elongate pockets projecting toward but not reaching each other. Anterior flagellum completely encircling body; posterior flagellum one body length.

Chromatophores oval, rich brown, radiating, many-tiered. Nucleus somewhat anterior of center, wider than long, with numerous, elongate chromatin corpuscles. Ingested bodies occasional; assimilate absent.

Woods Hole area: Great Pond; July, August.

This species is close to *Gyrodinium aureolum.* It is also quite like *Gymnodinium nelsoni,* differing in greater girdle displacement (so that it falls into the genus *Gyrodinium*), less dorsi-ventral flattening with no ventral concavity, and truncate rather than hemispherical epicone.

Gyrodinium uncatenum n. sp.

(Plate 4, Figures 1, 2, 3)

Body laterally flattened, elliptical to quadrangular in side view, elongate elliptical in ventral or dorsal view, with epicone and hypocone subequal. Length 40–54 μ, width 28–33 μ. Epicone in ventral and dorsal views helmet-shaped, taller than wide, broadly rounded at apex, its sides sloping gently, with slight concavities at girdle; in lateral view, sub-hemispherical to trapezoidal, wider than tall. Hypocone very similar but often slightly more truncate at antapex and more distinctly trapezoidal in side view. Girdle narrow, deep, displaced 0.33 body length, the right limb bending steeply posteriorly. Sulcus projecting slightly on epicone, curving to left in intercingular area, then sharply to right between right girdle end and antapex; carried across antapex all the way to dorsal side; deeply excavating hypocone, the anterior extent of excavation seen laterally as an oblique line running from dorsal end of sulcus toward ventral surface. Anterior flagellar chamber long, finger-like, projecting posteriorly and somewhat dorsally and rightward; posterior flagellar chamber a long extension of sulcal excavation, reaching nearly to anterior chamber. Anterior flagellum wide and strap-shaped, completely en-

circling body; posterior flagellum occasionally double, twice the body length; both flagella reaching all the way to the end of the chambers.

Chromatophores elongate, yellow-brown, radiating from center, leaving marginal area clear (lateral view). Nucleus spherical, in the epicone, with slightly elongate chromatin corpuscles. Crytoplasm showing vague radiating structure in peripheral region. Countless, dark assimilate bodies often present.

Woods Hole area: Great Pond, Uncatena Island; July, August, October.

This species is rendered distinctive by its lateral flattening, a rarity among gyrodinia. Very striking is the deep excavation in the hypocone, identical to that in *Gymnodinium stellatum* and *G. bifurcatum* (Kofoid and Swezy, 1921).

Gyrodinium **metum** n. sp.

(Plate 1, Figures 11, 12)

Body somewhat asymmetric, the right side more convex than the left, circular in cross-section, with hypocone slightly larger than epicone. Length 14.5–22 μ, width 11–16 μ. Epicone rounded-conical in outline; hypocone truncate with straight to convex sides. Girdle deeply excavated, displaced about 0.20–0.25 body length, its right limb curving steeply posteriorly. Sulcus sigmoid, not extending onto epicone, narrow in intercingular region, and wide and deep on hypocone, flattening or indenting the antapex. Anterior flagellar chamber produced inward and posteriorly; posterior flageller chamber a mere excavation in the sulcal floor. Anterior flagellum completely encircling the body; posterior flagellum 1.5 times body length.

Chromatophores absent. Cytoplasm gray, foamy in texture. Nucleus not seen.

Woods Hole area: Great Pond; May, June, July, December, February.

This species is distinguished by the Chinaman-hat shape of the epicone. A smaller-size variant was often seen. Its features are identical except respecting size—9.5–12 $\mu \times$ 9–7 μ.

Gyrodinium **glaebum** n. sp.

(Plate 1, Figures 17, 18)

Body elliptical, very slightly compressed dorsi-ventrally, with equal epicone and hypocone. Length 17–25 μ, width 12–19 μ. Epicone hemispherical but slightly asymmetric, with right contour more sloping or less fully curved than left. Hypocone similar, but broader, the asymmetry more marked, the left contour more sloping or less fully curved than right, often with oblique flattening in region of sulcus end. Girdle wide, rather deep, displaced two-three times its own width. Sulcus extending slightly onto epicone, narrow and leftward diverging in intercingular region, wide and deep in a straight course on hypocone. The two flagellar chambers overlapping each other, the anterior one a pronounced, posteriorly directed excavation, the posterior one an underhollowing of left girdle margin. Anterior flagellum only partially encircling body, posterior flagellum as long as body.

Chromatophores absent. Nucleus somewhat anterior of girdle, with large and relatively few chromatin corpuscles. Large, brown ingested bodies often present, as well as small, refractive assimilate bodies.

Woods Hole area: Great Pond; July, October.

Gymnodinium variabile (Herdman, 1924a) is close in shape but lacks any marked girdle displacement.

Gyrodinium undulans n. sp.

(Plate 3, Figures 7, 8, 9)

Body elliptical, somewhat dorsi-ventrally flattened with equal epicone and hypocone. Length 27–38 μ, width 21–31 μ. Epicone varying from sub-hemispherical to truncate-pyramidal, with rounded apex and straight but sloping sides. Hypocone asymmetric, truncate-pyramidal with rounded to flattened, oblique antapex, and sloping sides. Girdle wide, deep, displaced 0.20 body length, its right limb curving strongly posteriorly. Sulcus distinctive, forming a bi-sigmoid curve; very narrow on epicone, swerving leftward and then rightward to meet left girdle end, curving again to left in intercingular area and widening, then bending to right on hypocone to form large overlapping lobe, finally returning leftward to antapex. Floor of sulcus on hypocone extending straight to antapex; and right margin of sulcus "bearing away" laterally to form a swelling. Anterior and posterior flagellar chambers projecting toward, but not reaching, each other. Anterior flagellum completely encircling body; posterior flagellum one body length, occasionally double.

Chromatophores absent. Nucleus large, principally within epicone, of elongate chromatin corpuscles. Assimilate bodies sometimes in form of large blocks.

Woods Hole area: Great Pond; February, January.

Few Dinophyceae have either bi-sigmoid sulci or overlapping sulcal lobes.

Gyrodinium dominans n. sp.

(Plate 3, Figures 1, 2, 3)

Body broadly fusiform, circular in cross-section, epicone and hypocone subequal. Length 18.5–43 μ, width 10–22 μ. Epicone and hypocone conical to rotund-conical, their sides varying from convex to straight. Both epicone and hypocone occasionally with slight concavities near girdle. Surface with continuous striations, the number the same on epicone and hypocone, between 7 and 10 across ventral face. Girdle of moderate depth and width, displaced 0.25–0.33 body length. Sulcus sigmoid, deflected to left 0.25 transdiameter in intercingular region, reaching halfway up epicone, extending to posterior margin of hypocone on left side of antapex. Anterior flagellar chamber a posteriorly directed, finger-like projection from left end of girdle. Posterior flagellum inserted at posterior end of girdle (flagellar chamber not seen). Anterior flagellum not completely encircling body; posterior flagellum short, 0.50 body length.

Chromatophores absent. Nucleus anterior, in epicone, containing elongate, oriented chromatin corpuscles.

Woods Hole area: Great Pond, Falmouth Harbor, Salt Pond; April, July, August, October to December.

Gyrodinium dominans is an ally to three very similar species of *Gyrodinium: G. pingue* (Schütt, 1895, as *Gymnodinium spirale* var. *pinguis;* Wulff, 1916, as

Spirodinium varians; Kofoid and Swezy, 1921; Lebour, 1925), *G. obtusum* (Schütt, 1895, as *Gymnodinium spirale* var. *obtusa;* Kofoid and Swezy, 1921; Lebour, 1925), and *G. fissum* (Kofoid and Swezy, 1921). The chief distinction is the flexure of the sulcus in *G. dominans,* contrasting with the comparative straightness of the sulcus in the other three.

Gyrodinium spirale (Bergh) Kofoid and Swezy

(Plate 3, Figure 4)

Gyrodinium spirale (Bergh) Kofoid and Swezy, 1921, p. 332, pl. 4, fig. 43, text fig. DD, 14.

Gyrodinium spirale (Bergh) Kofoid and Swezy in Lebour, 1925, p. 56, pl. 8, fig. 1.

Body slender fusiform; circular in cross-section; somewhat twisted on its longitudinal axis; with epicone longer but narrower than hypocone. Length 66–96 μ, width 30–38 μ. Epicone narrowly conical, convex or straight along left and dorsal contours. Hypocone with sides parallel anteriorly, convex posteriorly; its antapex pointed, excentric, to right of ventral, longitudinal axis; with sulcal notch to left of antapex. Surface with continuous striations, 8–13 on epicone, 15–20 on hypocone. Girdle narrow, rather shallow, displaced more than 0.33 the body length, its right limb curving strongly posteriorly. Sulcus very narrow, from somewhat behind apex to left girdle end as a heavy line, a very narrow but distinct groove between girdle ends, widening and deepening from right girdle end to antapical notch. Anterior flagellum inserted at opening of a pustule, which is composed of a short, sac-shaped portion, extending posteriorly, and of a long, thread-like portion, extending somewhat anteriorly, then laterally, finally posteriorly along right contour to region of right girdle limb. Posterior flagellum likewise inserted at opening of a pustule having a long, thread-like extension anteriorly and leftward to region of left girdle limb. Anterior flagellum following girdle 0.33 or less of girdle length. Posterior flagellum short, about 0.25 of body length.

Chromatophores absent. Nucleus elongate-ellipsoidal on left side (ventral view), in intercingular area, without apparent structure.

Woods Hole area: Great Pond, Falmouth Harbor; December, April, May, August. Baltic Sea; Norway; Port Erin, Ireland; Plymouth Sound; Adriatic Sea; La Jolla, California; Indian Ocean; coast of Australia.

The organism defined here agrees closely with those described by Kofoid and Swezy and by Lebour. Distinctive characteristics are the slender form, twist of body, greater dorsal than ventral curvature, and a longer epicone than hypocone.

WARNOWIA Lindemann

Warnowia parva (Lohmann) Lindemann

(Plate 4, Figure 8)

Pouchetia parva Lohmann, 1908, p. 264, pl. 17, fig. 23.

Body elliptical, circular in cross-section, slightly tapered posteriorly, epicone somewhat larger than hypocone. Length 22.5–30 μ, width 15–18 μ. Epicone

hemispherical, hypocone similar but narrowed toward antapex and with sulcal indentation along left contour. Girdle shallow but very wide, displaced 0.50 the body length, with strongly descending right limb. Sulcus narrower than girdle, extending in sigmoid path from apex to antapex, bending from right margin toward center anterior to girdle, diagonal across ventral face between girdle ends, recurving from left margin toward center posterior to girdle. Anterior flagellar chamber an elongate pocket from left end of girdle limb, the posterior flagellar chamber not seen. Anterior flagellum not encircling body completely; posterior flagellum short, 0.33 body length.

Yellow bodies, resembling chromatophores in color and peripheral position but unlike in large size and irregular shape, sparse anteriorly but abundant posteriorly. Nucleus anterior, with elongate, oriented corpuscles. Melanosome near antapex, black, diffuse and spreading, or ellipsoidal and globular. Lens simple without evident laminations, projecting anteriorly on left side from melanosome. Nematocysts absent. Cell often within a pellice. Assimilate bodies frequent.

Woods Hole area: Great Pond; July. Baltic off Kiel.

The organism studied here is probably a close match to Lohmann's *Pouchetia parva,* but Lohmann showed no girdle and sulcus. It is close to *Nematodinium armatum,* but the tapered hypocone, large irregular chromatophores, absence of nematocysts, and cyst differentiate it, as well as the characteristics mentioned under *N. armatum.*

NEMATODINIUM Kofoid and Swezy

Nematodinium armatum (Dogiel) Lebour

(Plate 4, Figures 9, 10)

Pouchetia armata Dogiel, 1906, p. 36, pl. 2, figs. 48–49.
Nematodinium armatum (Dogiel) Lebour, 1925, p. 71, pl. 10, figs. 5a–5b
Nematodinium armatum (Dogiel) Lebour in Martin, 1929, p. 19, pl. 2, figs. 5–7.

Body elliptical, with equal epicone and hypocone. Length 33–53 μ, width 20–33 μ. Epicone evenly rounded, slightly asymmetric in ventral view, its right margin more sloping than the left; hypocone also slightly asymmetric with more sloping left than right side, the antapex either somewhat pointed and off-center, or, usually, obliquely truncate. Girdle deep, moderately wide, displaced 0.33 the body length, with strongly descending right limb. Sulcus extending from near apex in a sigmoid path to antapex, widening on the oblique margin of antapex, a portion of it curving sharply to the right, delimiting a knob-like protuberance on ventral face of the hypocone. Anterior flagellar chamber an elongate pocket from left end of girdle limb (posterior chamber not seen). Posterior flagellum one body length (anterior flagellum not fully studied).

Chromatophores yellow, circular or subcircular, few, and scattered. Nucleus large, anterior, of elongate chromatin corpuscles. Melanosome near antapex, black, diffuse and spreading, or ellipsoidal and globular. Lens single with concentric laminations, projecting anteriorly on left side from melanosome. Nematocysts present or absent, in region of right girdle limb.

Woods Hole area: Great Pond; August. Barnegat Bay, New Jersey; Plymouth Sound; Naples.

Some of the specimens were of full, ellipsoidal proportions, as in Dogiel's figure. Some were more slender, though not quite so slender as in Lebour's Figure 5a. Some had a slight tapering of hypocone and pointed antapex as in Martin's figures. They are all intermediate between the smaller *Warnowia parva* (Lohmann, 1908) without rightward curvature of sulcus on hypocone and with comparatively wider, shallower furrows, and the larger *Nematodinium lebourae* (Schiller, 1933; Kofoid and Swezy, 1921, as *N. armatum*), with rightward curvature of sulcus on hypocone and with comparatively greater transverse displacement of the sulcus.

POLYKRIKOS Bütschli

Key to Species

Chromatophores present:
- Body cylindrical ..... *P. hartmanni*
- Body laterally flattened ..... *P. lebourae*

Chromatophores absent ..... *P. schwartzi*

Polykrikos hartmanni Zimmermann

(Plate 4, Figure 7)

Polykrikos hartmanni Zimmermann, 1930, p. 436, figs. 8–9.

Colony cylindrical in form with rounded ends, consisting of two zooids, delimited by a slight constriction. Length 60–68 μ, width 42–47 μ. Epicone often smaller than hypocone in anterior zooid but equal to hypocone in posterior zooid. Girdles wide, rather shallow, displaced twice their width. Sulcus continuous from apex to antapex, roughly straight, narrowed at the constriction between zooids and in intercingular regions. Sulcus produced inward as anterior and posterior flagellar chambers, which extend posteriorly and anteriorly, respectively, their diverging ends overlapping. Anterior flagella incompletely encircling zooids; posterior flagella about 0.66 as long as the colony.

Chromatophores circular, small, numerous, yellow-brown in color. Nuclei always two, with elongate chromatin corpuscles. Several long nematocysts often, but not always, present in region just below anterior girdle. Innumerable black granules may fill peripheral cytoplasm of whole colony or may be restricted to posterior end.

Woods Hole area: Great Pond; August. Adriatic.

Slight differences between our specimens and Zimmermann's are the smaller size of ours (Zimmermann's 80–120 $\mu \times$ 55–75 μ) and the yellow-brown instead of yellow-green chromatophores. *Polykrikos barnegatensis* (Martin, 1929), also composed of two cells, is close to *P. hartmanni* but has a single nucleus and a more elliptical outline.

Polykrikos schwartzi Bütschli

Polykrikos schwartzi Bütschli in Kofoid and Swezy, 1921, p. 400, text fig. F, 4.
Polykrikos schwartzi Bütschli in Lebour, 1925, p. 67, pl. 10, figs. 2a–2b, text fig. 16c.

Woods Hole area: Great Pond; August. Arctic near Iceland; off coast of Norway; Skagerack; Baltic off coast of Denmark; Baltic off Kiel; North Sea off

Helgoland; Plymouth Sound; Atlantic off Concarneau, France; Mediterranean off French coast.

This species is distinguished by its many cells (zooids), averaging about eight, with four nuclei; by its cylindrical form; and by the absence of chromatophores.

Polykrikos lebourae E. C. Herdman

Polykrikos lebourae E. C. Herdman, 1924b, p. 60, fig. 6.
Polykrikos lebourae E. C. Herdman in Lebour, 1925, p. 68, pl. 10, fig. 3.

Woods Hole area: Salt Pond, beach sand; November. Port Erin, Ireland, in sand.

This species is quite distinctive in its lateral flattening. It has eight cells, two nuclei, and yellow-brown chromatophores. It is recorded from beach sand at Woods Hole by E. C. Herdman.

Diagnoses of New Species

Amphidinium **carteri** n. sp.

Corpus dorsali-ventraliter compressum, a fronte visum ovale; epicono minuto, asymmetrico et rostroformi; hypocono truncato-elliptico; sulco prope marginem dextrum, leniter curvato; chromatophoro uno, parietali, perforato, fulvo. Longitudo 12–15 μ, latitudo 8–9 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Amphidinium **wislouchi** n. sp.

Corpus simile *Amphidinio carteri* sed paullo majus; chromatophoris multis, ellipticis, paululum radiatim ordinatis. Longitudo 20–25 μ, latitudo 14–16.3 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gymnodinium **lazulum** n. sp.

Corpus globosum; epicono conico vel campaniformi; hypocono hemispherico vel campaniformi; sulco tenui; corpore sine striis; chromatophoris absentibus; cytoplasmate pellucido, subcaeruleo, saepe cum multis corporibus multi-coloratis. Longitudo 28–34 μ, latitudo 30–32 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gymnodinium **stellatum** n. sp.

Corpus lateraliter compressum; epicono quadrangulato et hypocono hemispherico; extremis cinguli 0.17 longitudinis corporis transpositis; sulco a vicinitate apicis ad cavernam profundam hypoconi extendente; corpore sine striis; chromatophoris absentibus; structura cytoplasmatis exterioris perspicue radiata. Longitudo 25–47 μ, latitudo 22–39 μ, crassitudo 13–25 μ. United States, in loco dicto Salt Pond, Barnstable County, Massachusetts.

Gymnodinium **striatissimum** n. sp.

Corpus globosum vel ellipticum; epicono et hypocono conico, aculeato, vel truncato; cingulo angusto, flexuram posterioram extremi sinistri habente; sulco a vicinitate apicis ad antapicem extendente; corpore cum striis, in hypocono pluribus quam in epicono ornato; chromatophoris absentibus. Longitudo 29–43 μ, latitudo 23–41 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **estuariale** n. sp.

Corpus ellipticum, paululum asymmetricum, margine dextro convexiore quam margine sinistro; epicono late conico vel sub-hemispherico; hypocono hemispherico; extremis cinguli 0.25–0.33 longitudinis corporis transpositis; sulco ad latus dextrum inter extrema cinguli multum deflecto; corpore sine striis; chromatophoris fulvis, 2–4, paulum intra peripheriam positis. Longitudo 11–16 μ, latitudo 9–12 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **aureolum** n. sp.

Corpus globosum; epicono et hypocono hemispherico vel conico; extremis cinguli 0.33 longitudinis corporis transpositis; sulco a vicinitate apicis ad antapicem extendente; corpore sine striis; chromatophoris multis, fulvis, plerumque omnibus plus minusve radiatim ordinatis. Longitudo 27–34 μ, latitudo 17–32 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **resplendens** n. sp.

Corpus late fusiforme, apice et antapice truncato, dorsaliventraliter compressum; extremis cinguli 0.20–0.25 longitudinis corporis transpositis; sulco in epicono augusto, tenui; ad latus dextrum curvato, in hypocono profundo et recto; corpore sine striis; chromatophoris multis, fulvis, radiatim ordinatis. Longitudo 36–62 μ, latitudo 32–48 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **uncatenum** n. sp.

Corpus lateraliter compressum, a late visum ellipticum vel quadrangulum; extremis cinguli 0.33 longitudinis corporis transpositis, parte dextra ad antapicem multum curvata; sulco in epicono tenui, ad sinistram prope extremitatem posteriorem cinguli deflecto; hypocono excavationem profundam antapicis praehente; corpore sine striis; chromatophoris luteo-fuscis, elongatis, radiatim ordinatis; cytoplasmate circa peripheriam structuram radiatam habente. Longitudo 40–54 μ, latitudo 28–33 μ. United States, in loco dicto Uncatena Island, Barnstable County, Massachusetts.

Gyrodinium **metum** n. sp.

Corpus paulum asymmetricum, latere dextro convexiore quam latere sinistro; epicono conico, multo latiore quam hypocono; hypocono truncato, multo longiore quam epicono; cingulo profundo, extremis cinguli 0.20–0.25 longitudinis corporis

transpositis; sulco S-curvato, in epicono absente, in hypocono lato; corpore sine striae; chromatophoris absentibus. Longitudo 14.5–2 μ, latitudo 11–16 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **glaebum** n. sp.

Corpus ellipticum, paulum asymmetricum; epicono hemispherico, hypocono late hemispherico; cingulo lato, extremis cinguli 2–3 latitudinibus cinguli transpositis; sulco in epiconum vix extendente, in hypocono lato et profundo; corpore sine striis; chromatophoris absentibus. Longitudo 17–25 μ, latitudo 12–19 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **undulans** n. sp.

Corpus ellipticum; epicono subhemispherico vel truncato-pyramidali; hypocono truncato-pyramidali; extremis cinguli 0.20 longitudinis corporis; sulco in duo S-formata curvamina facto, margine sinistro in hypocono marginem dextrum superposito; corpore sine striis; chromatophoris absentibus. Longitudo 27–38 μ, latitudo 21–31 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

Gyrodinium **dominans** n. sp.

Corpus fusiforme; epicono et hypocono conico; extremis cinguli 0.25–0.33 longitudinis corporis transpositis; sulco S-curvato, in epiconum extendente, in hypocono ad marginem sinistrum prope antapicem extendente; corpore cum striis, 7–10 a ventrale viso in epicono et hypocono; chromatophoris absentibus. Longitudo 18.5–43 μ, latitudo 10–22 μ. United States, in loco dicto Great Pond, Barnstable County, Massachusetts.

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Summary

1. Unarmored Dinophyceae were collected from very shallow embayments on the south shore of Cape Cod, Massachusetts.

2. Twenty-six species, distributed in eight genera, were studied. Twelve were considered as new species and nine showed extensions of range from Europe.

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THE NATURE OF CERTAIN RED CELLS IN DROSOPHILA MELANOGASTER

JACK COLVARD JONES [1] AND E. B. LEWIS [2]

In a stock of the spineless (*ss*) mutant of *Drosophila melanogaster,* some of the flies were observed to have bright red cells under the cuticle. The presence of these pigmented cells has been found to depend upon a recessive mutant gene located at 26.0 ± in the second chromosome. The mutant has been named "red cells," symbol, *rc*. This paper is a brief account of the location, histology, and cytology of the red-pigmented cells of the *rc* mutant.

Methods

A stock homozygous for *rc* and *ss* has been used for all studies unless otherwise specified. The *ss* mutant serves merely as a marker to check on contamination of the stock and does not have any obvious effect on the expression of *rc*. Under crowded culture conditions *rc* may overlap wild type. To obtain maximum expression of the *rc* mutant, it is desirable to rear the larvae on an abundant supply of yeast. In the present work, additional dried yeast or paper towelling saturated with a thick fresh yeast suspension was added to the standard culture medium on the fourth day after introducing the parents. To study the effect of trypan blue, the *rc* mutant was grown on standard culture media containing 1.5% trypan blue.

Larvae of the third stage, pupae of various ages, and young adults of both sexes were studied. Intact larvae were immersed in 0.85% NaCl or in water, covered with a cover slip, and their various tissues examined *in situ* under high power (970 X).

Pupae and anaesthetized adults were pinned to a paraffin dish and dissected in Beadle-Ephrussi saline or in ethyl cellosolve. While a variety of fixatives were employed, cellosolve-fixed specimens were chiefly used. Fixed specimens were transferred to methyl benzoate and finally embedded in paraffin. Serial, cross, and sagittal sections were stained principally with picric acid since this stain did not interfere with the recognition of trypan blue uptake nor of red pigment distribution. Several series were stained with methylene blue. One series was stained with hematoxylin and eosin, two series with Sudan III for fat, and two series according to the Bauer test for polysaccharides.

Observations

Third-instars and early pupae of the *rc* strain, whether examined as whole mounts or as sectioned material, do not show any pigmented cells. In older pupae,

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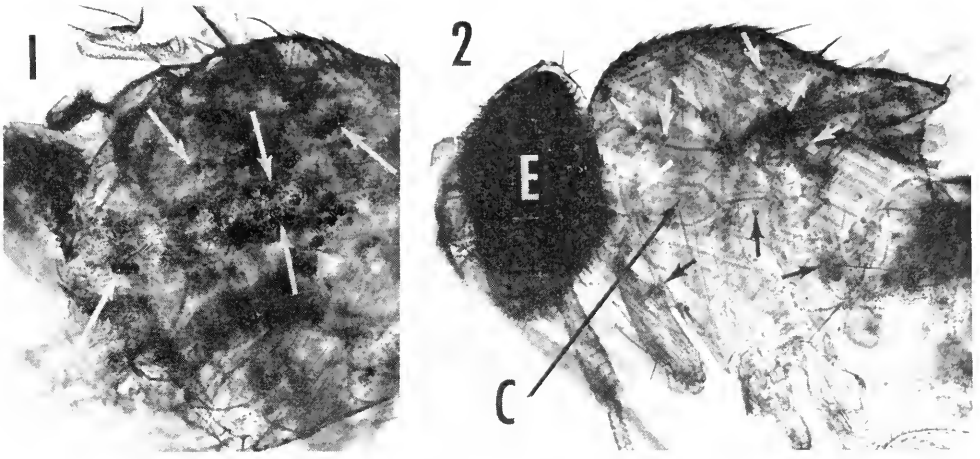

FIGURE 1. Dorsal view of thorax of adult *Drosophila rc* mutant showing alignment of red-pigmented cells. Unstained whole mount. × 87.

FIGURE 2. Lateral view of *rc* mutant showing clusters of pigmented cells. C = cardia, E = eye. Unstained whole mount. × 63.

a pale yellow substance appears in the eye and in many of the cells destined to contain red pigment granules. Red granules first appear in scattered cells in the head *before* becoming visible in the facet cells of the eye (Fig. 3). Whether yellow material is itself converted directly into red pigment has not been determined. As pigment in the eye changes from pale brown to dark brown and then to red brown, the number of red granules in the cells of the head and thorax increases from one or two to many per cell. Before the granules actually form, or when only one or two are present, these thoracic cells are conspicuous by their yellow tinge. By the time the eye pigment is formed, pigment is present in the cells of the head, thorax, and abdomen.

Living *rc* flies of both sexes show red-pigmented cells under the cuticle in the thorax and head (Figs. 1 and 2). While red-pigmented cells may be widely distributed throughout the body of young adults, they are most numerous and conspicuous in the head and thorax. In the thorax, loose aggregations occur in two longitudinal rows along the dorsal midline of the mesonotum and scutellum (Fig. 1), and there are less conspicuous groups on either side of these. Small clusters of pigmented cells are sometimes seen in the legs (coxa and trochanter) and isolated pigmented cells occur within the abdomen (Fig. 5).

The red-pigmented cells occur either singly or in definite groups of four or five or more, but they do not form syncytia (Fig. 4). When the cells are in definite locations, as in the supra-aortal masses and alongside the anterior part of the gut (Fig. 6), they are not bounded by connective tissue membranes and hence are extremely difficult to examine in fresh dissections.

The pigment granules are round, ovoid, or irregular in shape (Fig. 4) and they vary in size from less than 0.5 to about 4 microns. The number of granules per cell ranges from a few to 50 or more. The cells bearing the granules are round or ovoid (Figs. 3–6), measure about 20 microns in diameter, and have a single

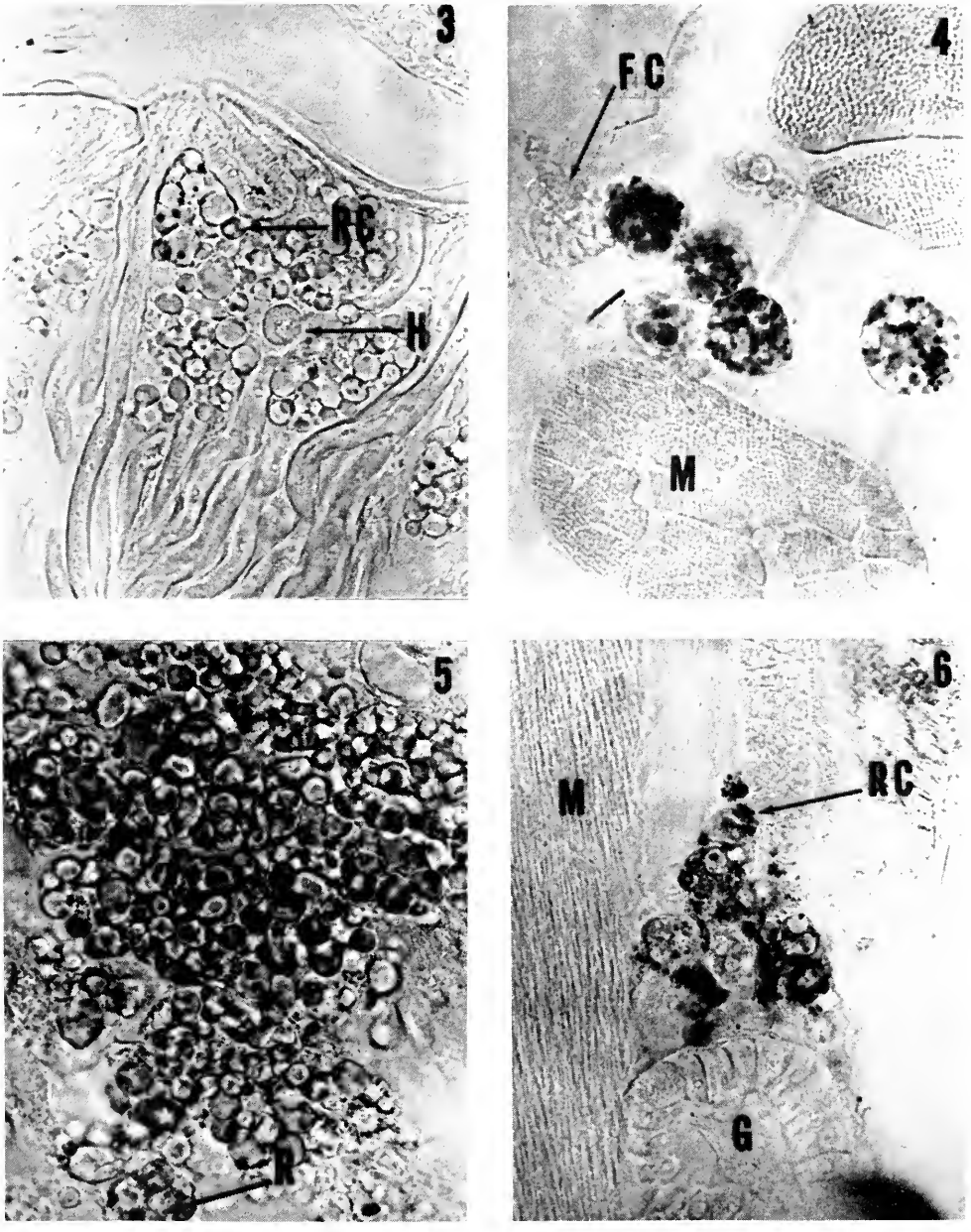

FIGURE 3. Horizontal section through head of young pupa before eye pigment has formed, showing a red pigmented fat cell (RC). Hemocyte is shown at H. Picric acid. × 940. Ref. no. 2173.

FIGURE 4. Typical red-pigmented cells in the thorax. Muscles are shown at M and an unpigmented fat cell at FC. Picric acid. × 1120. Ref. no. 2174.

FIGURE 5. Fat cells in the abdomen of an adult fly, showing a single cell with red granules at R. Picric acid. × 1120. Ref. no. 2172–5.

round nucleus. They are thus considerably larger than hemocytes which measure between 5 and 10 microns in diameter. The red cells, when examined in wet mounts, generally have large non-refringent droplets and other inclusions in their cytoplasm, in addition to pigment. Isolated pigment cells in saline do not send out lamellar extensions, and thus differ from certain kinds of hemocytes.

When larvae are reared on trypan blue media enriched with added yeast, the resulting pupae and adults have blue dye in the gut lumen, hemocytes, scattered thoracic fat cells, garland cells, and thoracic and abdominal nephrocytes. In the nephrocytes the dye appears in irregular diffuse granular masses and/or in dense aggregates. Sometimes large dye droplets are seen free in the hemocoele. Only occasionally do cells having red pigment take up the dye. Abdominal fat cells do not take up trypan blue.

Red-pigmented cells of the *rc* strain have large fat droplets (Sudan III) and smaller deposits of polysaccharide (Bauer-positive material), and are in the same size range as typical fat cells in the head and thorax. Fat cells in the abdomen of adults are distinctly larger (25–35 microns) than those in the head or thorax (14–21 microns).

Combinations of *rc* With Other Mutant Genes

The *rc* mutant was combined with mutants which affect the eye color in order to determine whether the red granules in the fat cells are related to the eye pigments. When *rc* is combined singly with mutants which remove the brown component of the eye pigment, namely, vermilion, cinnabar or scarlet, the homozygous double mutant combination has no pigment in the fat cells. When *rc* is combined with the brown mutant, which removes the red component and leaves the brown component of the eye pigment, the homozygous double mutant has typical red fat cells. It should be added that *rc* does not modify the eye color of wild-type nor of any of the above mutant types.

The *rc* mutant was combined with Microcephalus, a dominant mutant, locus 60.0 in the third chromosome which frequently results in a completely eyeless fly. Eyeless specimens thus obtained show the same degree of pigment development in the fat cells as do the cells of *rc* flies with normal eyes.

Discussion

The red-pigmented cells in *Drosophila* adults resemble typical peripheral fat body cells of the thorax and head in size, shape, general distribution, and possession of deposits of fat and polysaccharide.[3] However, they do not usually stain vitally with trypan blue, while some other typical head and thoracic fat cells without red pigment often incorporate the dye. Those relatively few red-pigmented cells present in the abdomen are usually peripheral in location and of about the same

[3] Dr. M. T. M. Rizki, who has examined our *rc* mutant, independently concludes from his observation of the red-pigmented cells that they are fat cells (personal communication).

Figure 6. Sagittal section through thorax of adult, showing clusters of cells lateral to the gut (G), some with trypan blue and others with red granules (RC). Picric acid. × 940. Ref. no. 2174–10.

size as the thoracic fat cells. Red fat cells are larger than typical hemocytes and do not send out lamellar extensions ("pseudopodia") in fresh dissections as many hemocytes do *in vitro*. None of the sessile or circulating hemocytes examined had red pigment. Indeed, so far as the authors are aware, there is no report of hemocytes with colored pigments among the insects.

Red pigment in fat cells first appears in the pupal stage at about the same time as pigment forms in the eyes. Combinations of *rc* with eye color mutants strongly suggest that the pigment in the fat cells is closely related to, or identical with, the brown eye pigment. Microscopically, brown eye pigment is red in color. That the pigment has not diffused out of the eyes and been secondarily taken up by fat cells is shown by the presence of abundant red fat cells in eyeless *rc* flies.

Summary

A mutant of *Drosophila melanogaster* possesses pigmented, stationary cells in the body cavity of the pupal and adult stages. The pigment is present as numerous red granules in the cytoplasm. By a number of criteria, the pigmented cells are a type of fat cell. The evidence suggests that the pigment is related to, or identical with, the brown component of the eye pigment and that it develops in the fat cells autonomously.

VASCULAR BUDDING, A NEW TYPE OF BUDDING IN BOTRYLLUS [1, 2]

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In the early days of the investigation on compound ascidians, it was believed that botryllids propagate either by stolonial budding alone or by stolonial *and* palleal (peribranchial) budding. It was Seeliger (1907) who, in his monumental treatise on ascidians, denied once and for all the occurrence of stolonial budding in botryllids. According to him, the supposed stolonial budding in these ascidians is palleal budding which was misinterpreted. After Seeliger, all the workers on ascidians agreed that the only budding in botryllids is palleal (*cf.* Huus, 1937; Brien, 1948; Berrill, 1950; *et al.*), except perhaps E. C. Herdman, who maintained as late as 1924 that in *Botryllus* buds are occasionally formed from the blood vessels of the test.

Since 1950 we often have had opportunities to observe that, in *Botryllus primigenus* at least, buds are formed from blood-cells gathered at the base of ampullae. It is proposed to name this type of budding "vascular" as distinct from "stolonial," for in *Botryllus* the blood vessels are generally called test vessels and not stolons, and, further, the buds are formed from blood-cells, and not from the mesenchymatous septum as in stolonial budding.

In this paper the process of vascular budding will be described in some detail and a comparison will be made between this and the ordinary palleal budding.

Historical [3]

Following are the various views concerning budding in botryllids, arranged in chronological order.

Savigny (1816), in his description of the marginal tubes (test vessels) in *Botryllus,* seems to have regarded them as an apparatus for the production of buds; this view, which was more fully elaborated and established by Milne-Edwards (1842), was generally adopted until Metschnikoff (1869) demonstrated that in *Botryllus* gemmation takes place from the sides of the ascidiozooids, *i.e.,* budding is palleal. This view of Metschnikoff was fully confirmed by later investigators such as Della Valle (1881), Oka (1892), *et al.*

Ganin (1870), however, maintained that the buds can be formed also "auf langen Stolonen und weit entfernt von dem Körper der Ascidien . . ." (p. 517).

[1] Contributions from the Shimoda Marine Biological Station, No. 92.

[2] The cost of this research has been partly defrayed from the Scientific Research Expenditure of the Department of Education of Japan.

[3] Because the wartime literature is only poorly represented in Japan, we consulted Prof. N. J. Berrill of McGill University, Montreal. He assured us in a letter that since 1940 no paper had been published on the stolonial budding in *Botryllus.* We wish to express here our thanks to Prof. Berrill for his kind information.

Giard (1872) also stated that in botryllids buds might be produced from blood vessels as well as from the body-walls of the ascidiozooids. In 1891, he still insisted that the inability of the test vessels to produce buds had not been sufficiently demonstrated.

W. A. Herdman (1886) described for *Sarcobotrylloides wyvillii* (and *Collela pedunculata*) that buds were produced intravascularly from aggregations of blood-cells. He went so far as to make the stolonial budding one of the characteristics of the family Botryllidae.

Bancroft (1903b) studied an aestivating colony of *Botrylloides gascoi* at Naples and found that many buds were formed in blood vessels apparently independently of zooids. He believed, however, that they were developed from zooids, not from blood vessels. He states (p. 151), "As no evidence in favor of an intravascular nor intra-ampullar origin of the isolated buds was detected, I feel convinced that they were developed from the zooids of the original colony before these had degenerated entirely. The buds must have severed their connections with the parent zooids, and must have been carried into the yellow lobe that was then being formed." According to the same author the buds described by Herdman (see above), too, might have been produced elsewhere and have migrated into the vessels.

Seeliger (1907) denied once and for all the occurrence of the stolonial budding in botryllids. According to him, the budding once described as stolonial is in reality typical palleal budding. As to how misinterpretation has come into existence, he says (p. 999): "Ich glaube, dass dieser Irrtum darauf zurückzuführen ist, dass die häufig in Rückbildung begriffenen Zooide, die bereits an Grösse abgenommen haben und mit den stoloähnlichen Mantelgefässen innig verwachsen sind, für neue Knospenanlagen gehalten wurden, die sich an und aus den erweiterten Gefässampullen entwickelt hätten."

E. C. Herdman (1924), however, could not reconcile herself to this view of Seeliger's and expressed herself in the following way (p. 4): "It is quite possible, however, that occasionally certain swollen knobs on the blood vessels of the test may give rise to buds." She adds, however, that "it is certainly not usual in *Botryllus* and there is no definite proof that it has ever occurred."

To sum up, it is today a well established fact that in botryllids buds are generally formed from the sides of ascidiozooids. Further, being organs for blood propulsion and respiration and perhaps also for excretion of test matrix, the ampullae are never transformed into new zooids. The problem is whether or not under certain circumstances buds are also formed from the walls of the blood vessels or the ampullae.

Materials and Methods

The following observations were made principally on living colonies of *Botryllus primigenus* Oka, occurring in the vicinity of the Shimoda Marine Biological Station, Shimoda, Japan. As is well known, the zooids in *Botryllus* are generally grouped into systems. In *Botryllus primigenus*, however, they are independent of one another, and each opens through its own atrial opening (*cf.* Oka, 1928).

To facilitate observation, colonies were fixed on glass slides. These, except at the time of observation, were set out in the bay. The technique employed for fixing the colonies was the same as that described in the paper of Oka and Usui (1944). The colonies grew well on glass slides.

Various developmental stages of the bud were observed and sketched under a binocular (magnification: $\times 72$) and an ordinary microscope (magnification: $\times 100$). Since the buds are formed from blood-cells, vital staining of these was tried with methylene blue and neutral red.

Observations on living materials were supplemented with examination of sections. In this case, strong Flemming's fluid was used as the fixative and the sections were stained with Heidenhain's haematoxylin and eosin. Staining with thionin (1% aqueous solution) was also tried.

All the observations were made at, or upon material obtained at, the Shimoda Marine Biological Station. It is our present duty to acknowledge our indebtedness to the Director and staff of that station for providing us facilities for carrying out this investigation. Thanks are also due to Miss Yoshiko Oshima who helped us in various ways.

Observations

Developmental cycle in the colony of *Botryllus*

In *Botryllus*, buds (palleal buds) appear very precociously, so that the constituting members of a colony are not single individuals, but aggregates of individuals belonging to three successive generations. They have been given the name "units" (Watanabe, 1953). Generally, a unit consists of more than three individuals. As a rule, two pairs of buds are formed by each individual, though not all of them develop. In the following text, as well as in Figure 1, each generation is represented by only one individual. An individual has a definite life-span. Its life has been divided into 11 developmental stages by Berrill (1941a).

A unit shows four different combinations of stages.

On the first day, a bud of stage 1 is seen on the lateral wall of a bud of stage 6, which in its turn is connected with a zooid of stage 9 by means of the connecting

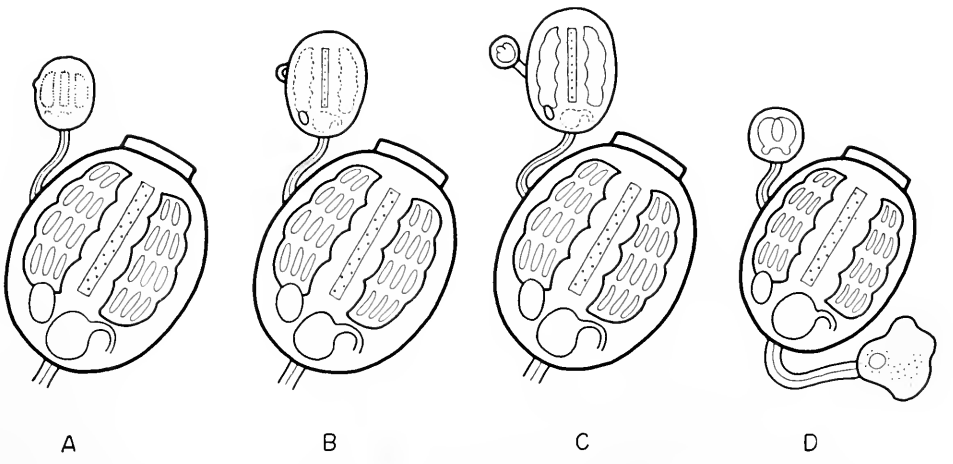


Figure 1. Developmental cycle of the unit in the colony of *Botryllus* (semi-diagrammatic). A, Phase A; B, Phase B; C, Phase C; D, Phase D.

vessel, *i.e.*, the colonial circulatory system. The combination of stages is 1–6–9. This phase is called phase A (Fig. 1, A).

On the second day, the bud of stage 1 has developed into a bud of stage 3. The bud of stage 6, on the other hand, has grown into a bud of stage 8. This latter is attached to the parent zooid which is still in stage 9. The combination of stages is 3–8–9. The phase is called phase B (Fig. 1, B).

Similarly, the phase on the third day is called phase C (combination of stages: 4–8–9) (Fig. 1, C), and that on the fourth day phase D (combination of stages: 5–9–11) (Fig. 1, D).

On the fifth day, the bud of stage 5 has grown into stage 6 with a new bud of stage 1 formed upon it. It is attached to the zooid of stage 9. The zooid of stage 11 has completely disappeared. The unit thus returns to phase A and starts a new cycle.

In each unit, the four phases are regularly repeated. Since all the units in a colony are exactly coordinated, we can also speak of the phases of the colony as a whole. A colony has four successive phases, which constitute a developmental cycle.

Formation of the vascular bud and its further development

The formation of the bud is initiated by gathering of particular blood-cells (diameter *ca.* 3–4 μ; see below) under the epidermis at the base of ampullae (Fig. 6). The number of cells is about 15–20. An intensive cell division follows, and, in one hour or so, a mass of cells (diameter *ca.* 20 μ) is formed (Figs. 2, 7). Since an ampulla is about 100–110 μ in diameter, the mass occupies about one-fifth of its width.

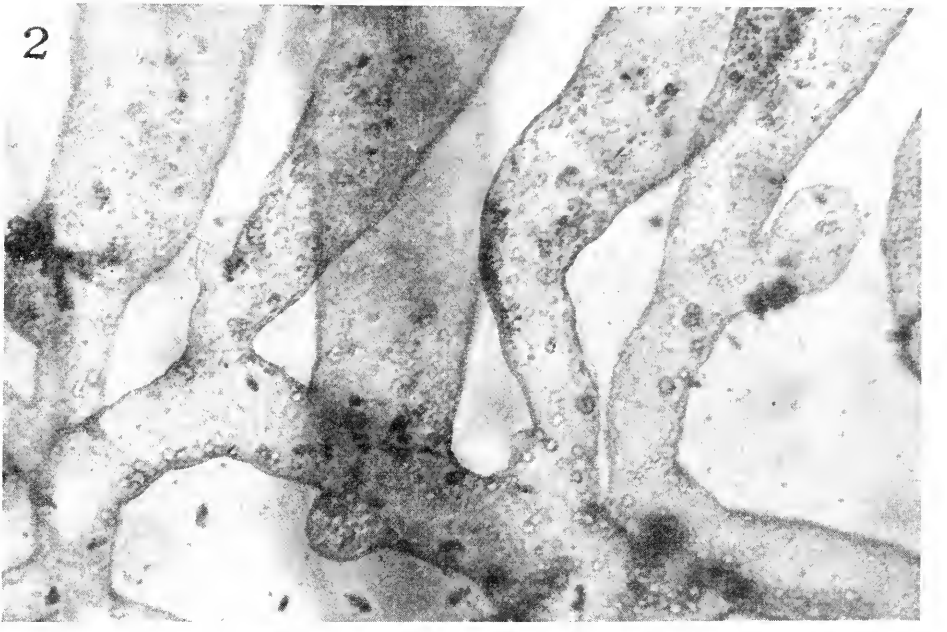
Active cell division continues, and, in two or three hours, a blastula-like structure is formed (Figs. 3, 8), At the same time, the side-wall of the ampulla that lies over the blastogenic mass begins gradually to protrude, and, in four to five hours, becomes distinctly visible as a bud (diameter 40–50 μ) (Figs. 4, 9).

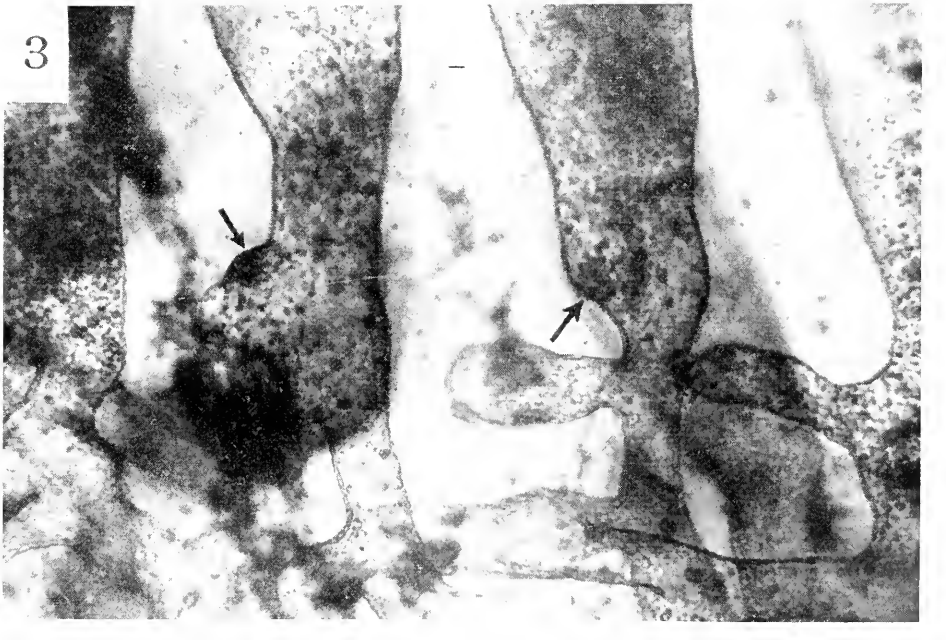
At this stage, which will be designated stage 3, the anterior wall of the inner vesicle that faces the epidermis of the ampulla is two or three cells thick, while the remaining walls are very thin. Morphologically, the vascular bud of this stage exactly corresponds to the palleal bud of stage 3 (diameter *ca.* 48 μ) except that it has no ova.

The cell layer constituting the outer vesicle or ectoderm of the bud is a direct continuation of the wall of the ampulla. The layer is at first closely applied to the inner vesicle, but later an ample space is formed between the two into which various kinds of blood-cells migrate, thus giving the impression that the inner vesicle is floating in the middle of the projecting part.

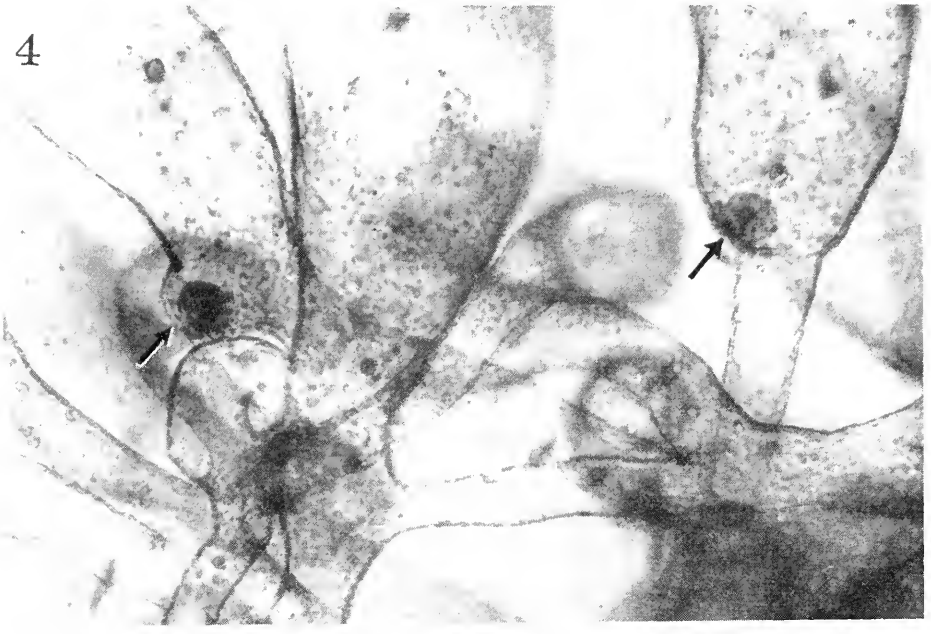
Development of the inner vesicle is as follows. As the anterior wall continues to expand, two vertical folds appear. These gradually extend backwards until they divide the vesicle into a median and two lateral chambers, which become later the central pharyngeal chamber and a pair of lateral atrial chambers. Next, three evaginations are formed, representing the heart, the neural mass and the intestine, respectively. Later development is primarily an elaboration of these unit regions.

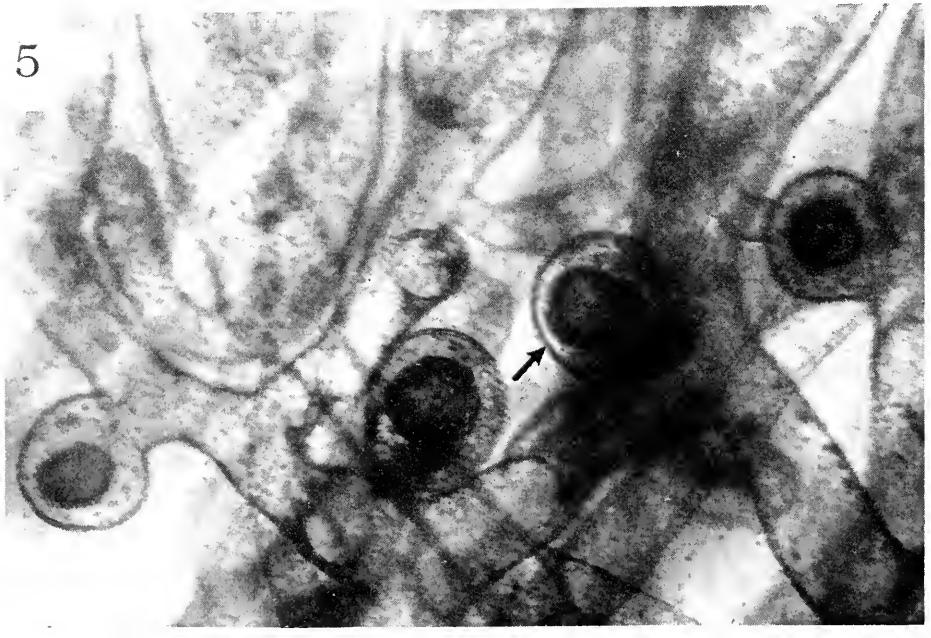
Thus, the blastogenesis in vascular buds is an exact replica of that in palleal buds.



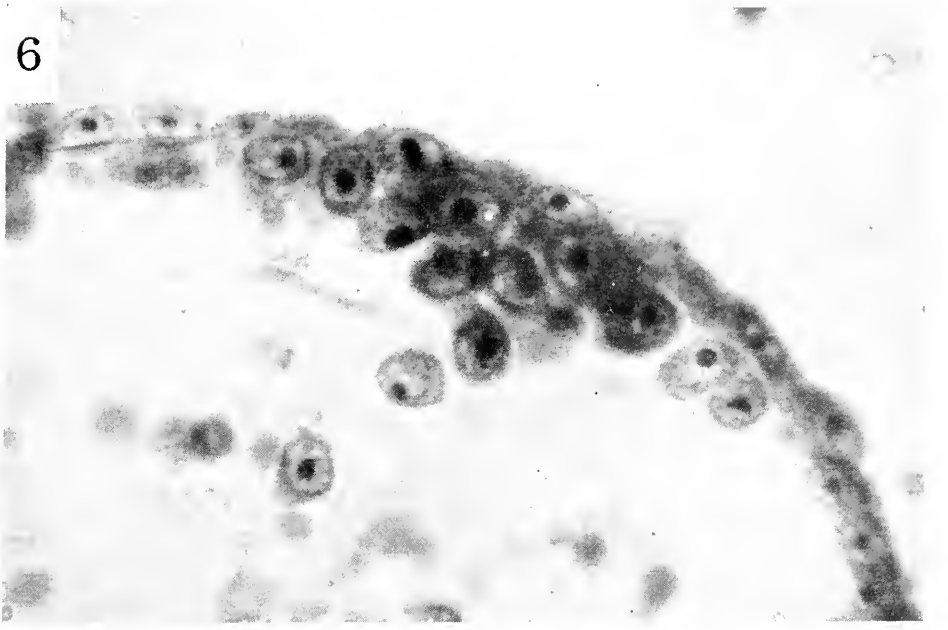


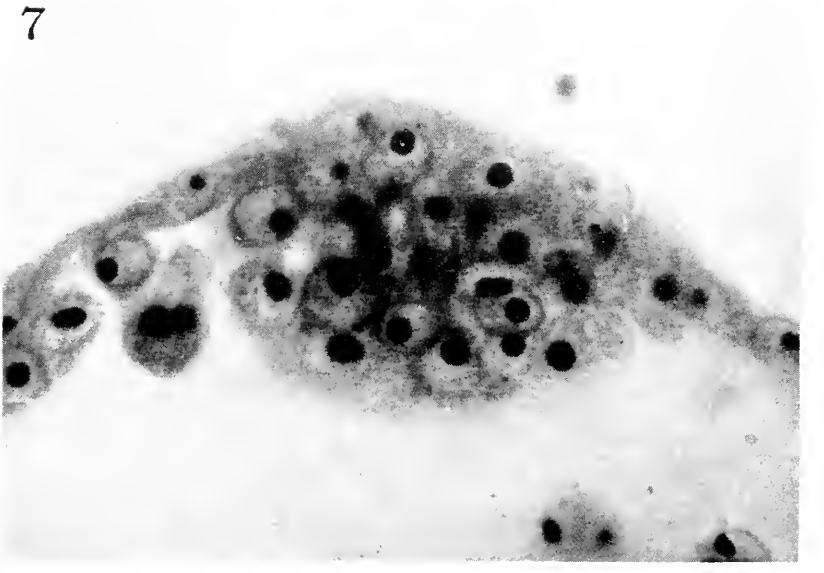
FIGURES 2–3. Development of the vascular buds. Figure 2 shows ampullae just before the appearance of vascular buds. × 120.



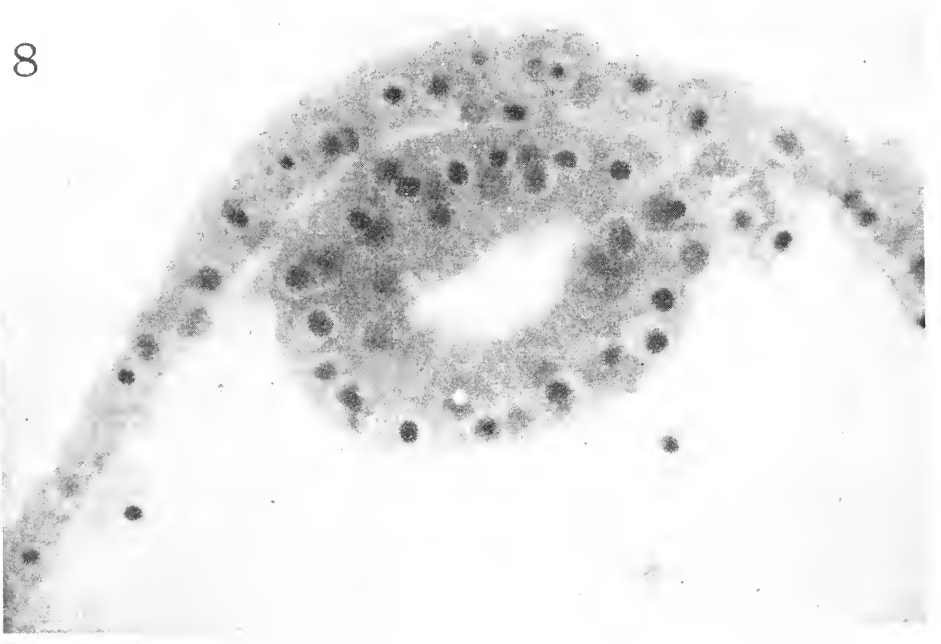


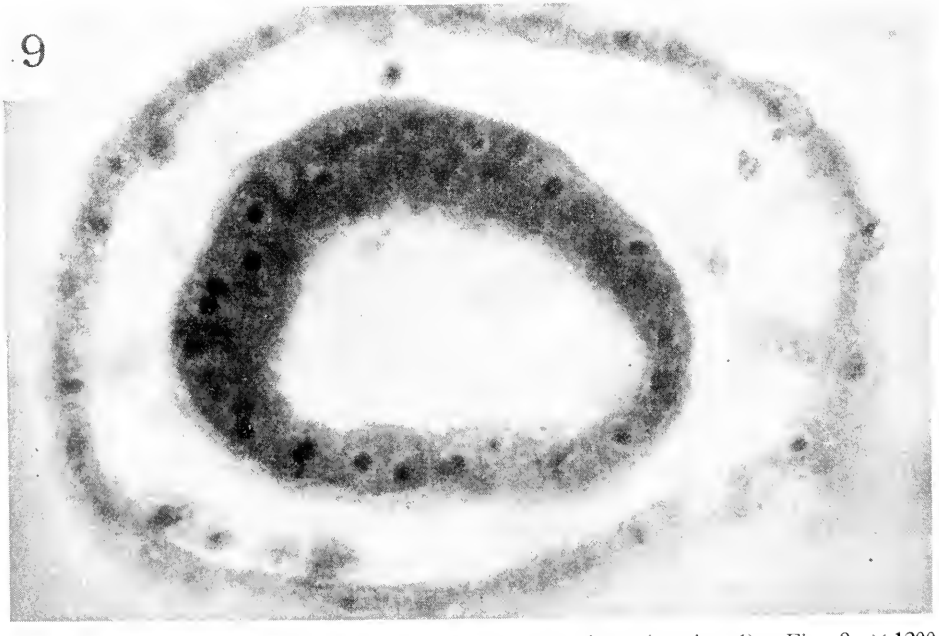
FIGURES 4–5. Development of the vascular buds (continued). × 120.





FIGURES 6–7. Development of the vascular buds; in sections. × 1200.





FIGURES 8–9. Development of the vascular buds; in sections (continued). Fig. 8, × 1200; Fig. 9, × 1000

Nature of the blood-cells

The blood-cells from which the inner vesicle originates are small, round cells. They show a strong affinity for thionin; further, they are more strongly stained by such vital stains as methylene blue (1/10,000 aq. sol.) or neutral red (1/10,000 aq. sol.) than other blood elements.

In sections, it is seen that the cells have a hyaline cytoplasm; their nucleus is filled with a dense network of chromatin and lacks a nucleolus. The total picture suggests that they are cells of a primitive nature. They are the lymphocytes in the terminology of Sabbadin (1955).

Time of appearance

The appearance of the vascular buds is limited to a certain phase in the life cycle of the colony. They appear only during a short period (about 10 hours), extending from later phase B to early phase C. At that time, the colony is at the maximum of its activity and, accordingly, the growth of the ampullae is also very active.

In other phases, no buds are formed even where they are expected to appear. In phase D we sometimes find small buds; however, they are not new buds but older ones that have appeared in early phase C and are now on the way to dissolution.

Site of appearance

In *Botryllus* colonies, numerous blood vessels traverse the test and terminate in contractile ampullae at the periphery of the colony; a flow of blood is maintained by them independently of heart action. The buds appear at the base of ampullae at a distance of about 0.6–0.7 mm. from the tip (Fig. 10).

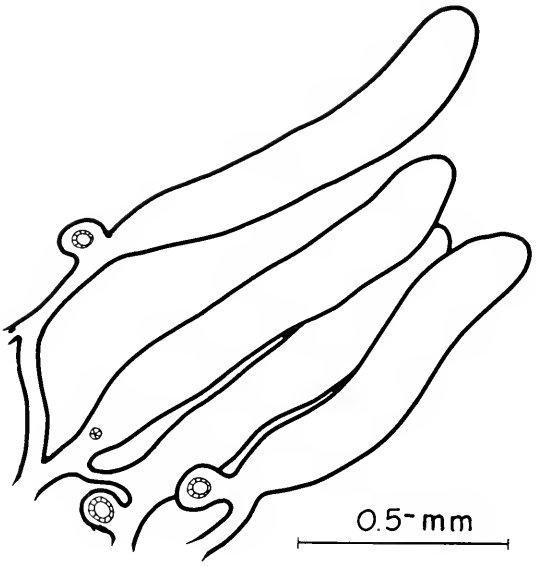


FIGURE 10. Vascular buds at the end of budding period. Note the various sizes.

On each ampulla, only one bud is formed at a time.

Not a single case has been recorded in which the buds are formed from the side-wall of the ordinary vessels.

So far as is known, the appearance of the vascular buds is restricted to the most actively growing edges of the colony where the ampullae are especially numerous and active.

Degeneration of the buds

At the end of the budding period (early phase C) we often find tens and hundreds of newly formed buds, but not all of them continue to develop. Only those which surpass a certain size can develop and form perfect zooids, while the remaining ones undergo involution without even approximately reaching the stage when the two vertical folds appear. For example, in a case out of 200 newly formed buds, only 70 developed into perfect ascidiozooids.

Table I shows an example of different sizes of the buds in a colony at the end of the budding period.

Of these 36 buds, those larger than 40 μ continued to develop (20 buds, or 56%), while those under 35 μ soon began involution and disappeared completely (16 buds, or 44%).

Relation between palleal and vascular budding

As is shown in Table II, the palleal bud appears in phase A. The vascular bud appears, as has already been pointed out, at the end of phase B, and attains stage 3 in phase C. It grows rapidly and becomes stage 5′ (a stage a little behind 5) in phase D and stage 6′ (a stage a little behind 6) in phase A of the following cycle. In phase B the vascular bud attains the stage 8, and from this moment on it develops exactly synchronously with the corresponding palleal bud. It also dies synchronously with the latter.

In brief, the life of the zooid produced by palleal budding extends over 12 days, while that of the zooids produced by vascular budding lasts for ten days. The development, especially in its early stages, progresses a little more rapidly in the vascular than in the palleal bud.

When the vascular bud has attained stage 6′ we see buds of stage 1 appearing on its peribranchial walls. Thus the vascular bud, once formed, propagates by palleal budding.

TABLE I

Various sizes of vascular buds at the end of the budding period

Diameter (in μ)	Number of buds
ca. 70	3
ca. 50	10
ca. 40	7
ca. 35	4
ca. 30	8
ca. 20	4
Total	36

TABLE II

Relation between palleal and vascular budding. Vascular buds are printed in bold-face type

Day	Cycle	Phase	Palleal budding	Vascular budding		
I		A	1			
II		B	3			
III	n	C	4	**3**		
IV		D	5	**5′**		
V		A	1–6	1–**6′**		
VI		B	3–8	3–8		
VII	n 1	C	4–8	4–8	**3**	
VIII		D	5–9	5–9	**5′**	
IX		A	1–6–9	1–6–9	1–**6′**	
X		B	3–8–9	3–8–9	3–8	
XI	n 2	C	4–8–9	4–8–9	4–8	**3**
XII		D	5–9–11	5–9–11	5–9	**5′**

Each vertical column represents the development of the same individual.

In the growing edges of a colony, the test vessels grow out continuously. The distal end of the ampulla grows out and forms the new tip, while its basal part is continuously transformed into the ordinary vessel. This means that the vascular bud, though first formed at the base of the ampulla, is gradually removed from the latter. By the time the bud has attained stage 4–8, it is already at some distance from the ampulla. At the same time we see that a new vascular bud is being formed at the base of the ampulla (Fig. 11). Thus an ampulla forms only one bud at a time, yet it can produce many consecutively.

As the vascular bud is formed a little later than the corresponding palleal one, it is at first smaller than the latter. At the end of development, however, no difference in size is perceptible between the two.

The full-grown zooids formed by vascular budding are the same as those produced by palleal budding in almost every respect, even in the number of tentacles

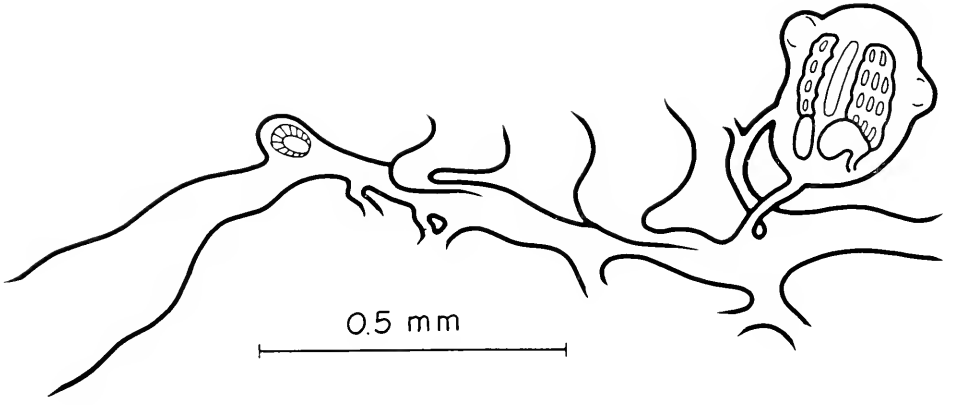


FIGURE 11. Two buds formed consecutively on the same ampulla.

or rows of stigmata. The only difference is that in vascular buds no gonads are formed. In palleal buds, the gonads segregate as a mass from the lateral walls of the bud at an extremely precocious period. Such a segregation has never been observed in vascular buds.

Discussion

Critical review of the earlier data

With this fully established vascular budding at hand, a critical review of the earlier data concerning budding in botryllids will be attempted.

Some of the earliest descriptions of the stolonial budding in *Botryllus*, such as those of Savigny or Milne-Edward, might have been founded on erroneous observations, but what Herdman observed in *Sarcobotrylloides wyvillii* and Bancroft in *Botrylloides gascoi* was in all probability vascular budding analogous to that described here. Some passages in Giard's paper (1872, p. 573) also make it probable that he really observed vascular budding in *Botryllus*.

According to W. H. Herdman (1886; pp. 59, 90), the buds were formed from three sources: large cells which became ova, blood corpuscles, and the wall of blood vessels. In the case of our buds, large cells which were to become ova could not be detected in the mass of cells.

The buds which were found in *Botrylloides gascoi* during aestivation, and which were entirely independent of parent zooids, are in our opinion certainly vascular buds, and not palleal buds developed from the zooids of the original colony and later carried into the yellow lobe, as was assumed by Bancroft. Bancroft met with the so-called yellow lobe only once, so he had no idea of its significance at the time when this was developing. He says (1903b, p. 152), "It is to be hoped that some future investigator will preserve and section the early stages of the development of the yellow lobe before the degeneration of the rest of the colony, and discover why in this case the buds separate from the parent zooids at such an early stage." These buds in all probability were formed *in situ* from blood cells.

There are some differences between our buds and those of Bancroft.

a) The buds described by Bancroft were formed after all the zooids had died away. Our vascular buds, on the contrary, are formed in an active colony simultaneously with palleal buds. In passing, it may be noted that in our opinion degeneration of zooids in the case of Bancroft was not a phenomenon of aestivation, but was caused by some unfavorable conditions in the aquarium.

b) The second difference is that in the case of Bancroft's material the usual coordination was entirely lost and the buds appeared so irregularly that it was practically impossible to tell to which generation they belonged, while our buds always appeared at the end of phase B of the colony.

c) The third difference is that the buds of Bancroft were scattered all through the colony, while ours are located strictly at the bases of ampullae. The examples of Bancroft perhaps show that the epithelial wall of the vascular system can develop into the ectoderm of the new buds, wherever these are formed. Why is it that normally the formation of the buds is localized to the base of ampullae? It is because the flow of blood is most sluggish there, thus giving the blood-cells the best opportunity for settling.

Vascular budding in the system of budding of ascidians

Whatever the type of budding may be, the initial stage of blastogenesis is a double-walled vesicle. The outer wall, called ectoblast, forms the epidermis, while the inner wall, called endoblast, forms the rest of the new zooid. The ectoblast invariably derives from the epidermis of the parent zooid, while the endoblast is variable in origin. According to this origin, the budding of ascidians is divided into three main groups: ectodermic, endodermic and mesoblastic. Vascular budding as described here, together with stolonial budding, belongs to the last group. Of all known cases of stolonial budding, that of *Perophora* comes nearest to the vascular budding of *Botryllus*. In *Perophora* the endoblast is formed from the mesenchymatous septum of the stolon.

Blood-cells in relation to budding

Generally, blood-cells are considered to have nothing to do with bud formation. Berrill (1951), for example, in a survey on regeneration and budding in ascidians states: (p. 468): "Trophocytes, 'cell-packets,' and the various blood cells, other than those lymphotic cells which at times arise from septal mesenchyme or the epicardial epithelium, apparently play no part in any morphogenetic or histogenetic processes, except as victuallers." Neither epicardium nor mesenchymatous septum does exist in *Botryllus*. But, since the mesenchymatous septum in other forms, notably in *Clavelina*, is known to gain or lose cells from or to the haemocoele as lymphochytes, the blood-cells of *Botryllus* forming new buds may possibly be conceived as mesenchymatous septum dissolved into its cellular elements and circulating with the blood stream.

According to the recent investigation of Sabbadin (1955), the blood-cells of *Botryllus* are of dual origin. One evolutionary series begins with a hemoblast. It has a vascular nucleus with a small amount of chromatin and cytoplasm rich in RNA-proteins. The other series begins with a lymphocyte. This is half the size of a hemoblast and is formed from the latter by division. Its nucleus is filled with a dense network of chromatin and lacks a nucleolus. Both series have their own leucocytes and vacuolated cells.

As is clear from the foregoing descriptions, the blood-cells partaking in the formation of vascular buds are lymphocytes as defined by Sabbadin. It seems rather strange that buds are formed from lymphocytes, not from hemoblasts which seem to have a still greater evolutionary capacity.

Size and morphogenesis

The relation between size and morphogenesis in palleal buds has been studied by Berrill (1941b). The palleal bud appears first as a thickened disc of atrial epithelium, which rapidly transforms into a closed sphere surrounded by epidermis. The new organism is formed by a process of folding and local evaginations of this expanding sphere.

Now there is seen a considerable variability in the size of the initial disc, and this determines, according to Berrill, the size and fate of the future zooid. Buds formed on too small a scale may fail to develop. Relatively small bud rudiments give rise to small zooids without gonads and with about six rows of stigmata, while

large rudiments form a greater number of rows of stigmata and produce testes and ripening ova; between these two there are many intermediate forms. In general, discs and spheres produced by early generations are relatively small compared with those produced by later generations. According to Berrill this accounts for the fact that juvenile colonies are asexual, somewhat older colonies have well-developed testes but no ova, and only at the last do both testes and ova appear.

The same explanation does not apply to the case of vascular buds. As has been demonstrated, the period during which the vascular buds appear is relatively short, extending from later phase B to early phase C. At the end of this period, we see buds of various sizes, but only those which surpass a certain size continue to develop, all the remainder degenerating.

We have studied in sections the number of blood-cells taking part in the formation of buds and found that variation is rather slight. The initial number of blood-cells, therefore, cannot be the cause of different sizes. The cause of the small size of some buds is to be sought in the belatedness of their appearance. Thus, small buds seem to have as full developmental capacity as larger ones. Their degeneration must, therefore, be understood as a manifestation of regulation of the colony as a whole.

The absence of gonads in vascular buds is also not the sequence of small size, for the average size of vascular buds is of the same order as that of palleal buds, in which well-developed testes and ova appear.

Regulating mechanism

Of all colony-forming animals, *Botryllus* is perhaps the one in which the zooids are most perfectly coordinated. We still know too little of the nature of the regulating mechanism, yet the fact that such a mechanism is working can be inferred from the following facts:

a) In a colony, the zooids are exactly coordinated in budding and development.

b) When two pieces of related colonies at different developmental phases are fused together, this difference is invariably equalized. This was first demonstrated by Bancroft (1903a) and is now being extensively studied by one of us (*cf.* Watanabe, 1953).

c) The vascular buds are formed a little later than the corresponding palleal buds, but they are soon synchronized with the latter.

d) Vascular buds formed too late are forced to degenerate, thus being eliminated from the colony.

Vascular budding in relation to the growth of the colony

In *Botryllus primigenus*, palleal and vascular budding coexist in a colony. Maintenance and multiplication of zooids are brought about by palleal budding, while localized, rapid growth of the colony seeking a new substratum is effected exclusively by vascular budding.

The colony can continue its existence by vascular budding alone. Such a state is realized when a piece of a colony containing no zooids but ampullae is isolated, or when all the zooids are experimentally removed, or again when, as in the case of Bancroft's material, all the zooids have died, owing to some unfavorable external

conditions. According to Bancroft (1899, p. 451) an isolated piece devoid of zooids never regenerated a colony, and none of the ampullae in such a piece showed the least tendency towards budding. On this point, therefore, we are quite at variance with him.

Significance of our discovery for the general theory of budding in ascidians

Our discovery is of significance in the following points:

a) In Stolidobranchiata, to which the genus *Botryllus* belongs, palleal budding has been considered the only way of asexual reproduction. We now know that *Botryllus* propagates also by vascular budding. This indicates that budding of a rather primitive nature, as the vascular, has not completely disappeared even in such a highly specialized group as Stolidobranchiata.

b) It is generally admitted that each species is given a single mode of asexual reproduction. Now, *Botryllus* propagates by two entirely different kinds of propagation, one ectodermic-peribranchial and one mesoblastic-vascular.

c) The lymphocytes are capable themselves of organizing new individuals.

Is vascular budding peculiar to Botryllus primigenus?

If we consider that of all the genera of compound ascidians it is *Botryllus* that has been most extensively studied till now, it is almost inconceivable that so typical a budding as the vascular has escaped the eyes of previous investigators. It is possible, although not very probable, that this type of budding is peculiar to our Japanese species. We have observed the same budding also in *Botryllus communis*, another Japanese species, which, however, in our opinion is homospecific with *B. primigenus*.

Summary

1. In *Botryllus primigenus*, it has been found that, in addition to palleal budding, new buds are formed also from aggregations of blood-cells at the base of ampullae.

2. The blood-cells partaking in the formation of buds are lymphocytes as defined by Sabbadin.

3. The formation of buds is possible only at a certain phase in the developmental cycle of the colony.

4. Even then, the buds are formed, not on all ampullae, but only on those lying in the most vigorously growing edges of the colony.

5. Our discovery is of significance in the following three points:

a. The ability to form new buds from the vascular wall has not completely disappeared in Stolidobranchiata.

b. A species can propagate by two entirely different kinds of budding. In our case, one is ectodermic-palleal and one mesoblastic-vascular.

c. The lymphocytes are themselves capable of organizing new individuals.

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OPTOKINETIC TESTING OF CYCLOPEAN AND SYNOPHTHALMIC FISH HATCHLINGS [1]

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Previous work on *Fundulus heteroclitus* embryos that were perfect cyclopeans, owing to treatment with ethyl alcohol, indicated that there is a strong tendency for the right side of the eye to function developmentally as a right eye would, in sending optic fibers to the left side of the brain, and for the left side of the eye to send fibers to the right side of the brain (Rogers, 1952). It seemed of interest to determine whether the right side of the cyclopean eye functions physiologically as a right eye would, and the left side, as a left eye. Optokinetic drum testing of optically abnormal amphibia (Sperry, 1944) suggested a means of making such tests. Even if positive optokinetic drum results were obtained with perfect cyclopean fish, it would be impossible to eliminate only one side of the retina surgically without damage to the other, but it might be possible to ablate one optic tectum to learn something further of the pathways involved. Nerve-stained sections could then be used to correlate the microscopic anatomy with the physiological results.

Pearcy and Koppanyi (1924) moved the left eye of a large goldfish to an artificial orbit in the top of the cranium, just to the left of the midline, without severing the nerve. The effect on equilibrium was noted and vision tested during four weeks. They stated that they had produced a real "experimental cyclops." This eye, however, has few of the characteristics of the cyclopean eye. It is not formed from the medullary plate material on both sides of the midline, it is not in the typical ventral position, and it did not develop its nerve connections while it was in close relation to the diencephalic floor on both sides of the midline.

Stockard (1909, p. 285) says in regard to cyclopean *Fundulus heteroclitus* treated with magnesium chloride solutions in the early stages: "Many embryos, showing the cyclopean defect in various degrees, hatched normally and were capable of swimming in a manner indistinguishable from ordinary two-eyed fish. These monsters gave many indications of ability to see. They went to the more brilliantly lighted side of the dish with the normal ones. They darted away in normal fashion when any object was placed in front of the eye, while similar objects put at equal distances from their tails caused no excitement." There appear to be no other reports in the literature of attempts to test cyclopean individuals for vision.

Materials and Methods

In the 1955 season 16,987 *Fundulus heteroclitus* eggs that successfully passed the early cleavage stages were treated with magnesium chloride solutions. Of

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11,912 embryos surviving at least until the eyes were well formed, 0.3% were perfect cyclopeans with an eye of close to normal size, and only three such embryos hatched (Rogers, 1956). In the 1956 season 190 bowls containing somewhat larger numbers of eggs than in the 1955 work were treated in the same manner with magnesium chloride. An estimate was made that about 25,000 embryos survived until the eyes were well formed, 76 embryos (about 0.3%) were perfect cyclopeans with an eye close to normal size, and again only three such embryos hatched and swam. In 1955 the effect of temperatures between 11° and 16° C. was not tested. In 1956 water in bowls kept on the water table between June 6th and 12th was never below 15° or above 16° C. At this temperature seven bowls of 19/60 *M* $MgCl_2$ solution, with a total of 1,020 eggs that were living when they reached the blastula stage, produced 7% optic abnormalities among the 560 embryos that survived until the eyes were formed. All 12 cyclopean embryos among them had reduced eyes and stunted bodies. Although the mortality rate rose to 45%, as compared with 20% in the 1955 series at 18° C., production of optic abnormalities was increased by only 1%.

In the material just described, three cyclopeans, three synophthalmics, and one anophthalmic in the 1955 series, and three cyclopeans and one synophthalmic in the 1956 series hatched and swam well enough to be tested in the optokinetic drum. In addition, from batches of *Lucania parva* eggs fertilized with *Fundulus heteroclitus* sperm, one perfect cyclopean in 1955, and another in 1956, hatched and were tested. At first, a very large striped drum was tried, mounted on a horizontally-placed bicycle wheel revolving around a stationary central platform to hold the dishes of fish. Normal adults responded only moderately well, and hatchlings, not at all. Feeling that the hatchlings might not be affected by movement at such a distance, Mr. Michael Baron, helping with the technical work in 1955, lined an oatmeal carton only 10.5 cm. in diameter with vertical black and white stripes of one-half centimeter to one centimeter in width. When this drum was turned smoothly by hand around a small beaker containing normal hatchlings, the fish readily turned with the drum and quickly reversed directions when the drum was reversed. The fish were kept 6 to 10 days after hatching, and tested a number of times. When most of their yolk was used up, selected ones were drawn with camera lucida while under urethane anaesthesia, and all were fixed, sectioned, and stained with a modification of the Bodian protargol stain in the manner previously described (Rogers, 1952).

Results of Optokinetic Tests

Synophthalmic fish 89–1 (bowl number—individual fish number) and 89–2 (Fig. 4) responded directionally to drum rotation in the same manner as normal control hatchlings. Fish 89–1 did not start to turn with the drum as readily, and did not turn as rapidly as control fish, but otherwise exhibited normal optokinetic reflexes. Fish 89–2 responded nearly as well as controls. Synophthalmic fish 341–1, similar in appearance to the preceding two, did not respond to the drum, although it frequently swam at times when there were no apparent external stimuli. The unusual synophthalmic fish with right eye much reduced, 76–5 (Fig. 5), was slightly but permanently flexed toward its left. It did not orient well and often lay on its back on the bottom of the dish. Nevertheless, in any orientation, it

could always be made to flex vigorously and repeatedly toward its own left with drum rotation in the *opposite* direction, whereas no movement could be elicited by drum rotation to its left. If nerve connections of such an eye are contralateral, as they normally are, the animal ought to flex or turn readily in the same direction as the drum when the drum is rotated toward the blind side, and turn hesitantly or not at all when the drum is rotated toward the seeing side.

The cyclopean "magnesium" fish 89–3, 105–1 (Fig. 1), 105–2, 340–2, 340–3, and 360–1, the cyclopean *Lucania-Fundulus* hybrids 263–1 (Figs. 2 and 3) and 478–1, and the anophthalmic "magnesium" fish 109–1 all failed to respond to any drum stimulation tried. Various tests were made on all these fish, but hatchling 105–1 was tested more extensively than the others, over a period of five days. Drums of various sizes with various widths of striping and turned at various speeds under various lighting conditions were used. The fish were sometimes positioned very close to the beaker side and the drum rotated so that its surface passed just outside the glass and very near the fish. Black-and-white, and red-and-white stripes, striped cones revolving under as well as around the beaker, and flat discs with alternate opaque cardboard and cut-away strips radiating from a central hub and rotated over a light bulb with the fish just above the disc, were also tried with uniformly negative results. Any movements on the part of the fish appeared to be fortuitous and unrelated to the drum rotation. When testing was not being carried on, the anophthalmic 109–1 appeared to swim around the dish at more frequent intervals than normal control fish.

The failure of the perfect cyclopean fish to respond to the drum in 1955 led to the second season's work with the hope of obtaining positive results or of making the negative results more certain. The lack of response also made of prime importance the question as to whether the perfect cyclopean fish can see at all. Unfortunately, all tests so far tried have failed to give rigorous proof. "Baiting" the fish from outside a glass container (Sperry, 1949) appears to be impossible with these hatchlings. Numerous attempts to observe the effect of a differentially lighted container (Stockard, 1909) failed to yield unequivocal results in the present work. The chromatophore response to visually perceived light in adult *Fundulus* (Butcher, 1938) was considered, but light does not have an obvious effect on the chromatophores of normal hatchlings. Potential-recording methods seem impractical with these small fish. Fish 89–3 was placed in a glass container within the large drum mounted on the bicycle wheel, and the drum rotated for five-minute periods with three- to four-inch-wide black-and-white stripes visible, and then for similar periods with the stripes covered by a blank white paper. The number of times the fish swam, and the total length of time in seconds it was swimming, were recorded. A number of tests made it clear that these data were too variable to afford rigorous proof of ability to see. The only evidence obtained consisted of the apparent ability of perfect cyclopeans 89–3, 105–1, and 105–2 to localize the tip of a fine forceps when it was placed in front of them. These observations were not considered conclusive and therefore no attempt was made to repeat them on the perfect cyclopeans of the second season. Fish 105–1 responded 5 or 6 times in succession to a forceps placed close in front of it, by jerking quickly away. On the two succeeding tries, however, the fish slowly and deliberately came off the bottom and moved forward, accurately placing its tubular mouth against the forceps.

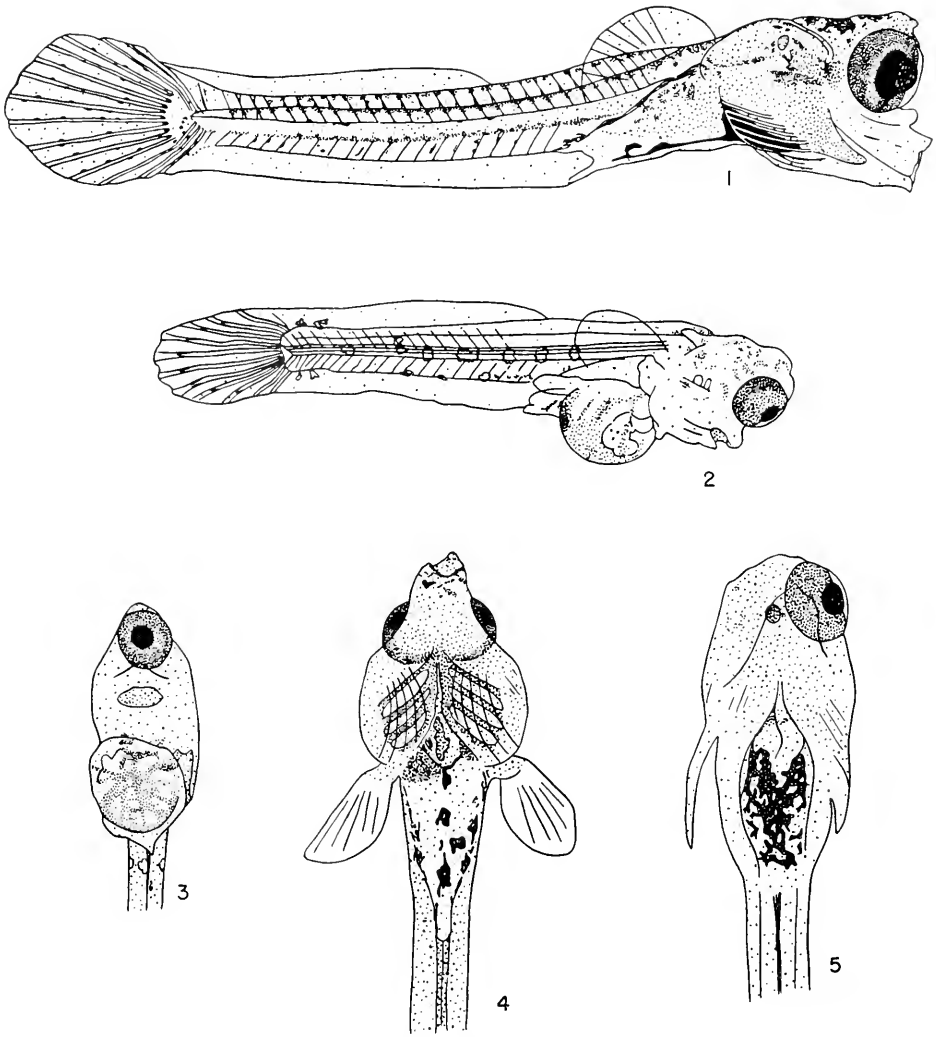


FIGURE 1. View of right side of cyclopean *Fundulus heteroclitus* hatchling 105–1. The anterior part of the body is flexed slightly to the right to show the eye. Drawn with camera lucida, urethane anaesthesia, × 20.

FIGURE 2. View of right side of cyclopean *Lucania parva—Fundulus heteroclitus* 263–1. Chromatophores are opaquely white rather than black as in *Fundulus*. × 20.

FIGURE 3. Ventral view of anterior part of 263–1, × 20.

FIGURE 4. Ventral view of anterior part of synophthalmic *Fundulus heteroclitus* 89–2, × 20.

FIGURE 5. Ventral view of anterior part of *Fundulus heteroclitus* 76–5, × 20.

It ignored the forceps when the latter was placed the same distance from its tail. When fish 105–2 had exhausted its yolk and become fairly inactive, it repeatedly exhibited following eye movements in the proper direction as a forceps tip was

moved across its front. Fish 89–3 responded to the forceps in front of it by moving away. All of these responses could conceivably be initiated by movement of water created by the forceps, but particularly in the case of the immobile forceps that was approached and accurately localized by fish 105–1, visual cues would seem to be a more likely explanation.

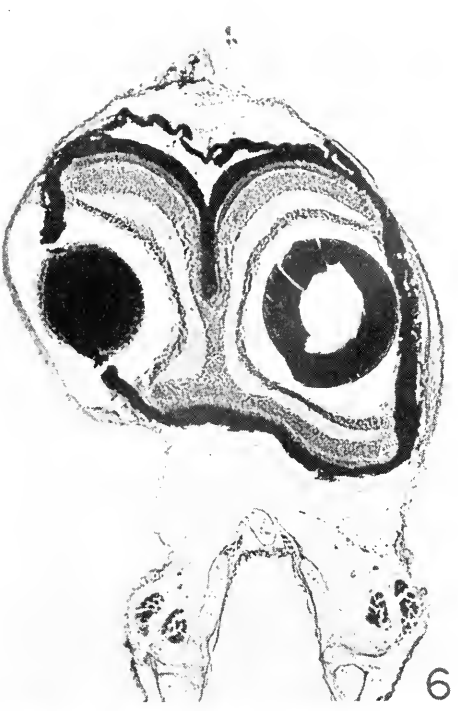
Histological Correlations

Synophthalmic fish 89–1, 89–2, and 341–1 each have two pupils and lenses, two arcs of sensory retina and pigment epithelium, and nerve bundles collecting from each retinal arc and passing out from the retina medially between the two arcs. Fish 341–1 has two choroid sacs and lacks an extensive fused area between the eyes, but 89–1 and 89–2 each have a single choroid sac enclosing the retinas, and a ventro-medial continuity of the sensory retinas (Fig. 6). The optic nerves in 89–1 and 89–2 pass posteriorly through the choroid coat in a single bundle and, possibly with a complete decussation, enter the floor of the diencephalon and proceed dorsally in two fairly equal bundles to distribute to the two optic tecta. Nerve fibers are so numerous, and they run parallel so far before turning up into the brain, that it is difficult to follow specific groups of fibers certainly enough to be sure there is complete decussation. The optic nerve in 341–1 collects from the retinal arcs and leaves the eyes in much the same manner, but after entering the diencephalic floor, it turns abruptly anteriorly and distributes to the telencephalon on the right side only. Fish 76–5 has a left eye that is fairly normal in form but that is displaced antero-medially over a cyclopean-type tubular mouth. The right eye consists of a small mass of sensory retinal cells, completely surrounded by a layer of pigment epithelium, and lacking a nerve. The left eye sends a large nerve entirely to the left side of the brain (Fig. 8). Part of this nerve passes into the left optic tectum and part passes anteriorly to distribute in the dorsal part of the diencephalon on the left side.

The form of the cyclopean eye is well known (Fig. 7). The eyes of perfect cyclopeans 105–1 and 105–2 each have a stalk similar to that of a normal eye. In both these fish, nerve fibers collect from both sides of the retina, leave the eye posteriorly, just behind a ventral retinal gap interpreted as the remains of a choroid fissure, run together in the optic nerve, sort out in the base of the diencephalon with a possible decussation, and distribute about equally to the two tecta.

Cyclopean fish 89–3 has no optic stalk, but instead the eye is continuous with the diencephalic floor over a wide area, the pigment epithelium appearing as a reflection of the diencephalic surface. The optic nerve collects from both sides of the retina but goes only to the right tectum. The eyes of the two hybrid cyclopeans, 263–1 and 478–1, have the same close relation to the diencephalon, with the stalk lacking, but in these cases the nerve distributes to both tecta. In 263–1 three-fourths of the optic fibers go to the left tectum and the remainder to the right. As they enter the diencephalon, some of these fibers certainly decussate but others do not. In 478–1 the fibers collect in a bundle and run parallel to each other for some distance before leaving the eye so that it is hard to tell whether they decussate, but about half go to each tectum after they sort out in the base of the brain.

The cyclopean eyes of 340–2, 340–3, and 360–1 lack optic stalks and are even more closely related to the diencephalon than the preceding cases, in that the sensory



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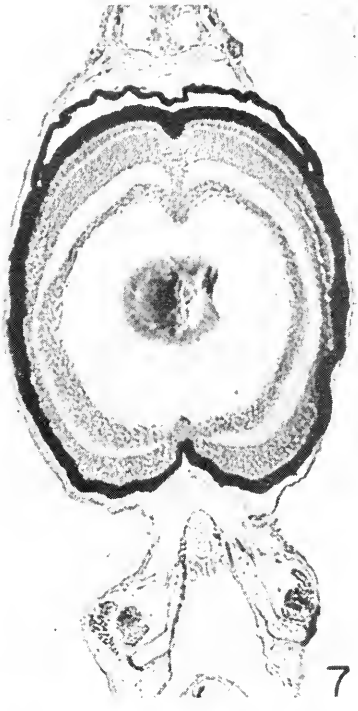

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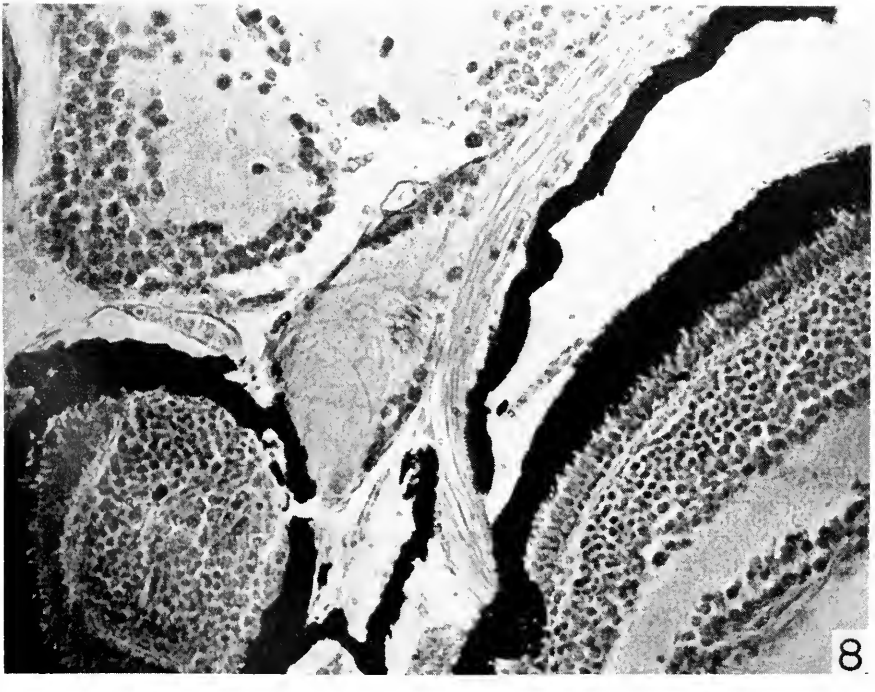

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retina projects posteriorly well into the third ventricle, instead of simply being continuous with the diencephalic wall. The pigment epithelium, however, is again a reflection of the diencephalic wall. In 340–2 the optic fibers clearly decussate as they leave the retinal tissue, with a large bundle from the right side of the eye going to the left tectum and a bundle half as large from the left side of the eye going to the right tectum. Fish 340–3 also has a large fiber bundle going to the left tectum and a small bundle to the right tectum, but fiber paths as they leave the eye cannot be followed clearly enough to see whether they decussate. All of the optic fibers of 360–1 go to the left tectum.

Anophthalmic fish 109–1 has no eye tissue identifiable in section.

Discussion

The two synophthalmic fish that responded directionally to the optokinetic drum in the same manner as control fish were found to have optic nerves that distribute equally to the two optic tecta after decussating at least in part, and possibly entirely. The synophthalmic fish of similar external appearance that failed to respond to the drum was found to have optic fibers distributing solely to the telencephalon on one side, and was probably blind.

The failure of the perfect cyclopean fish to respond to the drum is harder to explain. Until the histological sections of the first season were studied, it was thought possible that in the four cases tested the optic nerve fibers from each side of the eye just happened to distribute about equally to both optic tecta. Horizontal optokinetic reflexes might then be cancelled out. This cannot be the explanation, however, because two of the total of eight perfect cyclopean fish tested have optic nerve fibers distributing to the optic tectum of one side only. No other explanation for the failure to respond is apparent. A right or left eye, even when displaced far toward the midline (fish 76–5), will send impulses that will initiate reflexes in response to the drum. Therefore, the two pupils or two separate retinal arcs of the typical synophthalmic condition are not requisite for the response. There is slight evidence that the cyclopean fish can see objects placed close in front of them. Comparison of their behavior with that of anophthalmics when objects are introduced near them, leads to the belief that the cyclopeans can see, but does not afford rigorous proof. If they can see, the failure to respond to the drum remains perplexing.

The individual with only one functional eye in the synophthalmic position and nerve distributing only homolaterally, responded directionally to a rotating drum in the same way as one-eyed anurans in which the regenerating optic nerve had been forced to grow to the homolateral optic centers (Sperry, 1945). Thus, Sperry's results in a regenerative situation are extended to a primary developmental situation.

All figures are photomicrographs of Bodian preparations.

Figure 6. Transverse section through synophthalmic eyes of fish 89–2. Note ventral fusion of sensory retinal arcs. × 115.

Figure 7. Transverse section through perfect cyclopean eye of fish 105–1. Nasal pits are above, tubular mouth below. × 115.

Figure 8. Transverse section of portion of eyes of fish 76–5. The large left eye sends a normal-sized nerve to the left side of the brain. × 800.

Summary

1. Perfect cyclopean, closely synophthalmic, and anophthalmic fish hatchlings were obtained by magnesium chloride treatment or by hybridization.

2. Synophthalmic fish, with distribution of optic nerve fibers generally similar to controls, responded essentially normally to a horizontally rotating optokinetic drum.

3. A synophthalmic fish, with optic fibers distributing to the telencephalon of one side, failed to respond to the drum.

4. A fish with only one functional eye in the synophthalmic position, and optic fibers distributing entirely homolaterally, responded in the *opposite* direction when the drum was rotated toward the blind side.

5. Although there was other, somewhat inconclusive, evidence that they could see, eight perfect cyclopean fish failed to respond to a horizontally rotating drum.

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THE REVERSIBLE REPLACEMENT OF POTASSIUM BY RUBIDIUM IN ULVA LACTUCA [1]

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The substitution of rubidium ion for potassium ion in physiological processes has been studied for over 80 years. This subject is of special interest in view of the close chemical similarities of the two ions, rubidium being much more akin to potassium than is sodium. Permeability studies have revealed a high penetration rate of both rubidium and potassium ion (Brooks, 1932, 1939).

Studies on the replacement of potassium by rubidium have compared, on the one hand, the amount of physical replacement of potassium by rubidium within the cell, and on the other hand the suitability of rubidium as a substitute for potassium physiologically. Complete physical replacement of potassium by rubidium has been shown for *Chlorella* (Pirson, 1939), and yeast (Scott, unpublished data); partial physical replacement occurs in vertebrate muscle (Follis, 1943; Heppel and Schmidt, 1938; Mitchell, Wilson and Stanton, 1921) and in the duckweed *Lemna minor* (Pirson and Kellner, 1952). Rubidium can completely substitute for potassium in the repolarization of nerve (Feng and Liu, 1951; Gallego and De No, 1947), the restoration of heart beat in the frog heart (Ringer, 1884; Zwaardemaker, 1919), the activation of spermatozoa (White, 1953), the transport into the erythrocyte (Love and Burch, 1953), the activation of zymase (Giordani, 1932), the respiration of mitochondria (Pressman and Lardy, 1952), the bacterial production of pyruvic acid from malic acid (Lwoff and Ionesco, 1947), the activation of pyruvic phosphoferase (Kachmar and Boyer, 1953), and for growth in the bacterium *Streptococcus faccalis* (MacLeod and Snell, 1948). Rubidium is less effective than potassium in supporting assimilation, chlorophyll formation and cell division in *Chlorella* (Pirson, 1939), in supporting growth in *Lactobacillus casei* (MacLeod and Snell, 1948), or *Nitzschia closterium* (Stanberry, 1934), or for the secretion of adrenalin (Hermann, 1942). Rubidium is ineffective in supporting antibiotic activity in the subtilin-producing strain of *Bacillus subtilis*, although it promotes growth as well as does potassium in this bacterium (Feeney and Garibaldi, 1948). The element is definitely toxic at high concentration in certain bacteria, being antagonized by potassium (Scharrer and Schropp, 1933), and is fatal to rats when it is substituted for up to 50–66 per cent of the potassium in the tissues (Follis, 1943; Heppel and Schmidt, 1938; Mitchell, Wilson and Stanton, 1921; Zipser and Freedberg, 1952).

Previous work has been concerned with the extent of replacement of potassium

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by rubidium or the physiological results thereof, rather than with the kinetics of replacement. The present work is a kinetic study of the reversible exchange of rubidium for potassium and of potassium for rubidium.

Materials and Methods

The organism selected for this study was the green alga, *Ulva lactuca*. This marine organism, like most cells living in a high-sodium, low-potassium environment, normally accumulates potassium and partially excludes sodium. Consisting of large membranous fronds two cell layers in thickness, thus presenting a large surface area for interchange with the environment, this alga is particularly well suited for investigation of this nature.

Large fronds were collected from Perch Pond, near Falmouth, Mass., and conditioned for at least 24 hours in running sea water under incandescent illumination. Samples, cut from the same frond, were given a brief rinse in isotonic sucrose followed by a blot in absorbent toweling to remove most of the adherent sea water, then placed in large finger bowls with about 160 ml. per sample of an artificial sea water (Marine Biological Laboratory, Formulae and Methods IV, 1956), containing rubidium instead of potassium. The rubidium sea water was replaced with fresh rubidium sea water after 50 hours. Diffuse illumination was present during the experiment, which resulted in a slight drop in rubidium sea water pH. After 96 hours in rubidium sea water, the samples were placed in running sea water for 120 hours. Samples were removed in triplicate at various times, rinsed in isotonic sucrose for 30 seconds and blotted three times in absorbent tissue to remove extra-cellular sodium, potassium and rubidium ion. Wet and dry weights were taken and cell water calculated by difference. The samples were ground, extracted with 1 N HNO_3 for two hours at 110° C., and the extracts diluted to volume in 50-ml. volumetric flasks. The extracts were analyzed for sodium, potassium and rubidium ions by the Beckman flame spectrophotometer.

Results

The initial uptake of rubidium is both rapid and nearly complete within the first four hours, the time at which the first samples were taken. At 96 hours the rubidium ion concentration reaches a maximum of about 87 per cent of the control value of potassium; longer immersion in rubidium sea water does not result in an increase above this maximal concentration value. The potassium loss is initially rapid and then continues to decrease in a manner parallel to that of the control, reaching a minimum of about 13 per cent of the control (Fig. 1).

When the fronds are placed in running sea water, the kinetics of the replacement of rubidium by potassium are quite different from those involving the replacement of potassium by rubidium. After an initial sharp drop in rubidium for 10 hours and a sharp rise in potassium for 10 hours, the rates of loss of rubidium and rise of potassium decrease. After 120 hours in running sea water the potassium content of the alga has practically reached that of the control, whereas the rubidium content has been reduced to only 30 per cent of its maximal value. Longer immersions were not practical, as the alga begins to show signs of aging.

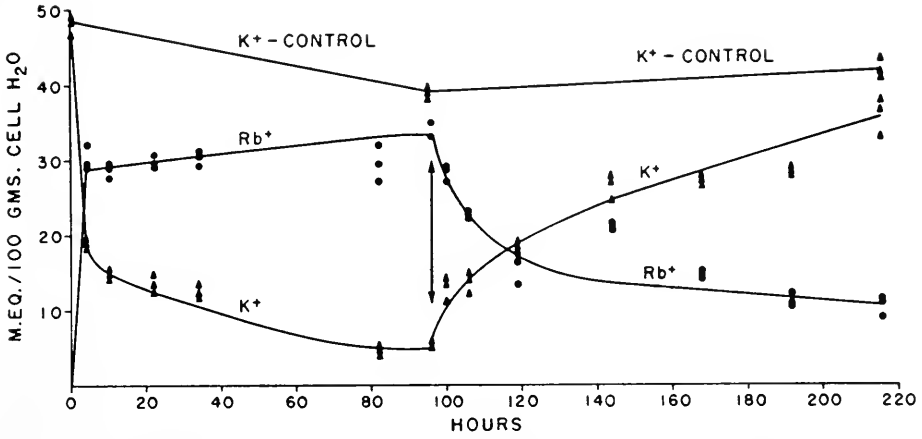

FIGURE 1. The replacement of potassium by rubidium within *Ulva lactuca* in rubidium sea water, and the replacement of rubidium by potassium on transfer of the alga to running sea water. The arrow indicates the time of transfer to running sea water. Concentrations are expressed on a cell water basis.

Throughout the experiment the sodium concentration of the samples remained essentially constant at 26 ± 4 meq. per 100 gm. cell water.

DISCUSSION

The initial uptake of rubidium is too rapid to be explained on the basis of a new, separate uptake mechanism for rubidium alone, for such a mechanism would have to be much faster than that previously demonstrated for potassium in this alga (Scott and Hayward, 1954b). Rather, the rate of rubidium uptake is entirely consistent with the known rate of potassium turnover at 20° (Scott and Hayward, 1954a). Therefore, rubidium is being transported by the same mechanism as is potassium. The cessation of rubidium uptake is probably the result of the establishment of an equilibrium between the rubidium and the potassium concentrations, for the total alkali metal base ($Na^+ + K^+ + Rb^+$) is constant within ± 5 meq. of that of the control (76–65 meq./100 gm. cell water).

Rubidium does not seem to affect the final level of potassium re-accumulation, but it would appear to have an effect on the kinetics of potassium re-accumulation. The re-accumulation of potassium is too slow to be accounted for solely on the basis of exchange of potassium ion for rubidium ion. Rather, it would seem that two factors might be operative: 1) the full maintenance of the potassium-accumulation mechanism is not supported by rubidium ion; 2) the age of the samples may be such as to render the postassium-accumulating mechanism sluggish. Since Ulva cycles from gametophyte to sporophyte generation on the order of every two weeks, it will be seen that the time course of the experiment (9 days) includes most of one phase of the cycle of the organism. (Whether a particular frond was gametophyte or sporophyte was not determined in this work, for the two generations are morphologically identical.)

In another experiment samples were left in rubidium sea water for as long as 144 hours before transfer to running sea water. The rate of potassium re-accumulation was identical to that found after 96 hours in the experiment reported above; hence the increased time in rubidium sea water was not progressively detrimental to the potassium-accumulating mechanism.

The presence of rubidium within this organism did not prevent the formation and discharge of a germinal ridge in some samples. These samples were not used in the analyses.

Summary

1. Rubidium ion replaces two-thirds of the potassium of *Ulva lactuca* within four hours after being placed in rubidium-containing sea water.

2. The rubidium concentration does not increase more than 5 meq. during the remainder of the experiment.

3. Potassium re-accumulation in running sea water is slower than the initial exchange of rubidium ion for potassium ion.

4. The disparity in the exchange kinetics is discussed.

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THE MORPHOLOGY AND LIFE-HISTORY OF THE DIGENETIC TREMATODE, MICROPHALLUS SIMILIS (JÄGERSKIÖLD, 1900) BAER, 1943 [1]

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Although significant observations had been reported earlier, the first complete life-histories of microphallid trematodes were worked out by Cable and Hunninen (1940) and Rankin (1940b). M'Intosh (1865) had described and figured larval worms from the tissues of the green crab, *Carcinides maenas* (Linnaeus), taken at St. Andrews, Scotland, but the life-cycles of trematodes were quite unknown at the time and M'Intosh described the structures as eggs, each of which contained a tiny worm that he surmised became a sexually mature distome in "such fishes as the Cotti, Gadi, and others," which feed on the crustaceans. He reported a specimen of *Cottus bulbalis,* about a foot long, with (p. 204) "two entire specimens of *Carcinus maenas,* each upwards of two inches across the carapace, in its stomach, besides the partially digested debris of others." His descriptions and figures identify the worms as members of the genus *Microphallus* and with considerable certainty as *M. similis* (Jägerskiöld, 1900). Although measurements were not given, his Figure 5 of an excysted specimen shows the suckers to be of approximately equal size and the "small globule" (seminal vesicle) anterior to the acetabulum, together with the size of the gonads, evidence full development of the metacercaria.

Levinsen (1881) described adult trematodes from the eider duck, *Somateria molissima,* taken at Egedesminde, Greenland, as *Distoma pygmaeum* and Brandes (1889) described similar worms from *Tringa alpinus* as *Distoma claviforme.* Jägerskiöld (1900) described specimens from Swedish gulls, *Larus argentatus* and *L. fuscus,* as *Distoma pygmaeum* var. *simile.* Other species have since been described from different hosts in other parts of the world. Ward (1894) described worms from American lake-fish as *Distoma opacum* and noted their similarity to *D. pygmaeum.* The parasites occurred in *Amia calva, Ictalurus punctatus* and *Perca flavescens,* and encysted larval stages were found in the crayfish, *Cambarus propinquus.* With the dismemberment of the old genus *Distoma,* Stossich (1899) erected the genus *Levinsenia* to include *Distoma brachysomum* Creplin, 1837, *D. macrophallos* von Linstow, 1875, *D. pygmaeum* Levinsen, 1881, and *D. opacum* Ward, 1894. Lühe (1899), but not Looss (1899), as has been claimed (cf. Looss, 1902: p. 704) designated *L. brachysoma,* and Jägerskiöld (1900) proposed *L. pygmaeum,* as type of the genus *Levinsenia.* Noting differences between *D. brachysomum* and *D. opacum,* Ward (1901) named the latter species type of a

[1] The experimental work was done at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the period May 1 to October 31, 1956.

new genus, *Microphallus*. In this paper he reported that the name *Levinsenia* Stossich is a homonym of *Levinsenia* Mesnil, 1897, a polychaete annelid, and that Stiles and Hassall were to propose the name *Levinseniella* to replace it. Although the paper by Stiles and Hassall did not appear until 1902, the name *Levinseniella* Stiles and Hassall in Ward, 1901 was validated with *L. brachysoma* as type. Jägerskiöld (1901), although aware of the announcement by Ward, proposed the name *Spelotrema* for the invalid name *Levinsenia,* with *S. pygmaeum* as type. Cable and Hunninen (1940), commenting on this action, wrote (p. 153), "If the law of priority should be applied to this case, *Spelotrema* Jägerskiöld, 1901 should be suppressed as a synonym of *Levinseniella* Stiles and Hassall in Ward, 1901, since Jägerskiöld stated subsequently (1904) '*Spelotrema* (= *Levinseniella*)' and therefore certainly regarded them as synonymous. His later (1907) conception of two distinct genera is valid, however, and must be accepted although he should not have retained for them names which he had regarded previously as synonyms. To suppress *Spelotrema* as a synonym of *Levinseniella,* and propose a new generic name for the species at present allocated to the genus *Spelotrema,* would probably increase rather than diminish the present confusion. For this reason, the writers are inclined to let the matter stand." As noted, *L. brachysoma* and *S. pygmaeum* are not congeneric and the characteristic features of the two genera were presented clearly by Rankin in two papers (1939, 1940a). The problem of nomenclature, stated by Cable and Hunninen, was resolved when Baer (1943) suppressed *Spelotrema* as a synonym of *Microphallus* and transferred all species from the former genus to the latter one. Baer described *Microphallus gracilis* from *Neomys fodiens* and formulated a key to thirteen members of the genus. Strandine (1943) and Rausch (1946a, 1946b, 1947) discussed morphological features and host-specificity of species in the genus *Microphallus.* Rausch and Locker (1951) described *Microphallus enhydrae* n. sp., from the sea-otter, *Enhydra lutris,* and recognized fourteen species in the genus. Stunkard (1951, 1953) described metacercariae from the horseshoe crab, *Limulus polyphemus,* and their development to sexual maturity in white mice, golden hamsters, and the herring gull, *Larus argentatus.* Although the worms agreed closely with the description of *M. claviformis* (Brandes, 1899), bionomic features seemed to preclude their allocation to that species and they were described as members of a new species, *Microphallus limuli.* Cable and Kuns (1951) erected a new genus, *Carneophallus,* and discussed the evolution and interrelationships of genera in the family Microphallidae.

Lebour (1905) reported grape-like masses of "sporocysts" in the liver of *Littorina rudis* (= *L. saxatilis*), filled with tail-less cercariae, doubled up in a curious manner. When extended, they measured 0.25 mm. in length and the figure shows them to be microphallid metacercariae. Miss Lebour regarded these larvae as identical with the encysted worms found by M'Intosh (1865) in the green crab, *C. maenas.* Brandes (1889) had suggested that the larva in the green crab is the encysted stage of *Distoma claviforme,* described by him from *Pelidna* (*Tringa*) *alpina* and *Aegialitis hiaticula.* Nicoll (1906) described specimens from the ceca and intestine of *Larus argentatus* taken at St. Andrews, Scotland, which he identified as *Levinsenia similis,* the species that Jägerskiöld (1900) had described as a variety of *Dist. pygmaeum* and (1907) raised to specific rank as type of *Spelotrema.* Nicoll noted that his specimens were somewhat larger than those of

Jägerskiöld and that worms from the ceca were larger than those from the intestine. He declared that the metacercariae from *C. maenas* are as likely to prove larvae of *S. similis* as of *S. claviforme*. The following year, Nicoll (1907) described the worms from *L. argentatus* at St. Andrews as a new species, *Spelotrema excellens,* distinct from *S. similis*. Other specimens from *Pelidna alpina, Totanus calidris* and *Aegialitis hiaticula* were described as a new species, *Spelotrema feriatum*. He gave a redescription of *S. claviforme* and in a footnote stated that the larvae from *C. maenas* are larger than the adults of *S. claviforme* and must belong to another species, perhaps *S. excellens*. The smaller metacercariae described by Lebour (1905) from the liver of *Littorina rudis* were suggested as the encysted stage of *S. claviforme*. Lebour (1908) designated the metacercaria from *C. maenas* as *Cercaria carcini* and recognized the difficulty of relating the larval forms of *Spelotrema* with any known adults. Nicoll and Small (1909) examined crabs at Millport on the Scottish west coast during August, 1908. They found three of four *C. maenas* and one of five *Cancer pagurus* infected with encysted metacercariae which they identified as a species of *Spelotrema,* most probably *S. excellens* Nicoll. At St. Andrews every green crab was infected, often with every tissue and organ riddled with cysts which occurred sometimes singly and sometimes in clusters in the liver, gonads, and along the blood vessels, nerves and intestine. They noted, as had Lebour (1908), that the cysts varied in size, shape and thickness of wall. They measured cysts of three size groups; the small ones were oval with thin walls and the largest ones were spherical with thick walls composed of inner concentric and outer radial layers. Referring to the gradual increase in size of the cysts, the authors stated (p. 239), "From these figures there seems no reason to suppose that these groups are other than stages in the growth of the same cyst, and such being the case it is evident that the cercariae increase considerably in size during their sojourn in the crab." They measured a cyst from the original material of Prof. M'Intosh which was 0.29 mm. in diameter. They suggested a possible error in the measurement given in his (1865) report, mentioned also by Guyénot *et al.* (1925).

Lebour (1912) proposed that larval trematodes should be classified on bionomic grounds and arranged the British marine cercariae in two categories, dependent on whether they were produced in sporocysts or in rediae. Among those which develop in sporocysts, she gave a more complete account of *Cercaria ubiquita,* a larva which she previously (1907) had described from *Paludestrina stagnalis* at Fenham Flats and Loch Ryan on the west coast of Scotland. She reported that this species occurred also in *Littorina saxatilis* (syn. *L. rudis*), and at Millport the usual host was *Littorina obtusata*. The cercariae resembled *C. cellulosa* and *C. pusilla* of Looss (1896) and Lebour reported that they entered *C. maenas* and *Cancer pagurus* where they developed into "Spelotrema-like cercariae." The encysted larvae occupied (p. 432) "almost every tissue of the crab, liver, muscles, gonad and outside the blood vessels. Having settled down it grows considerably and the cyst with it, but the latter however is still very thin-walled. The stylet is lost when the cyst measures about 0.30 mm. across. The ventral sucker and alimentary canal appear and the body spines begin to form. The worm stops growing when the cyst is about 0.35 mm. across and then the cyst wall becomes very thick, 0.02 mm. thick, and the real resting stage begins. The cercaria is now of the ordinary *Spelotrema* form. The usual size of the thick-walled cyst is

0.4–0.48 mm. across." After describing the excysted metacercaria, Lebour stated (p. 433), "From what has been said there can be little doubt that *Cercaria ubiquita* is the young form of *S. excellens* the first host thus being *Paludestrina stagnalis, Littorina obtusata* and *L. rudis*. The intermediate host *Carcinus maenas* and *Cancer pagurus* and the final host probably the herring gull, *Larus argentatus*." In this paper Lebour also gave figures of *Cercaria carcini* Lebour, 1908 and *Cercaria minor* sp. inq., both from *C. maenas*. These species were encysted and therefore metacercariae; furthermore, since the cyst walls were thin and the worms somewhat smaller, it is probable, as suggested by Nicoll and Small (1909), that these larvae are identical with the ones identified by Lebour as *Spelotrema excellens*.

Guyénot, Naville and Ponse (1925) described metacercariae which they found in a single, formalin-preserved specimen of *C. maenas* taken at Boulogne-sur-Mer, France. The cysts were oval, 0.40 by 0.35 mm., and the larvae were identified as *Spelotrema carcini* Lebour. These authors accepted Lebour's (1912) account, designating *C. ubiquita* as the larva of *S. excellens,* but noted that when computed from the stated magnification of the figure, the metacercaria portrayed by M'Intosh was only 0.13 by 0.16 mm. As noted earlier, M'Intosh gave no measurements and the presumed error in magnification may be explained by reduction in size of the drawing on publication. The French authors also described larger cysts, 0.90–1.2 mm. in diameter, in which the walls were weakened and the trematode larvae were filled with spores of a microsporidian, *Nosema* (*Plistophora*) *spelotremae* n. sp.

Stunkard (1932) described cercariae from *Littorina saxatilis* and *L. littorea* at Roscoff, France as *Cercaria ubiquitoides*. Although the larvae were very similar to *C. ubiquita,* slight differences between these specimens and Miss Lebour's description prevented their allocation to that species. In *C. ubiquita* Lebour described two ducts on each side, "which run up the body springing from two masses of large cells which occupy the greater part of the body." In her figure, Lebour showed six gland cells on each side with the two median ducts crossing to open on opposite sides of the body. The cercariae at Roscoff were described as having four penetration glands on each side of the body, with ducts which lead forward, three associated in a common bundle that passes along the lateral face of the oral sucker while the other duct lies more mediad and passes over the sucker. The penetration glands did not stain with neutral red but the secretory granules were clearly visible. These granules, colorless in the cell bodies, stained a deep blood-red in the terminal portions of the ducts and frequently accumulated there to form enlargements. Stunkard also described metacercariae from *C. maenas* and *Porcellana longicornis,* which were referred provisionally to the genus *Spelotrema,* but no attempt was made to relate them to *C. ubiquitoides*.

Rees (1936) described an ubiquitous cercaria from *L. rudis, L. obtusata* and *L. littorea* collected at Aberystwyth in February and March, 1935. He noted that the larvae were almost identical with those described by Stunkard (1932) from *L. rudis* and *L. littorea* at Roscoff. There were slight differences in measurement and other differences concerned the penetration glands, which in the specimens studied by Rees were, "more distinct, not lobed and the most anterior pair of cells is separated from the other three pairs." Rees declared (p. 621), "It is difficult to determine whether these differences are individual or specific because there are so few larval characters in cercariae of the Ubiquita group which can be

used for separating species. The problem is rendered more difficult by the close resemblance of the adult trematodes (species of the genus *Spelotrema*)." In general, the measurements given by Rees agree with or overlap those given for *C. ubiquitoides,* except for the length of the stylet which measured 28 microns against "about 25 microns" in *C. ubiquitoides.* The size, shape and relative position of the gland cells are altered by degree of maturity of the larva, by contractions of the body musculature, and by the emission of secretion into their ducts. Since the ducts from the most anterior pair of penetration glands are separate from the ducts of the other cells, the cell bodies may be somewhat removed. The observation by Rees that only the two anterior pairs of cells and ducts take up neutral red stain, whereas the others do not, is a significant contribution to knowledge of the species. Obviously, I failed to note this feature in *C. ubiquitoides,* but the staining reaction differs with the constitution, concentration, age and condition of the neutral red solution. Accordingly, I am disposed to regard the larvae described by Rees and *C. ubiquitoides* as specifically identical. Since each penetration gland has its own duct, the account of Lebour is not precise and the lateral one of the two reported ducts on each side of the body is almost certainly a bundle of three ducts. Furthermore, it appears that *C. ubiquitoides* can not be distinguished from *C. ubiquita* Lebour, 1907, and the name should be suppressed as a synonym. Lewis (1926) had reported *Spelotrema simile* as very common in gulls of the Aberystwyth area and the finding was confirmed by Rees (1936) who stated (p. 624), "The unusually high percentage infestation of *Littorina rudis* with this cercaria, together with the high percentage of gulls parasitized by *Spelotrema simile,* suggests that this may be the larval form of this species."

Cable and Hunninen (1938) described the successive stages in the life-cycle of a trematode which they identified as a new species, *Spelotrema nicolli.* The asexual generations were in the snail, *Bittium alternatum,* the metacercariae in the blue crab, *Callinectes sapidus,* and the sexual generation was developed in young herring gulls, *Larus argentatus.* These authors (1940) gave a more complete description of *S. nicolli,* the adult of which was compared with that of *S. pygmaeum, S. claviforme, S. simile, S. excellens* and *S. brevicaeca.* In size and size of organs, *S. nicolli* agrees closely with *S. simile;* the major difference is in the size of the male papilla, which is much smaller and similar to that of *S. pygmaeum.* The metacercariae were found only in certain slender fibers which extend from the viscera to the bases of the legs. Cysts increase from 0.05 to 0.50 mm. in diameter; the metacercariae become almost as large as the adults; the excretory formula is 2 [(2 + 2) + (2 + 2)], which persists in the adult stage. The cercaria agrees closely with the description of *C. ubiquitoides* and of the species described by Rees; the major difference is the shorter length of the stylet. *Spelotrema nicolli* is identified by the size of the male papilla, the location of the metacercariae, and the taxonomic position of the first and second intermediate hosts.

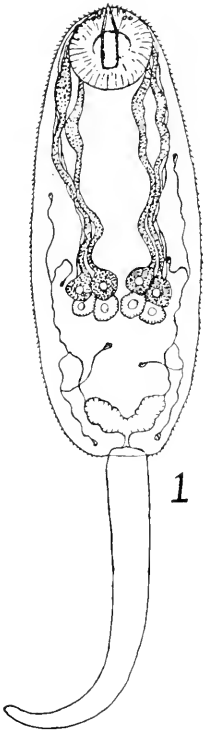
Timon-David (1949) reported metacercariae from the hepatopancreas of *C. maenas* in the Mediterranean at Marseille. The cysts were oval and on this feature and their size, they were identified as *Spelotrema carcini* Lebour.

The present report covers part of a project on clam investigation conducted by the U. S. Fish and Wildlife Service. Since the green or shore-crab, *C. maenas,* is a serious predator of *Mya arenaria* on the New England coast, a survey of its parasites was undertaken in an attempt to determine whether or not some of them

might serve as possible means of biological control. It has long been known that *C. maenas* in the Woods Hole area is infected by an undetermined, encysted metacercaria and experiments have been conducted to discover its identity, life-history, and biology. Metacercariae were fed to white mice and excysted specimens (Fig. 6) recovered after 24 hours. Other cysts were fed to recently hatched, uninfected birds, *Sterna hirundo* and *Larus argentatus*. Large numbers of worms were recovered, including all stages from juvenile to fully mature specimens. The structure of the metacercariae, especially of the smaller and recently excysted ones, suggested that they may be specifically identical with a minute, stylet-bearing cercaria (Figs. 1, 1a) reported by Stunkard (1950), which occurs in *Littorina obtusata, L. saxatilis,* and rarely in *L. littorea*. Small green crabs were exposed to these cercariae and became heavily infected; enormous numbers of worms entered their tissues and developed to metacercariae, identical with those of natural infections. Small crabs exposed continuously with six to eight infected snails died in 10 to 20 days, and on dissection, each one yielded thousands of larvae. The parasites were present throughout the body of the crab, but the heaviest concentration was in the digestive gland. When first encysted, the cyst is oval, the wall is very thin, and the stylet persists for a long time. Eggs of the parasite, recovered from the droppings of terns and gulls, and others teased from the uteri of gravid worms, were used to infect specimens of *L. obtusata,* collected near the laboratory from an area which is not frequented by birds and where examination of 200 snails showed no infection. The eggs were embryonated under running sea water for seven days and then spread on fronds of *Fucus* which were allowed to partially dry to ensure that the eggs would become attached to the slimy surface of the alga. These fronds were placed with 10 small specimens of *L. obtusata* in a gallon jar with a small opening and sharply curved upper shoulders, half filled with sea water and provided with a stream of fine bubbles from an air pump. The water was not changed for ten days and subsequently on alternate days the top water was siphoned off and replaced with fresh sea water. Since the eggs of microphallid trematodes do not hatch until they are eaten by a suitable snail, and since from the design of the experiment it is impossible to tell when eggs were eaten, the age of an infection is not known precisely. The snails were first exposed on July 31, and on October 1, five snails that were alive in the jar were crushed and examined. Three of them were infected; two with large numbers of small daughter sporocysts but not yet producing cercariae, and the other contained three primary or mother sporocysts (Fig. 2) but no free daughters. The early stages of the infections afford clear evidence of their experimental nature and the complete life-cycle was thus consummated by laboratory infection of both intermediate and final hosts.

The adult worms agree so completely with the descriptions of *Microphallus similis* (syn. *Spelotrema simile*) as given by Jägerskiöld (1900) and Odhner (1905), that they are assigned to that species. The experimental demonstration of the life-cycle confirms the suggestion of Rees (1936), that *Cercaria ubiquita* Lebour is the cercarial stage of *M. similis*. Actually, the adults were described by Jägerskiöld (1900), the metacercariae by M'Intosh (1865), and the cercariae by Lebour (1907). Whether the specimens described by Nicoll (1907) as *S. excellens* and *S. feriatum* are specifically distinct remains to be determined.

Attempts to infect *Limulus polyphemus* were unsuccessful. Three small horse-

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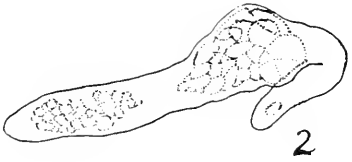
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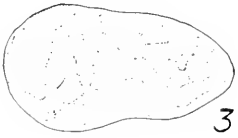
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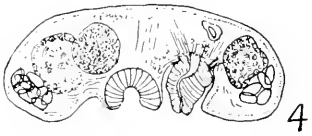
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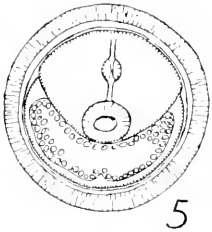
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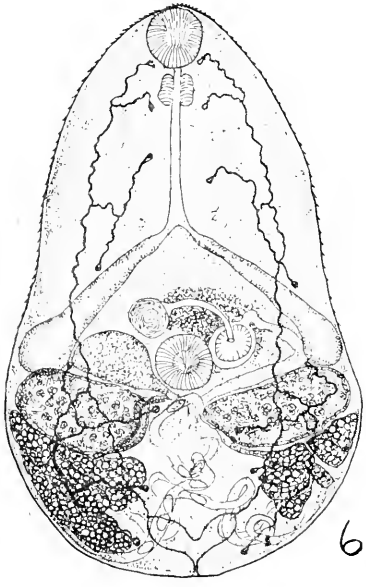
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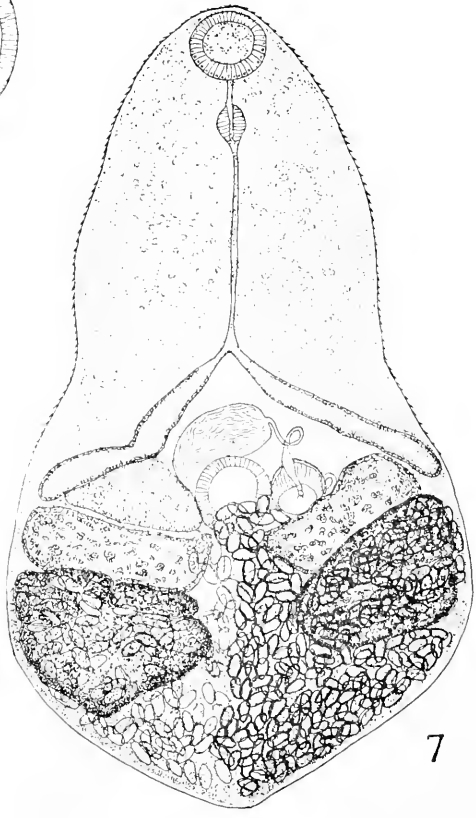
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shoe crabs with carapace widths of 17, 19, and 21 mm., collected near Orleans, Massachusetts, were exposed to cercariae for two weeks. On dissection, there were no recently entered, unencysted larvae although all three harbored mature cysts of *Microphallus limuli* in their livers. This finding supplements that of Stunkard (1953), who reported full-sized cysts in small *L. polyphemus*. In paragraph 3, line 4, of that report, 3 mm. should read 30 mm.

Description of Stages in the Life-Cycle

Measurements in millimeters

Adults (Fig. 7)

Egg-bearing specimens from gulls and terns are about the same size. Fixed, stained and mounted they measured: length, 0.36–0.7; width, 0.22–0.36; acetabulum, 0.048–0.065; oral sucker, 0.05–0.065; pharynx, 0.02–0.03; male papilla, diameter 0.038–0.058; testes, 0.10 × 0.06 to 0.16 × 0.09; seminal vesicle, 0.06 × 0.04 to 0.09 × 0.064; ovary, 0.08 × 0.05 to 0.1 × 0.062; eggs colorless on the ovarian side, yellow on the antovarian side, 0.022–0.027 × 0.011–0.012, often collapsed and somewhat distorted in fixed and stained specimens.

The body is oval to pyriform and either end may be wider; often there is a slight constriction between the more mobile forebody and the inert hindbody which is filled with reproductive organs and eggs. The dermomuscular wall is thin and weakly developed; it consists of the usual circular, longitudinal and oblique layers, but the fibers are delicate and relatively few. The edges of the forebody tend to turn ventrad, forming an adhesive cup. The cuticula bears flattened spines which are conspicuous on the anterior half of the body but become smaller and sparser posteriorly. The suckers are approximately equal in size; either may appear larger, depending on the degree of contraction by the sphincter and the size of the orifice. The esophagus is long and when the body is extended, it may be much longer than the ceca. The ceca are almost straight; they diverge at an obtuse angle and terminate at the acetabular level. The anterior tips are constricted and lined with cuticula, continuous with that of the esophagus. The excretory system is unchanged from the metacercarial stage and has the formula $2[\ (2+2)+(2+2)\ ]$. The testes are dorsal, lateral, opposite; the vasa deferentia arise at the medial, anterior faces, pass mediad and anteriad where they unite to form the seminal

Figure 1. Cercaria from *L. obtusata,* free-hand drawing of specimen stained with Nile blue sulphate; 1a, lateral aspect of stylet.

Figure 2. Primary sporocyst from *L. obtusata,* experimental infection; fixed and stained specimen, length 0.30 mm.

Figure 3. Daughter or secondary sporocyst from *L. obtusata;* natural infection; fixed and stained specimen, length, 0.375 mm.

Figure 4. Cross-section of a mature worm to show the relative position and size of the acetabulum and of the male papilla. The metraterm opens into the genital atrium near the base of the papilla and in front of the left testis whose anterior portion appears in the section; width of the worm, 0.28 mm.

Figure 5. Metacercaria from *C. maenas,* natural infection; cyst 0.46 mm. in diameter.

Figure 6. Specimen, much flattened to study the excretory system, from intestine of a white mouse, 24 hours after the cyst was eaten; length 0.55 mm.

Figure 7. Adult specimen from intestine of *Sterna hirundo,* experimental infection; one of the largest specimens, flattened under a cover glass, fixed and stained, length 0.69 mm.

vesicle, an oval sac which lies anterior and dorsal to the acetabulum. A coiled ejaculatory duct, enclosed in glandular cells, leads from the vesicle to the muscular male papilla which almost fills the genital atrium, situated at the left of the acetabulum. The ovary is oval to triangular, located on the right side of the body, between the seminal vesicle and the testis and cecum of the right side. The oviduct arises from the median posterior region, coils posteriad and ventrad where it expands to form a fertilization space from which Laurer's canal winds to the dorsal surface of the body, opening in the midline just behind the acetabulum. The oviduct then turns dorsad and anteriad, receives a short common vitelline duct and enlarges to form the ootype, lined with cilia and enclosed in the cells of Mehlis' gland. The vitellaria are large, lobed glands, situated below and behind the testes; ducts from the two sides pass mediad, anteriad and dorsad, uniting to form the common vitelline duct which discharges into the initial portion of the ootype. The uterus passes backward from the ootype almost to the posterior end of the body and then loops forward in coils on the ovarian side of the body as far as the end of the digestive cecum, then backward almost to the posterior end of the body where it crosses to the opposite side and forms a corresponding series of loops on the left side, with the terminal metratermal portion emptying into the left side of the genital atrium (Fig. 4). The extent of the uterus anteriorly is determined by the number of eggs; in certain specimens the uterine coils may be below and behind the testes whereas in others the coils may underlie the ends of the digestive ceca.

The egg and miracidium

Egg-production begins almost immediately after the metacercaria is eaten by the final host. Figure 6 shows a worm which, fed as a metacercaria to a white mouse twenty-four hours earlier, already has eggs in the initial portion of the uterus. At first the egg-shell is thin, flexible and transparent; but as the eggs traverse the uterus, the shells become thicker, harder, and bright yellow. This coloration of the shell obscures the larva in living eggs, but development can be followed by study of serial sections of gravid worms. The egg is operculate and the ovum is situated toward the opercular end of the egg. In eggs near the metraterm, the miracidium appears to be fully formed, but eggs used for infection experiments were kept for a week in running sea water to insure fully mature larvae. The miracidia emerge only after the eggs have been ingested by the snail host.

Sporocyst generations (Figs. 2, 3)

The amount of experimental material is limited, but three primary sporocysts were removed from a specimen of *L. obtusata* two months after exposure to eggs of *M. similis*. These sporocysts were sluggish, oval to cylindrical and 0.25 to 0.40 mm. long. One of them, fixed and stained, is shown in Figure 2. The presence in them of recognizable daughters identified them as sporocysts of the mother or primary generation. Daughter sporocysts obtained from two experimental infections were young, small, and very numerous. Daughter sporocysts of natural infection measured 0.10 to 0.60 mm. in length; they are oval, occur in

large numbers, more than one hundred in a single snail. The wall often contains a yellowish pigment; it is thin and in older sporocysts change of shape results from movement of contained cercariae. Figure 3 was made from a daughter sporocyst, 0.375 mm. long.

Cercaria (Figs. 1, 1a)

Body length, 0.1–0.22 mm.; width, 0.02–0.05 mm.; the larva is very thin, delicate, colorless. The tail is 0.01–0.012 mm. wide at the base; contracted it is 0.05 mm. long, with fine cuticular annulations; extended it may be 0.25 mm. long and very slender. There is no acetabulum; the larvae move by strokes of the tail but are unable to creep. They swim upward and sink when motionless. In swimming the body is contracted, bent ventrad, while the tail is extended and lashes violently. The body is covered with cuticular spines and the dorsal wall of the sucker bears a stylet, 0.023–0.026 mm. long and 0.009 mm. wide. The stylet (Fig. 1a) is asymmetrical in lateral aspect. The oral sucker measures 0.025–0.032 mm.; other parts of the digestive system are not yet developed. The body contains numerous cystogenous glands and on either side, near the middle, there are four penetration glands. The two anterior cells on each side differ from the posterior ones; the difference is demonstrated by the use of neutral red or Nile blue sulphate solutions, especially the latter, which stain the secretory granules of the anterior cells selectively while the contents of the posterior cells do not take the stain. Ducts from the anterior pair of cells pass forward beside those from the other cells for a short distance, but about halfway to the mouth they separate from the others and pass more mediad, crossing the dorsal side of the oral sucker, while the ducts from the other three penetration gland cells form a bundle that continues forward and passes at the side of the sucker. The ducts from the two anterior pairs of cells open to the surface ventrally, below the tip of the stylet, whereas the ducts from the posterior pairs open more anteriorly and at the sides of the tip of the stylet. The excretory system is shown in Figure 1; the vesicle is U- or V-shaped and the flame-cell formula is $2[\ (1+1)+(1+1)\ ]$. The cercariae are carried by respiratory currents into the gill chambers of *C. maenas* where they enter the body at the bases of the gills and possibly at other non-cuticularized places, pass by way of the vascular system to all parts of the body, and localize principally in the connective tissue of the digestive gland.

Metacercaria (Fig. 5)

The cysts increase in size and measure from 0.05 to 0.55 mm. in diameter; when the cercariae encyst they are bent ventrally; the cyst is oval and the wall is very thin and flexible. The stylet is retained for some time and readily identifies the species. As the metacercaria grows, the digestive tract and actabulum are formed and the number of flame-cells is doubled. As the worm grows, the cyst becomes spherical and the wall increases in thickness. In a full grown cyst, the cavity may be 0.35–0.40 mm. in diameter and the wall consists of two layers, an inner hyaline one which may be resolved into strata and attains a thickness of 0.02 mm., and an outer radially striated layer which increases to a thickness of 0.06 mm. The substance of this layer, after digestion in a pancreatin solution,

appears to consist of parallel prisms. The worms become almost full grown as metacercariae; the adults increase in size only by the further activity of the reproductive organs and the accumulation of eggs in the uterus. When younger and smaller metacercariae are eaten, the worms become gravid at a smaller size than when the metacercariae are older. The excretory vesicle of older metacercariae contains spherical concretions, the excretory wastes accumulated during the period of encystment.

Summary

1. The life-history of *Microphallus similis* has been worked out by experimental infection of both intermediate and final hosts.

2. Encysted metacercariae from *Carcinides maenas* developed to sexual maturity in *Larus argentatus* and *Sterna hirundo*. Eggs of the parasite developed in these hosts were used to infect *Littorina obtusata*. Two generations of sporocysts were recovered.

3. *Littorina saxatilis* and *Littorina littorea* also harbor the asexual generations at Woods Hole, Massachusetts.

4. The cercariae are minute, stylet-bearing monostomes and small green crabs, *C. maenas*, exposed to these cercariae became heavily infected; enormous numbers of larvae entered the tissues and developed into metacercariae identical with those of natural infections. Small crabs, each exposed continuously to the cercariae from six to eight infected snails, died in ten to twenty days and on dissection each yielded thousands of larvae.

5. The stages in the life-cycle of the parasite agree with descriptions by European investigators of corresponding stages: the metacercariae with metacercariae from *C. maenas*, described but not named by M'Intosh (1865); the adults with *M. similis* from Swedish gulls, described and named by Jägerskiöld (1900); and the cercariae with *Cercaria ubiquita* Lebour, 1907. The identity of these parasites as stages in the life-cycle of a single species is predicated.

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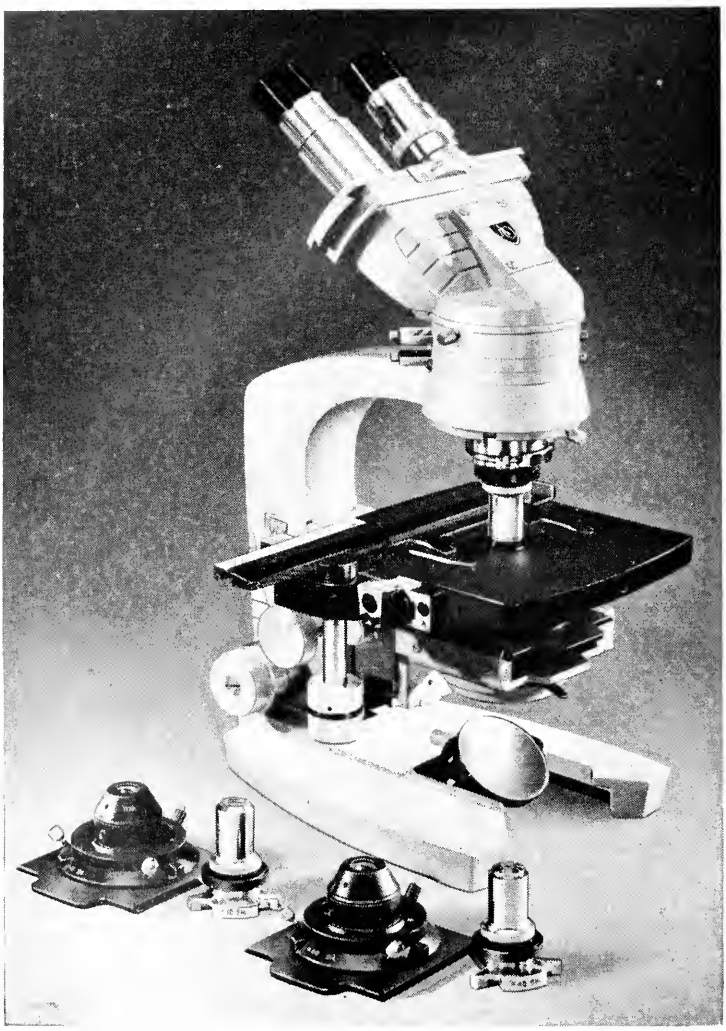

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PERSISTENT TIDAL CYCLES OF SPONTANEOUS MOTOR ACTIVITY IN THE FIDDLER CRAB, UCA PUGNAX [1]

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Early in the present century, it was reported that a number of littoral organisms showed cycles of behavior which persisted under so-called constant laboratory conditions with tidal frequencies and with phases adaptively related to tidal events of the areas from which the organisms were collected (Gamble and Keeble, 1903, 1904; Bohn, 1904, 1906). Later, Gompel (1937) found that several species of animals display tidal rhythms of O_2-consumption which also persist under constant laboratory conditions. These reports were not very successful in convincing the majority of biologists of the reality of persistent tidal rhythmicity. However, during the past few years, a number of studies have again pointed out that many organic processes, *e.g.*, color change, spontaneous activity, and O_2-consumption, in a rather wide variety of plants and animals do indeed vary with primary lunar or tidal frequency under constant conditions.

Rao (1954) found that the filtering rate of species of *Mytilus* was greatest at the times of high tides in the areas of collection when the mussels were maintained in the laboratory. This rhythm of behavior was clearly apparent day by day. However, many of the lunar or tidal cycles that have been described are apparent only by statistical analyses of 15 or 29 days of continuous data (Brown, Freeland and Ralph, 1955; Brown, Webb, Bennett and Sandeen, 1955; Brown, Shriner and Ralph, 1956).

The results of the work to be described demonstrate that the fiddler crab, *Uca pugnax*, does have an overt rhythm of primary lunar frequency, a rhythm of spontaneous motor activity.

Materials and Methods

In both 1955 and 1956, males of the species *Uca pugnax* were used in these studies. The crabs were collected from Chapoquoit beach or Sippiwisset beach on the Buzzards Bay side of Cape Cod. Tidal events on these two beaches occur roughly 10 minutes later than they do at New York City. In the laboratory the animals were kept in white-enamelled pans in a small amount of sea water until they

[1] These studies were aided by contracts between the Office of Naval Research, Department of Navy, and Northwestern University, NONR 122803.

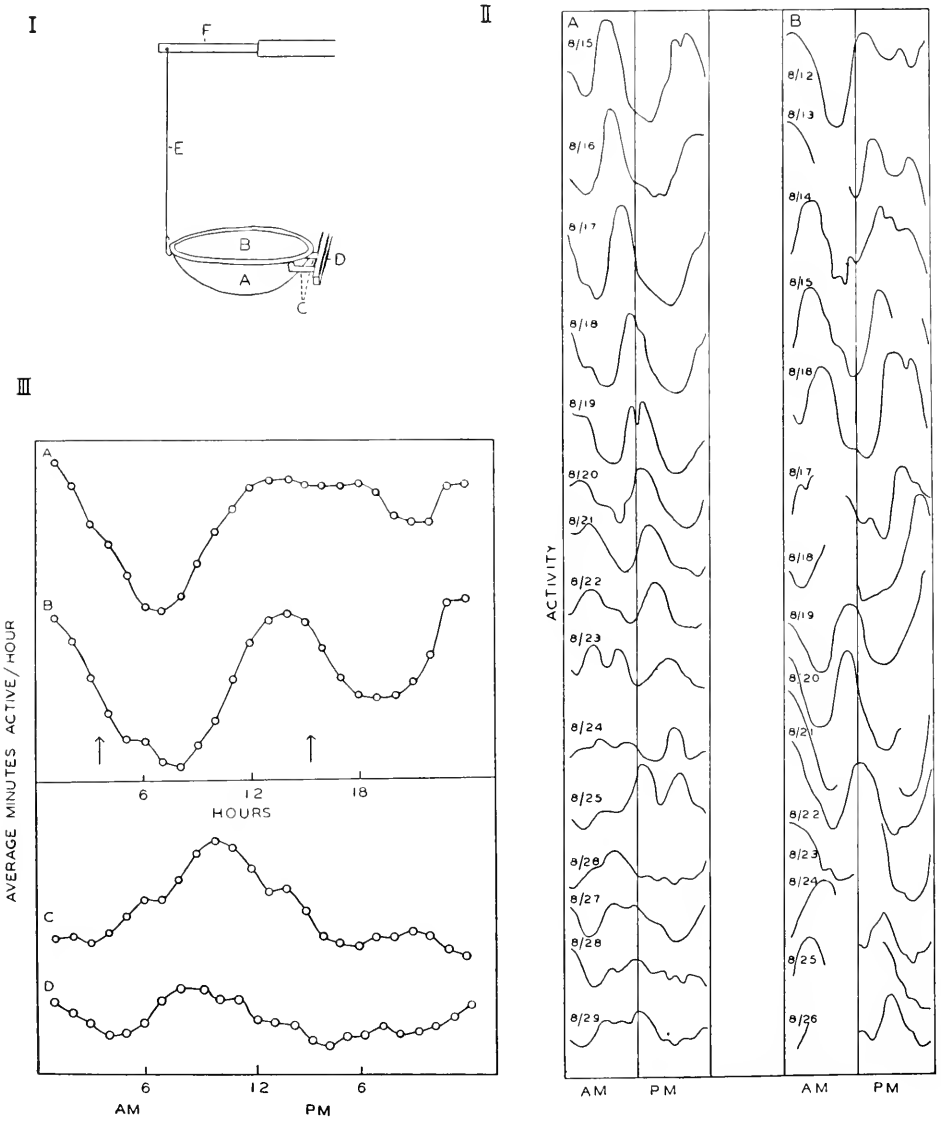

FIGURE 1. I. An illustration of part of the apparatus used to record the motor activity of an individual fiddler crab. For explanation of letters, see text. II. A, the average cycles of activity for a group of 20 crabs for the 15 consecutive days from August 15 through August 29, 1955. B, the average cycles of activity for a group of 20 crabs for the 15 consecutive days from August 12 through August 26, 1956. III. A and B, the mean, 29-day, tidal cycles of activity of fiddler crabs for July 6 through August 3, 1955 and August 2 through August 30, 1955, respectively. The arrows indicate the relative times of low tide at Chapoquoit Beach. C and D, the mean, 29-day, solar cycles of activity of fiddler crabs for July 6 through August 3, 1955 and August 2 through August 30, 1955, respectively.

were placed in the recording apparatus. This was done usually within two days of their collection.

Part of the recording apparatus employed is illustrated in Figure 1, I. A single crab was placed with a small amount of sea water in a plastic saucer (A), and covered with a circular piece of cardboard (B) which fitted the saucer tightly. Each of 10 saucers was supported on one side by metal bands (C) which were in turn fastened to a rigid horizontal bar (D). To the opposite side of each saucer was attached a nylon thread (E) which was fastened to the lever of a spring balance recording system (F) equipped with an ink-writing pen. The movements of the crabs in the finely balanced saucers were recorded on a kymograph which made one complete revolution every 24 hours. The spring balances and kymographs were of the type described by Brown (1954a).

The experiments were carried on in an inner room in a brick building at the Marine Biological Laboratory in which the light intensity at the level of the recording apparatus was at all times essentially constant and less than two ft. c. The containers with the inclosed crabs were shielded from movements and shadows in the laboratory. The air temperature in the room varied non-rhythmically from 21° to 24° C. through the summer months.

During the summer of 1955, the activity of 10 crabs was recorded continuously from July 6 through August 13, and that of 20 crabs was recorded from August 14 through 30. Freshly collected crabs were placed in the recorders on July 5, July 14, and August 14. In 1956, 20 crabs, which were collected on June 16 were placed in the recorders on that day, and their activity was recorded through the afternoon of June 26. On August 11, another group was collected, and the activity of 20 of these was recorded from that day through August 28.

The data recorded in 1955 were analyzed in the following manner: the number of minutes of each hour that each animal was active was determined from the kymograph recordings. From these figures was calculated the average number of minutes per hour that the population (either 10 or 20 individuals) was active. The hourly data were converted into three-hour moving means as given in Table I. Mean, 29-day solar and lunar cycles of activity were analyzed by methods used extensively in our laboratory (Brown, Bennett, Webb and Ralph, 1956; Brown, Freeland and Ralph, 1955) by which any possible solar or lunar cycles are synchronized day by day.

The results for 1956, given in Table II, are in terms of activity units per hour. Activity units were derived as follows: for each hour, the number of animals of the group of 10 that was active, *e.g.,* 8 out of 10, was recorded from the kymograph records. From these hourly values were calculated three-hour moving sums. The sums for the two groups of 10 each that were observed concurrently were added.

For the periods August 14–30, 1955, June 17–26, 1956, and August 12–28, 1956, when the motor activity of 20 individuals was recorded, the average values per hour for the two groups of 10 crabs each were correlated on an hour-by-hour basis. However, in this report, all data are given as the average for the entire population that was observed at any particular time.

Results

The hourly values of spontaneous motor activity for *Uca pugnax* in 1955 are found in Table I, and those for the two periods of 1956 in Table II. By merely

scanning these data, it is possible to observe that within many solar days there are two periods, each several hours in duration, of relatively high activity which are separated from one another by 12 to 13 hours, *e.g.*, on July 7, 1955 (Table I), there was high activity during the first two hours of the day and again during hours 12 and 13. By noting the situation on two consecutive days, *e.g.*, July 16 and 17, 1955 (Table I), it can be seen that the two periods of high activity occur later in the solar period on the second day than on the first. It is also evident from inspection of the data that there is a considerable range in the values, 0 minutes per hour active to 45

TABLE I

The average number of minutes active/hour for groups of fiddler crabs for 1955

	Hour																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
July 6	33	30	23	14	7	8	9	19	27	37	38	34	32	26	21	13	12	12	17	17	18	21	23	26
7	29	29	22	19	10	11	8	16	23	32	34	39	39	33	25	17	16	—	—	—	24	17	13	11
8	14	23	28	29	23	—	—	—	—	15	16	18	14	18	18	17	11	8	8	8	10	8	10	12
9	17	20	16	17	12	10	6	4	3	3	6	10	17	20	14	9	6	6	5	4	8	9	8	7
10	10	14	15	13	13	11	8	6	6	10	10	13	11	14	13	13	9	6	5	8	10	11	6	5
11	6	9	12	14	13	10	9	7	7	6	6	4	4	7	11	10	8	5	6	5	6	6	8	5
12	4	4	6	8	10	12	14	12	10	6	4	2	1	1	4	6	7	8	6	6	2	4	4	5
13	3	3	3	7	10	10	7	5	—	—	—	13	10	7	5	1	6	11	13	8	4	3	3	4
14	3	2	2	3	7	9	10	7	—	—	32	17	13	13	19	23	29	34	36	31	26	22	15	12
15	12	8	10	24	36	45	41	36	28	21	15	9	6	3	7	13	20	22	28	30	32	28	24	20
16	14	13	11	18	23	31	34	36	31	24	15	9	10	8	8	5	8	14	19	24	22	18	14	15
17	15	9	6	6	10	18	22	25	23	20	16	13	8	7	6	7	7	5	7	11	14	15	13	14
18	14	14	11	9	8	8	11	15	20	21	22	18	—	—	—	6	6	5	3	6	8	11	13	18
19	17	15	8	6	8	8	8	7	13	15	19	14	12	5	2	1	1	4	4	5	3	5	9	11
20	15	16	16	10	7	6	7	9	12	19	16	14	11	13	10	5	2	2	2	2	1	1	3	6
21	7	5	5	6	12	12	12	8	5	3	5	7	—	—	—	9	5	3	2	2	4	4	5	4
22	5	8	12	12	14	12	12	10	12	10	6	5	7	8	11	12	12	7	5	3	4	3	3	2
23	4	6	9	15	17	19	12	9	7	8	9	6	4	4	4	4	5	5	3	1	3	6	9	7
24	5	4	8	6	5	3	10	18	19	18	15	15	10	6	6	8	9	10	12	11	5	1	1	2
25	5	6	6	5	3	4	3	11	23	31	26	17	8	8	7	7	7	7	7	9	9	12	9	8
26	5	5	4	3	4	8	11	20	19	21	13	8	3	1	—	—	—	—	2	3	7	8	8	6
27	6	6	3	2	2	5	10	11	11	9	11	10	7	5	2	3	2	2	3	3	3	5	7	7
28	5	4	4	5	—	—	—	—	—	11	12	18	18	10	4	1	0	1	—	—	—	—	—	—
29	—	—	—	—	—	—	—	—	—	—	—	24	21	16	11	8	5	3	2	3	6	11	15	11
30	8	4	5	5	7	6	6	3	4	11	16	19	19	19	16	11	6	4	3	4	5	6	4	3
31	3	4	7	12	15	18	16	16	14	13	15	19	21	23	19	14	9	8	9	9	7	7	8	11
Aug. 1	11	9	5	6	9	13	12	13	12	12	12	14	17	19	22	22	19	14	17	18	14	10	5	3
2	3	5	6	7	6	6	6	10	11	8	3	5	12	19	22	20	20	20	18	16	11	10	6	7
3	6	7	8	10	11	—	—	—	—	—	13	9	6	10	11	19	24	30	30	25	23	20	13	11
4	12	12	12	11	14	15	14	12	7	10	12	12	9	7	10	8	10	11	11	11	9	12	10	11
5	11	12	12	9	6	5	6	7	6	5	4	4	3	4	6	8	9	10	11	11	10	9	10	11
6	10	9	5	5	4	6	7	7	8	8	8	4	3	4	5	4	3	3	5	6	6	7	9	9
7	8	7	6	7	5	8	7	8	5	6	10	14	14	9	8	10	11	11	9	9	11	8	7	5
8	9	8	7	5	7	10	8	8	7	8	9	8	7	5	3	5	7	8	7	6	9	11	11	10
9	9	7	4	5	8	12	10	7	8	7	7	3	3	6	6	8	11	16	15	9	4	6	6	13
10	15	19	18	17	15	13	11	8	6	3	2	3	7	10	13	15	17	17	17	15	11	5	10	15
11	16	14	12	15	19	21	20	18	18	16	10	5	3	3	5	8	16	16	11	4	1	2	1	2
12	7	11	11	9	11	16	18	19	19	17	12	7	7	6	9	9	11	14	16	16	15	13	11	7
13	5	10	10	9	10	6	9	8	9	9	8	8	9	9	9	7	5	7	6	9	10	12	12	9
14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	19	17	21	23	20	25	26	26
15	23	21	16	15	20	30	40	40	34	22	13	10	8	6	5	7	16	20	33	31	36	34	29	22
16	17	15	13	11	14	19	35	40	37	26	19	15	14	13	10	11	10	16	19	25	29	31	31	31
17	29	19	12	10	6	7	14	26	37	37	32	22	14	10	8	6	5	3	6	11	17	22	23	29
18	25	19	14	13	12	7	7	9	17	27	31	27	24	17	11	7	5	4	4	6	12	18	21	28
19	24	23	23	20	11	8	7	6	10	18	26	20	28	23	16	11	4	3	3	4	6	12	14	18
20	19	20	20	16	14	12	11	10	6	13	16	24	24	23	18	14	10	8	6	4	5	6	13	16
21	19	19	21	22	18	16	12	10	7	5	8	13	20	21	20	16	12	8	7	6	6	4	5	8
22	10	14	17	17	16	13	11	11	9	7	7	10	14	19	20	17	12	7	6	5	5	4	6	7
23	10	9	15	16	17	11	12	15	15	10	6	5	6	9	11	13	14	14	10	8	6	5	5	5
24	8	10	10	11	13	12	11	10	12	12	11	8	7	6	6	7	11	17	15	7	7	7	9	8
25	8	6	5	8	10	11	10	10	11	13	18	26	27	23	16	13	17	22	24	20	15	12	12	9
26	10	11	14	16	16	18	20	22	20	17	15	13	13	13	12	13	12	11	12	13	13	13	16	20
27	19	15	10	9	11	16	19	20	19	19	20	18	15	14	13	11	9	7	8	10	12	15	19	20
28	21	16	12	9	9	11	14	13	14	16	17	17	15	13	13	13	11	12	10	12	12	12	10	7
29	6	5	5	9	12	13	12	13	13	12	15	15	14	12	9	6	6	5	7	6	7	8	10	9
30	10	10	10	8	7	6	7	7	8	9	11	13	14	14	11	9	6	7	4	—	—	—	—	—

TABLE II

The activity units/hour for groups of fiddler carbs for 1956

	Hour																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
June 17	20	28	36	41	37	30	27	23	22	18	18	17	18	24	30	37	35	33	30	29	26	24	25	28
18	26	24	29	36	41	37	31	26	23	22	19	17	17	18	17	24	30	37	35	30	25	24	28	29
19	28	26	25	31	38	44	42	—	—	—	—	—	26	20	16	13	15	20	21	23	21	23	23	24
20	26	27	29	30	32	34	36	36	33	30	25	19	17	19	22	20	16	17	21	30	32	32	31	28
21	24	18	19	21	21	20	25	31	33	31	28	26	20	16	11	12	10	13	19	26	29	30	28	28
22	27	26	25	22	24	23	24	28	31	33	32	33	28	20	14	17	23	23	22	24	30	30	26	25
23	27	29	27	26	29	28	25	24	30	42	42	40	30	25	20	18	16	12	9	13	22	23	30	27
24	26	25	24	21	24	24	24	21	24	32	33	31	28	23	18	11	10	9	10	8	19	26	35	30
25	28	25	27	27	27	24	24	21	21	25	29	30	28	25	17	12	7	11	14	12	11	14	26	34
26	33	30	29	27	27	26	23	19	15	20	23	27	27	26	20	16	11	—	—	—	—	—	—	—
Aug. 12	57	56	53	51	43	35	28	27	25	28	37	50	55	56	54	50	48	47	52	50	48	44	49	54
13	58	56	53	48	44	—	—	—	—	—	36	32	39	48	52	49	42	41	40	43	46	42	35	29
14	32	38	48	49	49	40	35	24	24	21	30	28	33	37	42	47	44	44	39	40	40	37	31	24
15	28	37	46	—	—	—	36	29	28	24	18	18	22	32	41	47	45	38	—	—	—	—	38	32
16	24	20	24	32	37	40	35	27	16	13	13	11	9	9	15	29	42	44	43	39	43	36	26	20
17	17	26	24	29	29	—	—	—	—	22	17	13	13	14	10	7	14	24	30	31	27	26	22	21
18	15	13	13	17	21	25	—	—	—	—	—	13	9	11	11	13	12	14	18	27	35	42	40	34
19	24	20	16	14	8	8	10	20	28	30	31	26	20	14	11	10	10	12	17	20	25	34	41	40
20	34	27	22	16	14	14	16	25	34	40	36	29	20	14	8	7	6	12	14	—	—	—	44	41
21	39	35	29	24	17	13	9	10	—	—	—	—	—	—	—	—	14	9	9	7	7	11	23	35
22	38	34	26	23	19	16	10	10	15	23	28	32	31	29	23	16	11	8	10	10	11	16	23	30
23	36	35	34	27	24	20	21	17	17	17	19	—	—	—	—	39	21	15	13	12	10	11	13	28
24	19	24	28	32	36	36	36	31	—	—	—	15	14	20	22	26	20	15	11	11	9	12	12	17
25	21	28	33	33	29	25	—	—	—	—	13	12	—	—	—	32	30	22	18	13	13	11	11	11
26	16	20	23	—	—	—	—	—	—	—	—	22	22	19	23	29	34	33	28	23	21	17	17	19
27	25	25	27	26	31	32	38	37	32	30	28	25	19	16	22	23	25	25	27	27	26	23	20	17
28	16	18	21	20	25	27	32	32	33	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—

minutes per hour active in 1955, and 8 activity units per hour to 58 activity units per hour in 1956. Near the end of a particular recording period, both the mean daily activity and the amplitude of the cycles are lower than at the beginning of the same period.

These results are shown graphically in Figure 1, II A and B, in which are plotted the cycles of motor activity for groups of 20 crabs for August 15 through 29, 1955 and August 12 through 26, 1956. In Figure 1, II A, one can follow the moving of the two peaks of activity across succeeding solar days from August 15 to 22. In this instance, the peak that occurred between hours 7 and 8 on August 15 occurs between hours 14 and 15 on the 22nd, and the evening peak of August 15 has moved to the early morning on August 22. For the cycles of August 23 through 29, it is more difficult to distinguish maxima or the movement of these maxima very precisely. There seems to be a warping and diminution of a peak as it moves into the afternoon and evening hours. The peak of the morning hours which can be identified on August 20, can be traced through August 29; however, it, too, shows warping and broadening.

Generally, the same characteristics are to be seen in Figure 1, II B, especially for the cycles of August 12 through 16. After this time, the data are incomplete, and it is not advisable to complete the curves from only the available data. A period of high activity seen just after 0 hour on August 12 is identified as a rather sharp maximum at 12 on August 22, and similarly the afternoon high (hour 14) on August 12 is identified between hours 3 and 4 on August 25. Again, the warping and broadening of maxima, observed in 1955, are evident.

In order to demonstrate the reality of the movement of peaks of activity across solar days at the average primary lunar or tidal rate of 51 minutes per day, the rate of movement for specific peaks was determined for 20 different intervals from the data of 1955 and 1956. The intervals used were in no case less than two days or more than 11 days. The average rate for these periods was 50.1 ± 3.58 minutes per day.

Tidal phase relationships as well as frequency relationships are to be noted in these rhythms of activity. It was observed that in 1955, the peaks of activity occurred two to three hours before the times of low tide on the beaches from which the crabs were collected. In 1956, the maximum activity was, on the average, four to five hours before low tides.

This phase relationship is shown in Figure 1, III in which are plotted the mean, 29-day tidal cycles of locomotor activity for July 6 through August 3, 1955 (A) and August 2 through 30, 1955 (B). These curves are plotted so that the times of low tides at Chapoquoit Beach, as indicated by arrows, lie directly under one another, although the tidal events actually occurred at different times on the two days with which the 29-day analyses were begun. The times of low tide were: 3:22 and 15:19 on July 6 and 1:34 and 13:37 on August 2. In these figures, the form of the tidal cycle, discussed previously, is again apparent. In A, a sharp peak of activity is seen at hour 1, or 2 hours and 22 minutes before low tide. The second maximum takes the form of a broad peak from hour 13 through 18, extending from 2 hours before until 3 hours after low tide. Activity fell rather steeply from 1 until 7 while the activity following the second maximum did not decrease so steeply nor to so great a degree. The cycle for August 1955 (Fig. 1, III B) was very much like that illustrated in Figure 1, III A, although the second period of high activity does not last so long in the former as in the latter. Again, the activity persisted at a higher level following the second maximum than it did following the first peak.

In Figure 1, III C and D are plotted the mean, 29-day, solar cycles for the same periods of time as the lunar cycles. It is apparent that the amplitude of the solar cycles is less than that of the lunar cycles. From the lowest to the highest values there was a 2.4-fold increase in the tidal cycle for July 6 through August 3, and a 2.2-fold increase in the tidal cycle for August 2 through 30, whereas the increases for the solar cycles for the same periods were 1.6-fold and 1.3-fold, respectively. Although the amplitude of these solar cycles is low, the cycles indicate that activity between hours 6 and 12, other factors equal, is higher than at other times of the solar day. This tendency can be seen in Figure 1, II A by comparing the heights of the two peaks, *i.e.*, the morning peak is higher than the afternoon peak from August 15 through 17.

Coefficients of correlation for the activity of two groups of crabs, the activities of which were recorded independently during the same periods of time, were: + 0.768 ± 0.028 for August 14 through 30, 1955; + 0.410 ± 0.058 for June 17 through 26, 1956; and + 0.687 ± 0.034 for August 12 through 28, 1956.

Discussion

The cycle of spontaneous motor activity for the fiddler crab, *Uca pugnax,* described in this report, appears to be the first example of a clearly overt locomotor rhythm of primary lunar or tidal frequency. The occurrence of two peaks of ac-

tivity, 12 to 13 hours apart, within one solar day, and the movement of these peaks across succeeding solar days at an average tidal rate establish the reality of a tidal cycle persisting under laboratory conditions. It must be pointed out that the day-to-day preciseness of this rhythm decreases somewhat after the crabs have been in the recording containers seven or eight days. The warping and broadening of peaks, as well as the diminution of activity, discussed previously, cannot be explained satisfactorily at the present time. It is tempting to postulate that these phenomena are indications that an internal timing mechanism is not able to maintain a precise cycle longer than a week or so under constant conditions, and that after this time unknown external signals alone maintain only a less regular rhythm. Evidences for both endogenous and exogenous components of persistent rhythmicity in fiddler crabs have been reported recently (Brown, Webb and Bennett, 1955; Brown, Webb, Bennett and Sandeen, 1955).

The difference between the phase relationships of actual tidal events and the peaks of activity observed in 1955 and 1956 brings up questions regarding the setting of phases of tidal cycles, and to what extent behavior cycles under constant conditions parallel those of the organisms in their natural environments. The lunar or tidal cycles described previously indicate that although many species have cycles of 12.4 and/or 24.8 hours, phase relationships are species specific. For example, the spontaneous activity of quahogs is low during the times of low tides when this species may not be covered by water (Bennett, 1954), while the pigment in the melanophores of the fiddler crab, *Uca pugnax,* is most dispersed shortly before the times of low tide when these animals are typically active on the beaches (Brown, Fingerman, Sandeen and Webb, 1953). In these cases, the phases of the persistent tidal cycles seem to indicate some adaptiveness of the cycles to conditions which obtain under natural field conditions. On the other hand, Fingerman (1956) has reported that fiddler crabs, *Uca pugilator* and *Uca speciosa,* collected from regions of the Gulf coast where there is but one low tide per day, have persistent tidal cycles of color change characterized by two peaks of pigment dispersion during each solar day, one shortly after low tide, and the second 12.4 hours later or shortly after high tide.

The relationship observed between the fiddler crab activity and the times of low tide in 1955, *i.e.,* maximum activity two to three hours before low tides, might suggest that the behavior of the crabs in the laboratory is much the same as that of the crabs on the beaches. Typically, these crabs begin to emerge from their burrows as the water recedes following a high tide, and great numbers of these crabs are usually running on the beaches until shortly before low tide. However, the observations for 1956, *i.e.,* that the peaks of activity occurred four to five hours before low tide, suggest that the phase relationships of persistent tidal cycles change, and may not always reflect adaptive behavior of the species in its natural, changing, physical environment. It is possible that the difference in the rhythmic behavior of the crabs between the two years may reflect differences noted in field observations between these same two years. In 1955, as was usual, the crabs were collected easily shortly before the times of low tides. In 1956, the animals were difficult to collect since there were few running on the beaches before low tides. Many of the crabs used in 1956 had to be dug from their burrows. It is possible that stimuli other than those resulting from emergence and running of the crabs may set the phases of the observed tidal cycles.

That the phases of a tidal cycle do shift in the absence of experimental modifications is shown clearly in the report of Brown (1954b). In this work, it was found that the maxima of the tidal cycle of oysters collected from New Haven Harbor and shipped to Evanston, Illinois correlated with the times of high tide in the native habitat during the first two weeks of maintenance under constant conditions. During the next month, the same group of oysters showed a rhythm with maxima at the times of lunar zenith and nadir. Two other reports contain information regarding the experimental shifting of the phases of tidal cycles (Brown, Fingerman, Sandeen and Webb, 1953; Rao, 1954). However, since the problem of the setting of phases of persistent tidal cycles is one that has not been investigated to a great degree as yet, much more work must be done before any definite statements can be made.

The overt tidal rhythm described here promises to be one by which not only the problem of phase setting but also experimental modifications of tidal cycles can be studied. The facts 1) that the cycle of locomotor activity repeats itself rather precisely on a day-to-day basis for at least a week after the crabs are placed under constant conditions, and 2) that the cycles of two independent small groups correlate significantly to a high degree do point to its usefulness in such studies.

Summary and Conclusions

1. The spontaneous locomotor activity of groups of fiddler crabs, *Uca pugnax,* was recorded during the summers of 1955 and 1956 under constant laboratory conditions.

2. This species shows an overt rhythm of activity of primary lunar or tidal frequency. Within solar days, there are two peaks of activity which are 12 to 13 hours apart. These maxima move across succeeding solar days at an average tidal rate. The cycles are precise for at least a week under constant conditions, but after this time, some warping and displacement of maxima occur.

3. A low amplitude solar rhythm of activity is apparent upon analysis of 29 days of continuous data. This rhythm is characterized by high activity between hours 6 and 12 of the solar day.

4. There was observed a difference in phase relationships of the tidal rhythms between 1955 and 1956. The state of the problem of the setting of phases of persistent tidal cycles is discussed.

5. Since this rhythm is precise for at least a week under constant conditions, and since the cycles of two groups of crabs recorded independently correlate to a high degree, this cycle appears to be an excellent one with which to study experimental modifications of persistent tidal rhythms.

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STUDIES OF THE METABOLISM OF PHOSPHORUS IN THE DEVELOPMENT OF THE SEA URCHIN, STRONGYLOCENTROTUS PURPURATUS [1]

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The eggs of sea urchins contain a group of acid-soluble phosphorylated compounds whose barium salts are soluble in alcohol. The magnitude of this fraction is unusually large in comparison with other animal tissues. The unfertilized eggs of various sea urchins and asteroids have been reported to have from 9.3% (Mende and Chambers, 1953) to 39.7% (Whiteley, 1949) of their acid-soluble phosphorus in the form of barium-soluble, alcohol-soluble compounds. In various vertebrate tissues these compounds comprise about 1 to 8.3% of the acid-soluble phosphorus (LePage, 1948; Sacks, 1949). Aside from quantitative measurements, very little is known about these compounds or the part they play in the metabolism of the echinoderm egg. Lindberg (1943) reported finding a compound in this fraction from the egg of the heart urchin, *Brissopsis*, which he subsequently (1946) identified as 1, 2-propanediol phosphate, whose metabolism he studied. Hörstadius and Gustafson (1947) reported some animalizing effect by both synthetic propanediol phosphate and a natural compound, and Borei (1948) has described the effect of this ester on egg respiration. However, Rudney (1952, 1954) has reported that the barium salt of propanediol phosphate is not soluble in alcohol, and the significance of the above observations, therefore, is not clear. Mende and Chambers (1953) have shown that some of the phosphorus of the barium-soluble, alcohol-soluble fraction of *Asterias forbesii* and *Strongylocentrotus dröbachiensis* is acid-labile.

This paper supplies information relative to the extent to which the barium-soluble, alcohol-soluble fraction serves as a storage material for metabolism, and the rate of turnover of the compounds of this fraction in the eggs and embryos of the sea urchin, *Strongylocentrotus purpuratus*. The possibility has been explored that there are special periods in the developmental process when the metabolic activity of these compounds varies. The existence of propanediol phosphate as a component of this fraction has been examined, and preliminary chromatographic determination of the number of esters in the fraction has been made. As an outgrowth of the turnover studies, an unusual pattern of permeability of embryos to inorganic phosphate during larval development has been found.

[1] This investigation was supported in part by the Research Fund 171 of the University of Washington. A report of these studies was given at the meeting of the Western Society of Naturalists, December, 1952.

[2] Currently on duty in the Navy.

Materials and Methods

Embryological. The sea urchins used in these experiments were *Strongylocentrotus purpuratus* (Stimpson), collected from San Juan Island, Washington. Animals were induced to spawn by the injection of isotonic KCl (Tyler, 1949). Batches of eggs less than 95% fertilizable were not used. "Dry" sperm were diluted to a 1% suspension immediately before use. After insemination, excess sperm were removed from eggs by washing with fresh sea water. Filtered sea water was used throughout.

In experiments on embryos older than the two-cell stage suspensions of developing embryos were cultured in a four-liter Erlenmeyer flask which lay at an angle of about 30° in a water bath held to 11.0 ± 0.1° C. by a thermostat. Egg suspensions, ranging in concentration from 0.3 to 0.9% by volume, were gently agitated by rotating the flask at 28 rpm. For egg counts, 10-ml. aliquots were taken and, after appropriate dilution, ten individual counts were made and averaged.

Aliquots of 300 ml. were taken from the stock suspensions at different stages of development for analysis of phosphates and for separate incubation with radioactive phosphate. Carrier-free radioactive phosphate was added to give a final radioactivity of 0.04 μc./ml., and incubation was continued in the same manner as described above, normally for 60 minutes. Duplicate 10-ml. aliquots were then taken for analysis of total phosphorus. The embryos were removed from the remainder of the suspension in a lucite centrifuge patterned after the Foerst plankton centrifuge. After washing with fresh sea water the embryos were frozen and stored for later analysis.

In experiments with eggs prior to the first cleavage the incubation times and radiophosphate concentrations varied and are given with the results. These samples were analyzed immediately without freezing.

Analytical. The extraction of the barium-soluble, alcohol-soluble material from the embryos followed the procedure of Sacks (1949) and that of Umbreit, Burris and Stauffer (1949). In some instances the other phosphate fractions identified below were also prepared. The sample was thawed and homogenized in a Potter-Elvehjem type of homogenizer in 2 ml. of 0.5 N $HClO_4$. This and the subsequent steps were carried out near 0° C. The homogenate was centrifuged and the residue (acid-insoluble fraction) re-homogenized twice with 1 ml. of 0.5 N $HClO_4$. The three supernatants were pooled, four volumes of re-distilled 95% ethyl alcohol added, and the mixture set aside for one hour. The extract was centrifuged, the residue (acid-soluble, alcohol-insoluble, probably polysaccharides) washed twice with acid-ethanol, the combined supernatants adjusted to pH 8.2, and 1 ml. of 25% barium acetate added. The precipitate (barium-insoluble plus barium-soluble, alcohol-insoluble fractions) that formed after one hour was centrifuged and washed with ethanol adjusted to pH 8.2. The supernatants were brought to 50.0 ml. This is the barium-soluble, alcohol-soluble fraction.

Phosphorus was determined by the method of Berenblum and Chain (1938), and, in a few cases, by a modified Fiske and SubbaRow (1925) method with ferrous sulfate as reducing agent. The dried samples, blanks, and standards were digested in 70% $HClO_4$. The blue isobutanol extracts resulting from the phosphorus determinations were used for radioactivity assay. Aliquots were pipetted into planchets, dried, and counted with a Geiger-Müller counter equipped with an end-window tube

with window thickness of 3.26 mg./cm.^{2} Samples were counted to an error of less than 1%. In experiments with pre-cleavage stages, where phosphate was analyzed by the Fiske and SubbaRow method, aliquots of eggs or extracts were dried directly on planchets.

Paper chromatographic analysis for phosphate esters was carried out using a chromatographic system the details of which will be published elsewhere. The solvent system contained n-butanol, n-heptyl amine, and water. The descending method was used with washed Whatman No. 1 filter paper. The chromatograms were run at 1° C. for about 10 hours and developed by spraying with the Hanes-Isherwood molybdate reagent, heating at 80° C. for 5 minutes (Hanes and Isherwood, 1949), and irradiating with ultraviolet light at wave-length 2537 Å (Bandurski and Axelrod, 1951).

Results

If the phosphorus-containing compounds of the barium-soluble, alcohol-soluble fraction serve as a reserve of phosphorus, energy, or precursors of other substances during development, an indication of this function would probably be given by a decrease in the concentration of the phosphorus in the fraction as development progresses. Two experiments, each involving the eggs of a single sea urchin, were carried out to determine if this occurs. In each a large batch of fertilized eggs was cultured at 11° C. to the early pluteus stage. Aliquots were taken at intervals for determination of total phosphorus and barium-soluble, alcohol-soluble phosphorus. The results are given in columns three and four of Table I, and are shown in

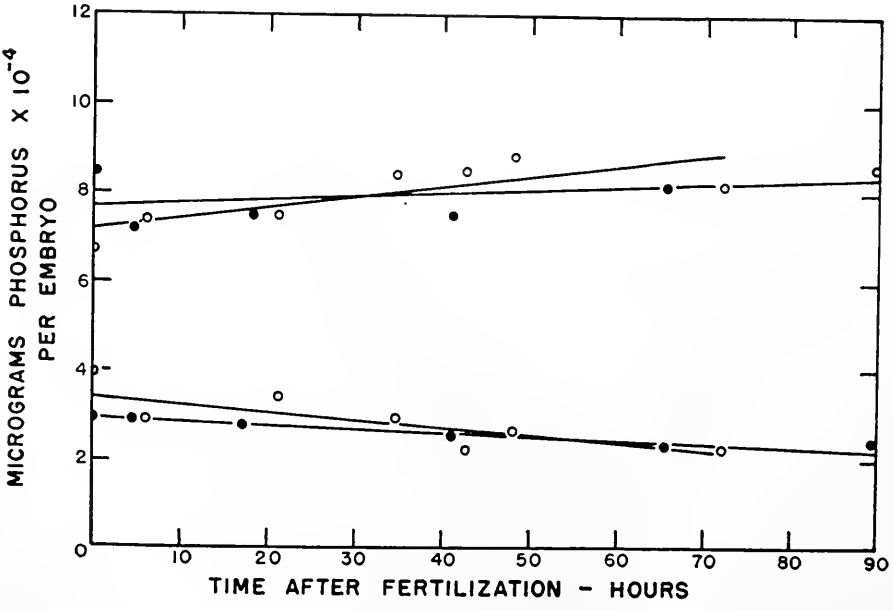

Figure 1. Total (upper curve) and barium-soluble, alcohol-soluble phosphorus content (lower curve) of developing embryos of *Strongylocentrotus purpuratus*. Solid circles are data from Experiment 1 and open circles are from Experiment 2 of Table I.

TABLE I

Amount of phosphorus and rates of incorporation of radioactive phosphorus in embryos and barium-soluble, alcohol-soluble compounds of embryos of the sea urchin, Strongylocentrotus purpuratus

Embryos		Phosphorus content μg./egg		Radioactive phosphorus content (cts./min./egg/hr.)		% BSAS-P^{32}	Specific activity (cts./min./μgP/hr.)		% BSAS-P^{32}
Age	Stage	BSAS-P	Total P	BSAS-P^{32}	Total P^{32}		BSAS-P^{32}	Total P^{32}	
Experiment No. 1									
0 hrs.	UF*	3.0×10^{-4}	8.5×10^{-4}	—	—	—	—	—	—
4½	2-Cl	2.9	7.2	6.5×10^{-2}	120×10^{-2}	5.4	225	1710	13.2
17	UB	2.8	7.6	15.2	221	6.9	532	2910	18.3
41	EG	2.5	7.6	2.8**	36**	7.8	108**	472**	22.9
65½	LG	2.3	6.2	13.8	193	7.2	595	2240	26.6
89½	EP	2.4	8.6	6.4	140	4.6	262	1550	16.9
Experiment No. 2									
0	UF	3.9	6.7	—	—	—	—	—	—
6	4-Cl	2.9	7.4	3.9	56	7.0	134	732	18.3
21	UB	3.4	7.6	13.3	241	5.4	384	3090	12.4
34½	EG	2.9	8.4	24.8	392	6.4	863	4520	19.1
42½	EG	2.2	8.5	19.0	332	5.7	868	3760	23.1
48	MG	2.6	8.8	17.3	248	7.0	657	3360	19.6
72	PR	2.2	8.2	7.2	141	5.1	324	1670	19.4
Experiment No. 3									
43	EG	3.0	12.0	29.6	559	5.3	1120	5420	20.6

* Eggs from a single female used for each experiment. UF = unfertilized, 2-Cl = 2-cell stage, 4-Cl = 4-cell stage, UB = unhatched blastula, EG = early gastrula, MG = mid-gastrula, LG = late gastrula, PR = prism, EP = early pluteus.

** These data low, presumably by a factor of 10, due to a technical error. Experiment No. 3 was run at 43 hours to check this point.

Figure 1, the curves of which are fitted by the method of least squares. The quantity of barium-soluble, alcohol-soluble phosphorus decreases at a uniform rate throughout the non-feeding stages of larval development. The rate of utilization in Experiment 1 is 7.4×10^{-7} microgram/egg/hour and in Experiment 2 is 20×10^{-7} microgram/egg/hour. The per cent of the fraction present at the beginning of development utilized in reaching the prism stage (72 hours) is 18.5% and 40.7% for Experiments 1 and 2, respectively (based on the fitted curves). It seems safe to conclude that the utilization of the material contributed appreciably to the general metabolism since it involves mobilization on the average of 13.6% of the total phosphorus of the unfertilized egg. No significant variations in the rate of utilization correlated with visible aspects of morphogenesis are apparent in either experiment.

TABLE II

Distribution of radiophosphorus in barium-soluble, alcohol-soluble and other phosphorus-containing fractions in unfertilized and fertilized eggs. The per cent of radiophosphorus in each fraction is calculated with acid-insoluble plus acid-soluble equal to 100%

Fraction	Unfertilized eggs*			Fertilized eggs**		
	cts./min./ml.	Per cent P^{32}	Specific activity cts./min./μg P	cts./min./ml.	Per cent P^{32}	Specific activity cts./min./μg P
Total egg	—	—	—	188,500	100.3%	240
Acid-insoluble	24,050	29.2%	28.3	14,690	7.87	33.4
Acid-soluble	58,600	71.0	75.1	172,100	92.0	486
Barium-insoluble plus barium-soluble, alcohol-insoluble	60,100	73.0	95.5	160,500	85.9	596
Barium-soluble, alcohol-soluble	1,180	1.40	4.37	1,350	0.72	11.6

* Incubated 9 hours in P^{32} concentration of 0.04 μc/ml.

** Incubated one hour, before first cleavage, in P^{32} concentration of 0.02 μc/ml.

Among analyses done by both of the present authors, as well as those by others, the variation in the quantity of the barium-soluble, alcohol-soluble phosphorus is broad. Data of the present authors include values of 3.9, 3.0, 1.05 and 0.98×10^{-4} micrograms per egg, and many other analyses for which egg counts are not available indicate that the fraction from unfertilized eggs contains from 12.0% to 58% of the total phosphorus. Variations in multiplicate analyses rarely are of appreciable magnitude, usually amounting to only a few per cent; it is believed the large differences from batch to batch represent true biological variations. Sacks (1949) reported variations of comparable magnitude in this fraction extracted from rat liver. Mende and Chambers (1953) found different batches of eggs of *Asterias forbesii* to contain 322, 153, and 138 μg. P/ml., and *Strongylocentrotus dröbachiensis* to contain 59 and 44 μg. P/ml.

During the period of development covered in these experiments there was an increase in the total egg phosphorus. In Experiment 1 this amounted to an increase of 9.1%, and in Experiment 2 of 23%, as calculated from the fitted curves.

Although the barium-soluble, alcohol-soluble fraction shows an appreciable decrease during development, the possibility exists that there is a simultaneous synthesis of some of the compounds in the fraction. The question of synthesis was examined in unfertilized eggs and in embryos by adding radioactive inorganic phosphate to cultures and determining the radioactivity of the fraction and of the whole eggs after appropriate time intervals.

Unfertilized sea urchin eggs take up radioactive phosphate from sea water at an extremely low rate. To obtain sufficient activity in the phosphate fractions of such eggs, they were incubated for 9 hours at 12.0° C. in sea water containing 0.04 μc. radiophosphate/ml. The egg concentration was 0.4% by volume. Of a small sample inseminated after this incubation, more than 95% fertilized and developed to the early pluteus. The data in Table II show that the incorporation into the barium-

soluble, alcohol-soluble fraction prior to fertilization is very low; even after prolonged exposure to radiophosphate only 1.4% of the total activity of the egg was in this fraction. In a second experiment the figure was 0.78%. In confirmation of other investigators most of the activity enters the fraction containing inorganic phosphate, nucleotide phosphate, and labile esters, though in the 9-hour experiment very much more is found in the acid-insoluble fraction than has been reported before (Chambers and White, 1954).

The same low rate of incorporation of phosphate into this fraction persists directly after fertilization, before the first cleavage. This is apparent from the second experiment of Table II, in which a 1% suspension of eggs inseminated 12 minutes earlier was incubated for 60 minutes with 0.02 μc./ml. of radiophosphate. Although considerably more phosphate penetrated into these than into unfertilized eggs, the proportion in the barium-soluble, alcohol-soluble fraction is still only 0.7% of the whole. In comparable experiments, neither mono-iodoacetate nor 2,4-dinitrophenol, added with the radiophosphate, changed this pattern markedly, though both inhibited the uptake of phosphate by the egg.

The subsequent embryonic period was examined from the two-celled embryo to the early pluteus stage at 90 hours in the experiments of Table I. In these the time of exposure to radiophosphate at each stage examined was 60 minutes. Columns 5 through 10 of Table I present the pertinent determinations of radioactivities. Of the radiophosphorus that enters the embryos during one hour, an average of 6.5% (4.6 to 7.8%, Column 7) is incorporated into the fraction. There is no consistent pattern of change in this value during the rest of the development. Considered in terms of specific activities, the fraction attains a level that is about 19% (12.4% to 26.6%, Column 10) of the specific activity of the total egg phosphorus, again with no consistent change during the rest of the development. This contrasts with 5% for the freshly fertilized eggs in the experiment of Table II. Although the components of this fraction are metabolically very inactive in the unfertilized egg and before the first cleavage, it is concluded that one or two hours after fertilization at least part of the barium-soluble, alcohol-soluble fraction becomes moderately stimulated metabolically relative to phosphorus compounds of the egg as a whole, and this increased level of metabolic activity is maintained with approximate constancy until the pluteus stage.

The specific activities of the total egg phosphorus and of the barium-soluble, alcohol-soluble fraction are plotted against age of the embryos in Figure 2. It should be noted that these curves are not cumulative uptake curves, but rather are rate curves, each point representing the counts per minute per microgram of phosphorus per 60 minutes exposure at the particular age indicated. The rate of incorporation into the fraction is seen to increase to a maximum at about 40 hours, and then decline subsequent to this time. The peak of activity at 40 hours probably does not represent a special stimulation in metabolism within the fraction leading up to gastrulation, because, as was pointed out above, the activity of the fraction, when expressed as percentage of the whole embryo, is constant. Rather the peak reflects closely the uptake curve for the whole embryo, and is probably due to a change in permeability of the embryo to inorganic phosphate from the sea water.

An unanticipated finding in these experiments is that the rate of uptake in both the total phosphorus and the barium-soluble, alcohol-soluble fraction varies markedly in the different phases of development. The rate of uptake which begins to increase

15 or 20 minutes after fertilization (Abelson, 1947; Brooks and Chambers, 1948; Whiteley, 1949) continues to increase markedly until the stage of early gastrulation, at 34 to 43 hours in the different experiments. The rate then begins to decrease

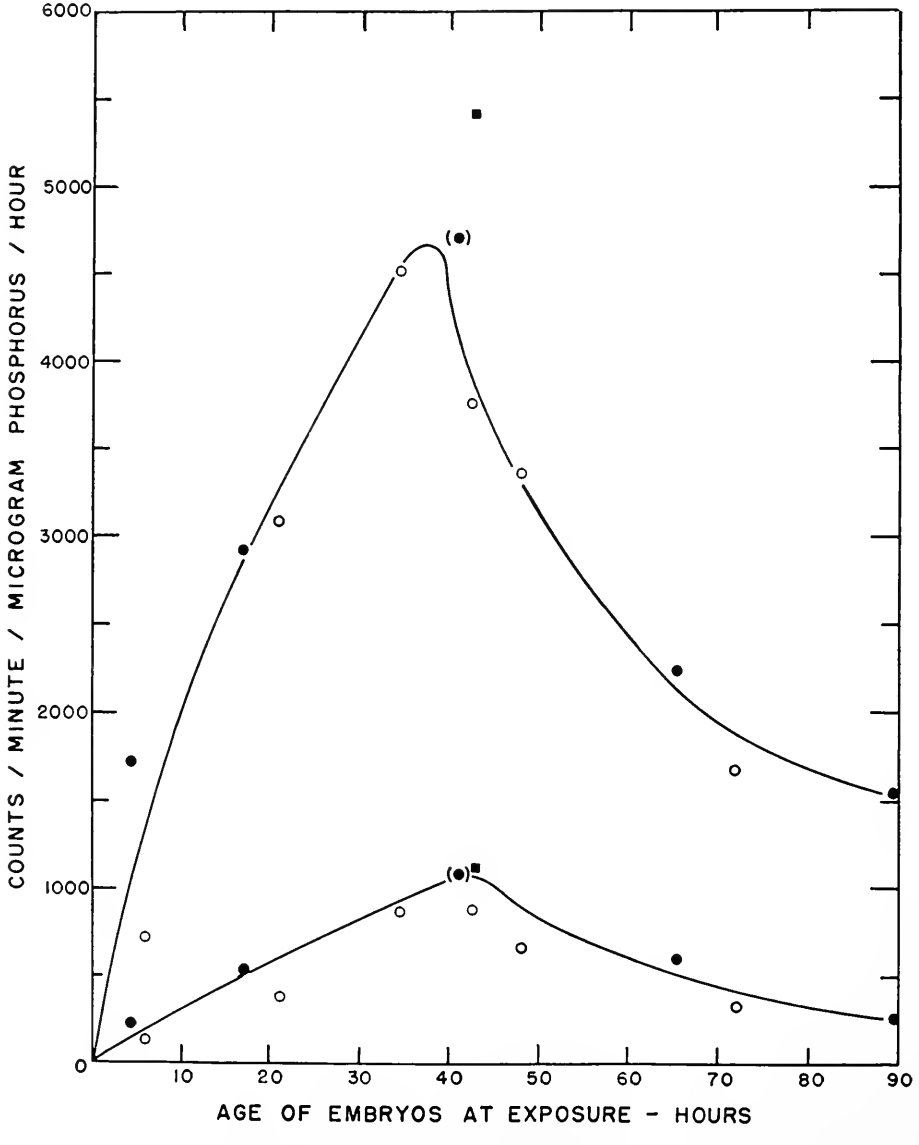

FIGURE 2. Rate of uptake of radiophosphate into the embryos of *Strongylocentrotus purpuratus* (upper curve) and into the barium-soluble, alcohol-soluble phosphate fraction (lower curve) of these embryos. Solid circles are data from Experiment 1, open circles are from Experiment 2, and squares from Experiment 3 of Table I. The bracketed solid circles at 41 hours have been multiplied by 10 (see footnote to Table I). Specific activities are in counts/min./μg. P/hour.

sharply, tending to level off in the prism and early pluteus stages. As nearly as could be determined, the inflection in the rate coincides with the onset of gastrulation. This pattern of uptake is shown when the data are considered either on the basis of counts per minute per egg or of specific activity.

This change of permeability to orthophosphate is not, however, universal for all phosphate compounds. The permeability of embryos to propanediol phosphate, determined in an experiment similar to those with orthophosphate but using labeled ester instead, reaches a maximum rate earlier in development (mid-blastula) and thereafter does not decrease through the pluteus stage. Even in the absence of information needed to calculate permeability constants for the ester and for orthophosphate, it appears that the ester penetrates much more slowly. Different mechanisms for the penetration of the two substances probably exist.

The composition of the barium-soluble, alcohol-soluble fraction in this material is unknown. Lindberg (1943) had reported the identification of 1,2-propanediol phosphate in this fraction in sea urchin eggs, but Rudney (1952, 1954) presents cogent reasons for believing this ester separates into the barium-soluble, alcohol-insoluble fraction in rat liver. Sea urchin eggs possess very large amounts of barium-soluble, alcohol-soluble phosphates and of polysaccharides. Interference by the polysaccharides with the clean separation of esters might account for different distribution of propanediol phosphate reported by Lindberg and Rudney. To determine the solubility of propanediol phosphate under the exact conditions used in this investigation, fractionations were made of sea urchin egg homogenates containing known amounts of synthetic 1,2-propanediol phosphate labeled with radioactive phosphorus.

Two separate lots of propanediol phosphate labeled with P^{32} were synthesized by the method described by Lampson and Lardy (1949), using 0.188 and 0.150 millicurie P^{32} in the reaction tubes for the two syntheses. The lead salts obtained were converted to the free acid with dilute sulfuric acid in the first case and H_2S in the other, and remaining traces of lead removed with Dowex-50 in the hydrogen form. The solution of lot 1 was neutralized to pH 7.0. This product contained no inorganic phosphate. The specific activity of the ester was 1439 counts per minute per microgram phosphorus under the standard counting conditions. The second lot, subjected to paper chromatographic analysis, showed a component with the same R_f as a commercial preparation (Nutritional Biochemicals Corp.) and a very faint unidentified second component with much less than 1% of the total activity. No inorganic phosphate was present. The specific activity was not determined.

In each of two separate experiments,[3] a mass of approximately a million unfertilized eggs having an estimated barium-soluble, alcohol-soluble phosphorus content of 300 micrograms was homogenized with cold 0.5 N $HClO_4$. Three hundred to 350 micrograms of phosphorus in the form of labeled propanediol phosphate were added to increase by a significant amount the content of supposed propanediol phosphate already in the eggs. The fractionation was completed in the usual manner and the radioactivity in the various fractions measured with the results given in Table III. Essentially the entire amount of radioactivity was found in the barium-insoluble, plus barium-soluble, alcohol-insoluble fraction. It is concluded, in agree-

[3] We are pleased to acknowledge the help of Miss Kathryn Eschenberg in one of these experiments.

ment with Rudney, that the barium-soluble, alcohol-soluble fraction does not contain propanediol phosphate. This leaves us with no specific information as to the composition of this very large fraction of these eggs.

A preliminary examination of the fraction has been made by paper chromatography. From a number of experiments in which, after isolation, it was subjected to various desalting pre-treatments, evidence for at least three components was derived. In all of these pre-treatments the samples were desalted with Dowex-50 in the hydrogen form and concentrated by evaporation. In some cases the phosphates precipitable by basic lead acetate were chromatographed, and in one the sample was treated with Dowex-2-OH. With different treatments, and depending on the

TABLE III

Recovery of phosphorus-labeled 1,2-propanediol phosphate when added to homogenates of unfertilized eggs in perchloric acid and subjected to barium and alcohol fractionation

Fraction	Experiment 1		Experiment 2	
	cts./min.	% of total activity	cts./min.	% of total activity
Acid-insoluble	31	0.3	38	0.4
Acid-soluble				
Alcohol-insoluble	104	1.0	60	0.7
Barium-insoluble and barium-soluble, alcohol-insoluble	10,203	96.4	8,825	97.8
Barium-soluble, alcohol-soluble	247	2.3	100	1.1
Totals	10,585*	100%	9,023	100%

* 11,174 counts were added to the homogenate. Recovery 94.6%.

amount of sample, one or two clear spots were detectable. Compounds with R_f's of 0.12, 0.29, and 0.71 were found. With this system of chromatography, inorganic phosphate has an R_f of 0.54 and propanediol phosphate, either commercial or prepared by us, has an R_f of 0.73. The similarity between this R_f and that of the unknown at 0.71 is not taken as evidence of identity because other substances, for example glycerol phosphate, have the same R_f in this system.

DISCUSSION

The results of the present investigation indicate that there are at least three components in the barium-soluble, alcohol-soluble fraction. From its magnitude it is possible that one of these could serve as a storage compound of some kind, and the gradual, uniform utilization of the material during development would be in accord with this. The experiments with radioactive orthophosphate demonstrate an appreciable, though not great, synthesis in the fraction, probably in one or more components other than the storage ones.

This synthesis is extremely low prior to fertilization and in the first hour thereafter, but increases demonstrably beginning with the first cleavage. Cleavage initiates an increase in activity in this fraction that is greater than the average increase

for the phosphorus compounds of the egg; before the first cleavage the specific activity in the fraction is 5% of that in the total egg phosphorus, while in subsequent stages it is about 19% of the total. The percentage of radioactive phosphorus that is incorporated into the fraction in embryos of different stages of development after cleavage starts is rather constant, despite large differences in total amount of radiophosphorus that enters the embryo. This suggests that the limiting factor for the synthesis of the component is the availability of phosphate rather than the level of activity of the synthesizing enzymes. Chambers and White (1949) supply evidence that the eggs of this species have a very small inorganic phosphate pool, especially after fertilization, which would be in accord with this idea. The constancy indicates that there are no special periods during development after cleavage in which turnover in this fraction is especially rapid relative to the turnover of the other phosphorus compounds of the embryo.

The evidence used by Lindberg (1946) for identification of propanediol phosphate isolated from cow brain is extensive, but it is not clear in his 1943 or 1946 papers on what basis propanediol phosphate is considered to be a component of the barium-soluble, alcohol-soluble fraction, other than that the fraction has a high stability toward acid and alkaline hydrolysis. His identification of the ester in sea urchin eggs is also based on these features. LePage (1948) identified a phosphate compound in the barium-soluble, alcohol-soluble fraction of rat carcinoma as 1,2-propanediol phosphate on the basis of the phosphorus and lead content of its lead salt and its stability to hydrolysis in 1 N HCl. Against these observations, however, the experiments by Rudney (1952, 1954) and those reported here in which 96% to 98% recovery of labeled, synthetic ester was obtained in the barium-soluble, alcohol-insoluble fraction, demonstrate in a manner that seems unequivocal that this substance is not a component of the barium-soluble, alcohol-soluble fraction. With the demonstration that this ester is not in the barium-soluble, alcohol-soluble fraction, there is, at present, no evidence that propanediol phosphate exists in sea urchin eggs.

The existence of more than one compound in the fraction is further supported by the chromatographic studies which show a minimum of three components. In the related sea urchin, *Strongylocentrotus dröbachiensis,* and in the star fish, *Asterias forbesii,* Mende and Chambers (1953) found that 23% and 60%, respectively, of the phosphorus of this fraction was hydrolyzed to orthophosphate in three hours at 100° C. in 1 N HCl. Further identification or characterization of these components has not been attempted.

In several investigations of the permeability of sea urchin embryos to inorganic phosphate a greatly increased rate of penetration after fertilization has been reported, but subsequent changes in rate have not been followed beyond seven hours in *S. purpuratus* and four or five hours in *Arbacia punctulata.* In these early stages the uptake proceeds at a uniform rate. The experiments reported here show that during later cleavages and blastulation the rate of penetration continues to rise markedly, but that coincident with the onset of the first major form change, gastrulation, the rate shows an abrupt and considerable drop which continues at least to the early pluteus. It will be of interest to determine if this new pattern reflects some profound change at gastrulation either of the metabolism within the cells of the embryo, or of a surface transport mechanism for phosphate correlated with the differentiation of the ectoderm.

Summary

1. The quantity of phosphorus in the barium-soluble, alcohol-soluble fraction of the acid-soluble phosphate compounds of the embryos of the sea urchin, *Strongylocentrotus purpuratus* (Stimpson), was measured until the formation of the early pluteus stage. In two experiments the phosphorus in the fraction decreased by 18.5% and 40.7% in reaching the late prism stage (72 hours).

2. During this period, the total phosphorus of the embryos increased an average of 14.4%.

3. The rate of penetration of radioactive phosphorus from sea water into the embryos during these experiments increased very greatly during cleavage and blastulation, reached a maximum at the onset of gastrulation, and decreased subsequently to a middle level at the prism stage.

4. The rate of uptake of radioactive phosphorus into the barium-soluble, alcohol-soluble fraction was extremely low in unfertilized eggs and in fertilized eggs before the first cleavage, amounting to 0.7% to 1.4% of the total uptake.

5. The rate of uptake of radioactive phosphorus into this fraction in cleaving eggs and embryos mirrored that of the total phosphorus, and the percentage of the labeled phosphorus in the fraction was relatively constant at all stages, averaging 6.5% of the total.

6. Chromatographic examination of the fraction has indicated the existence of at least three components.

7. It is concluded that there are several components in the fraction, at least one of which is a stored material that is steadily metabolized during development, and one or more others synthesized at a rate that is governed largely by the supply of available phosphate.

8. Synthetic radioactive propanediol phosphate added to a perchloric acid homogenate of eggs separated to the extent of 97% into the barium- and alcohol-insoluble, rather than the barium-soluble, alcohol-soluble fraction. It is concluded from this, in contrast to previous reports, that the latter fraction does not contain propanediol phosphate and that no evidence remains for the existence of this ester in sea urchin eggs.

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RESPONSE OF A LIVING ORGANISM, UNDER "CONSTANT CONDITIONS" INCLUDING PRESSURE, TO A BAROMETRIC-PRESSURE-CORRELATED, CYCLIC, EXTERNAL VARIABLE [1]

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Many overt biological rhythms are known which exhibit cycles of 24 hours with such great precision, at least statistically, that the phases of the cycles do not become significantly altered with respect to external day-night even after weeks or months in conditions of constant darkness and temperature. Such rhythms have been reported in a wide variety of organisms and for many processes (see reviews by Welsh, 1938; Kleitman, 1949; Webb, 1950; Caspers, 1951). Less commonly known is the fact that persistent cycles of lunar-day frequency appear also widespread (*e.g.*, Gompel, 1937; Brown, Fingerman, Sandeen and Webb, 1953; Rao, 1954; and Ralph, 1956). One of the most striking properties of these rhythms appears to be the temperature-independence of their frequency (Brown and Webb, 1948; Brown, Webb, Bennett and Sandeen, 1954; Pittendrigh, 1954). To account for these rhythms, either 1) there must be an astonishingly precise, temperature-independent clock-mechanism present within the organisms, or, 2) they are receiving signals with the same average frequencies from external sources and which serve in some manner as pacemakers, or, 3) some combination of these two. Fluctuations in cycle-length from day to day under constant conditions in such regular overt cycles as running in the mouse (Johnson, 1939), and crab color change (Brown, Webb and Bennett, 1955), despite the great precision of the average lengths of the cycles, suggest strongly the operation of an external pacemaker. Proof for the existence of at least a reasonably precise internal clock has been advanced (Brown, Webb and Bennett, 1955; Renner, 1955) through studying the results of rapid east-west geographic displacement of animals, but it is still not demonstrated that this is sufficiently precise to serve as the exclusive mechanism. Since there are known 24-hour physical cycles which appear to operate on universal time (*e.g.*, atmospheric electrical potential), it may be argued that there is still lacking definitive proof that regular pacemakers are inoperative even during such rapid longitudinal displacement of organisms.

In the course of seeking possible effective external rhythmic signals of the appropriate frequencies, average rhythms of O_2-consumption of primary solar and primary lunar frequencies have been found in all organisms so far examined for them. These have included organisms as widely diverse as seaweed, snails, crabs, Triturus, worms, carrots and potatoes (Brown, Bennett and Webb, 1954; Brown, Webb, Bennett and Sandeen, 1955; Brown, Freeland and Ralph, 1955). Studies of the

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, NONR-122803.

activities of quahogs, oysters and rats indicate that these, too, possess the same general types of average solar and lunar rhythms (Brown, 1954a; Brown, Shriner and Ralph, 1956; Brown, Bennett, Webb and Ralph, 1956).

The fact that there are known to exist average rhythms of barometric pressure of solar- and lunar-day frequencies led to attempts to correlate barometric pressure changes with the observed biological cycles, and some definite correlations were found not only between the hourly rates of barometric pressure change and of concurrent O_2-consumption, or activity, but also between mean daily levels of pressure and mean daily metabolic rates (Brown, Freeland and Ralph, 1955; Brown, Webb, Bennett and Sandeen, 1955). There were shown, furthermore, to be such distinct similarities between the general forms of the daily pressure changes and daily variations in oyster and quahog activity (Brown, Bennett, Webb and Ralph, 1956) as to suggest strongly more than a fortuitous relationship. This resemblance was sometimes a direct one and other times one of a mirror image. The experiments to be described herein were initially planned to resolve the problem of a possible direct role of barometric pressure variations.

Materials and Methods

Potatoes, *Solanum tuberosum*, which were purchased at a local grocery store constituted the organism. With a cork-borer, cylinders 22 mm. in diameter and about 1½ cm. high, each bearing an eye, were cut and the injured surfaces allowed to heal. In the experimental situation of constant temperature and constant very low illumination these developed sprouts. They were replaced during the experiments only after long periods when they grew too large for the respirometer vessels.

The respirometers were of the type designed by Brown (1954b) which permitted continuous automatic recording of O_2-consumption. The instrument was modified, however, in such a manner as to permit four respirometers to record as a unit, and the recorder was small enough to be sealed along with the respirometers in a barostat (Fig. 1). The barostat first successfully used was an 11 × 24-inch vertical autoclave sealed with an O-ring. Since it was not possible to view the interior once this was sealed, a simple barograph was included in the system to assure an absence of leakage. Later, five simplified barostats were constructed. These consisted of 1/32-inch copper cylinders, 10¼ inches in diameter and 22 inches deep, with dished bottoms and a 1-inch-wide, ⅛-inch-flat, ground-brass rim. These, covered by twelve-inch vacuum desiccator covers with an electrical inlet for the recorder motor and a glass stopcock passing through a rubber stopper, served as excellent barostats. A ⅜-inch brass tripod ring, fitting snugly within the cylinder served as a platform to support the recorder, with its divers hanging into an enclosed, 10-inch-deep water bath. Each copper cylinder was supported in a 55-gallon steel drum full of water maintained at constant temperature. The temperature settings for the five baths all lay in the range 19.6 to 19.9° C.

The apparatus was shielded from all light except that from a row of 7½-watt, opalescent, incandescent lamps which provided a continuous illumination of about 1–2 ft. c. at the desiccator-cover surface, and obviously some far lower constant illumination at the surface of the organisms.

One cylinder of potato was placed in each of the four respirometers of a recording unit and the whole lowered into a barostat, sealed, and the barostat as-

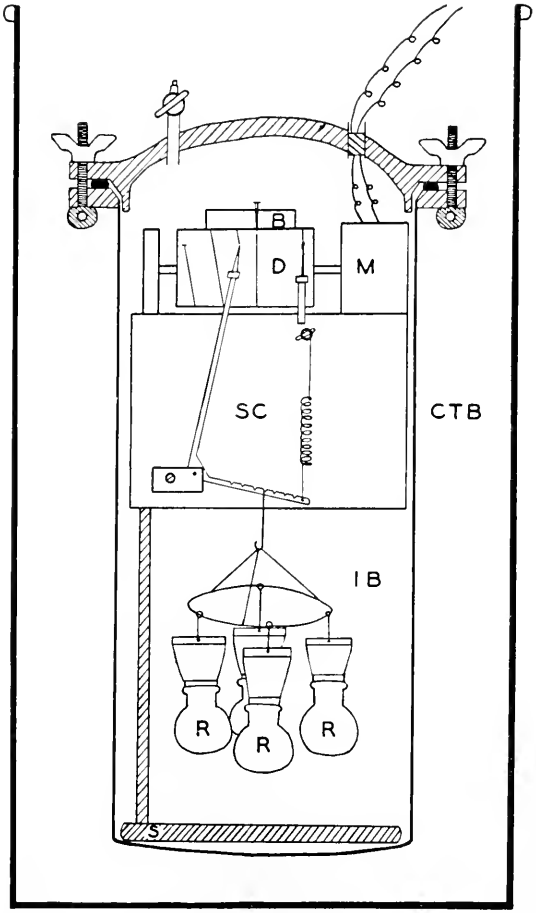

FIGURE 1. Four respirometers (R) hanging into a water bath (IB) from a spring scale (SC) ink recording system registering on a 24-hour drum (D) activated by an electric clock motor (M). A barograph (B) also registers on the same drum. The whole is supported by a stand (S) in a sealed copper-and-brass container which sets deeply in a constant-temperature bath (CTB).

pirated to reduce the internal pressure to 28.00 in Hg. This was sufficiently below the lowest normal external pressure in Evanston, Ill. (ca. 28.5) that the cover would always retain its seal. A barometer in each barostat permitted one to be continuously assured of the absence of leakage. Usually the apparatus was then left undisturbed for three to five days. This was the length of time the recording needle required to spiral down the five-inch-length of the three-inch-diameter brass drum to the recording base-line, with the drum turned one revolution per day with an electric time-clock mechanism. The paper was a smooth-surface, bond, writing paper held in place with rubber cement. The pens were small L-shaped glass capillaries with polished tips and contained a 50–50 glycerine-water mixture, colored with

neutral red. In more recent work, millimeter graph paper and commercially available thermograph pens (counterbalanced) and ink have been found simpler to use.

The springs used were wound from 0.014-inch, 8-18, stainless-steel wire, and were 5 cm. long and about ½ cm. in diameter. Over the recording range (10 gms./cm.) in this mechanical recording system, there was only about a 10% variation from linearity from the first to the last day of recording. There was, undoubtedly, also, a very minor, gradual reduction in pressure in the barostat over a recording period as the O_2-reservoirs of the respirometers were depleted, but this was not significant in view of the large size of the ratio: barostat gaseous volume/O_2-reservoir volume.

Respirometers were always loaded sometime between 8 A.M. and 8 P.M. In the analyses to follow, except when specifically stated otherwise, no data were used before the first midnight after the sealing of a barostat, nor of the day they were opened. Hence, there were usually complete, undisturbed, calendar days of data. All times are central standard.

From April 1 through April 26, 1955, only one barostat was in operation. From April 28 through June 8, five were in continuous operation.

Results

It was apparent even after the first day of recording that the rate of O_2-consumption in the potato is by no means uniform even under constant temperature, illumination, oxygen, CO_2, humidity and pressure. There were fluctuations usually with more than one conspicuous maximum a day. Eight sample days picked at random from the data, illustrating the variation in the two to five separate barostats in which recordings were complete on that particular day, are illustrated in Figure 2. These

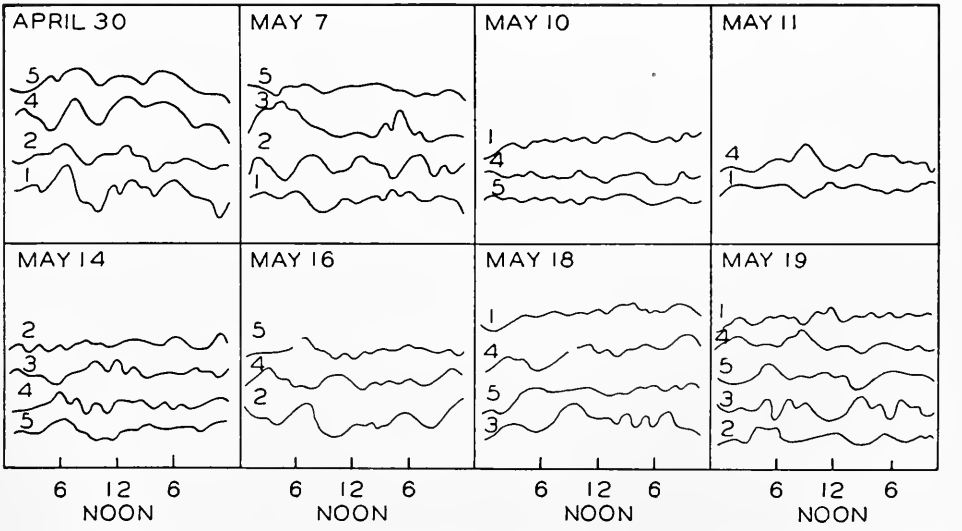

Figure 2. The forms of the daily fluctuations in oxygen consumption (three-hour sliding averages) measured on eight randomly selected days in all those (1 to 5) barostats from which records were complete for that day. The ordinate scales are comparable for all, but the patterns have been separated for clarity.

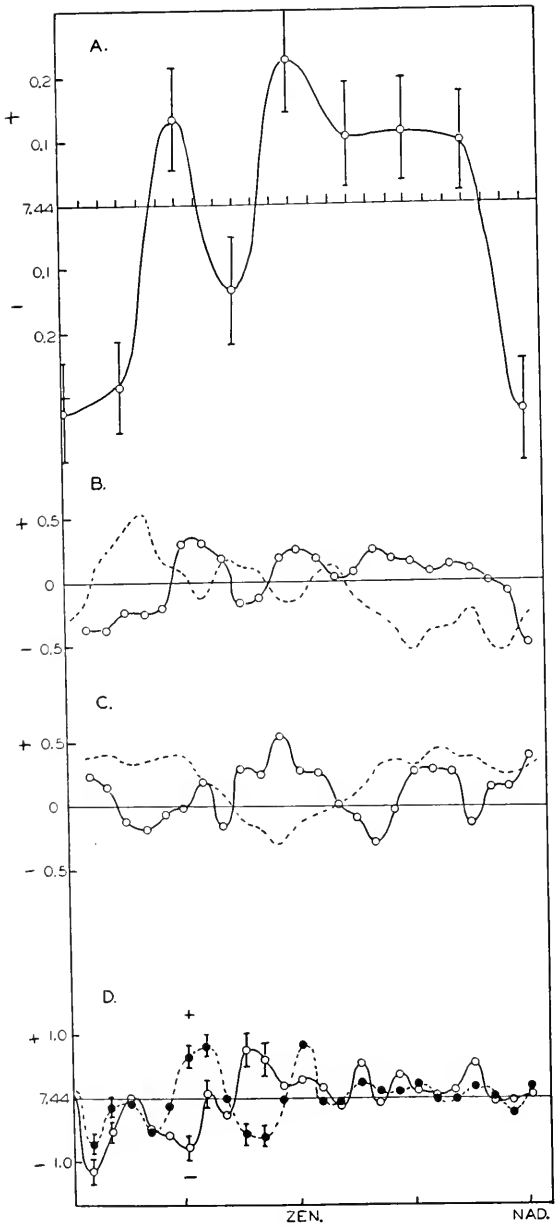

FIGURE 3. A. The mean rates of O_2-consumption for the 8 three-hour periods of the solar-day for all the data of the 154 barostat-days. These are expressed as deviations from the daily means.

B. Moving three-hour means of the daily variation for 100 randomly selected days in 1955 (solid line), compared with the comparable mean daily cycle obtained at the same time of year in 1954.

TABLE I

The percentage increase from lowest to highest values of the daily cycles illustrated in Figure 2

Unit	Date							
	Apr. 30	May 7	May 10	May 11	May 14	May 16	May 18	May 19
1	133	38	71	33			73	49
2	37	73	26		35	84		31
3		67			35		77	52
4	100			46	38	34	89	54
5	67	23	10		80	45	72	32
Av.	82	50	35	40	47	54	78	45

are three-hour moving means. Table I includes the percentage increase from lowest to highest values of the days illustrated. One hundred and fifty-four complete barostat-days of data, including the 29 illustrated in Figure 2, were obtained, together with numerous fractional barostat-days.

Inspection of the daily patterns revealed that there were clearly as many detailed patterns as there were days of data. But there appeared to be a suggestive generic similarity in the records obtained for any given day in entirely independent barostats with respect to amplitude of the fluctuations, gross trends, and approximate times of the major maxima and minima. There seemed, apparently too commonly to be attributed simply to chance, a tendency for one or more of the records to show on any given day an inversion of some of, or even the greater part of, the daily pattern relative to the other concurrent ones. In nearly every instance, however, if one obtained the average daily pattern for a three- to five-day period of continuous recording in one barostat, there was a distinct maximum about 6 or 7 A.M. and a minimum about 9 or 10 A.M.; also, low values nearly always characterized the early morning hours.

In Figure 3A is plotted the mean daily cycle for all barostat-days of data. This is plotted as the mean differences from the mean hourly rate for the whole day for each of the eight three-hour periods of the day. The mean daily fluctuation is seen to be about 8%. The mean hourly value for all these data was 7.44 with the range extending from about 5 to 14. For each value in the figure is indicated the standard error of the mean.

The mean daily pattern is in large measure a mirror image of the one obtained without the use of a barostat during the 29-day period, May 12–June 9, 1954 (Brown, Freeland and Ralph, 1955) involving both inversion of the major 24-hour cycle and the secondary fluctuations superimposed on the larger cycle. Moving three-hour averages of 100 randomly picked days in 1955 and the 1954 cycle are seen in Figure 3B. In 1955 the highest rates for the day occurred in the afternoon; in 1954 the highest rates were found in the morning.

C. The mean lunar day cycle for the potato for the month of May, 1955 (solid line), compared with that obtained in May, 1954 (broken line).

D. The mean daily cycles with sample standard deviations of the means, for the 99 positive barostat-days of O_2-consumption, and the 55 negative ones.

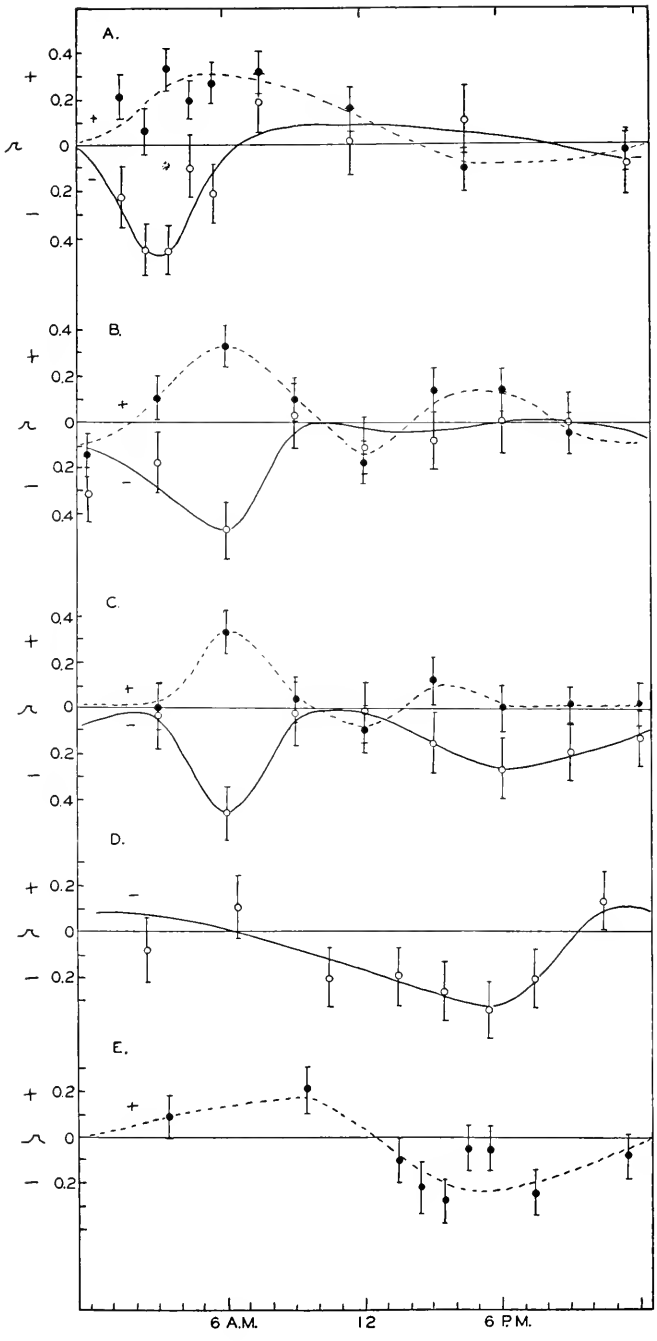

FIGURE 4. A. The correlations between the rate of O_2-consumption during the 4 to 7 A.M. period of a day and the mean rate and direction of barometric pressure change for the same day

There was also an apparent mean lunar-day cycle in the potatoes. It, too, showed a general major inversion relative to the comparable lunar-day cycle obtained in 1954. When only 29 consecutive days of data in May were used, in order exactly to randomize a daily cycle and hence to obtain the minimally distorted form of a lunar-day cycle, the results seen in Figure 3C were obtained. In the same figure is the form of the lunar-day cycle obtained in 1954 without the use of a barostat. These are both three-hour moving means.

Brown, Freeland and Ralph (1955) have shown that for the potato in respirometers subjected to normal fluctuations in pressure in 1954, there was present, even after all appropriate corrections for pressure changes had been made, a correlation coefficient of -0.58 ± 0.035 between the hourly values of O_2-consumption and the concurrent rates and direction of barometric pressure change over a month. That this demonstrated a direct response to some external factor fluctuating with barometric pressure changes was ascertained by obtaining the coefficient for the same monthly period using metabolism on the hours of day n and the pressure changes for day $n + 1$. Now, the value dropped to 0.217 ± 0.038, a value not significantly different from the auto-correlation of hourly pressure changes on two consecutive days. For reasons discussed in that paper it was considered unlikely that the response was a direct one to pressure on the part of the plant. This last presumption is supported amply by this work in which the pressure was kept constant during the recordings.

Although the earlier work had shown a correlation between the hourly rates of O_2-consumption in the potato and the concurrent rate and direction of barometric-pressure change in organisms subjected to barometric-pressure fluctuation, using data available in the current experiments, no correlation with the hourly pressure change in the external environment was found. With 2374 hours of data, a value of r, 0.020 ± 0.021 was obtained.

An attempt was made to test an hypothesis that there was not a correlation with the barometric pressure changes in these data obtained in 1955 because of abrupt 180°-shifts of the phases of at least one important component of daily rhythmicity in the plants. The 154 barostat-days of daily patterns of respiration were, by inspection, divided into two categories on the basis of whether a maximum and minimum occurred about 6 and 9 A.M., respectively, or the cycle at these hours was ap-

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and the two preceding days for various two-hour periods of the day, for both the positive and negative groups of potatoes.

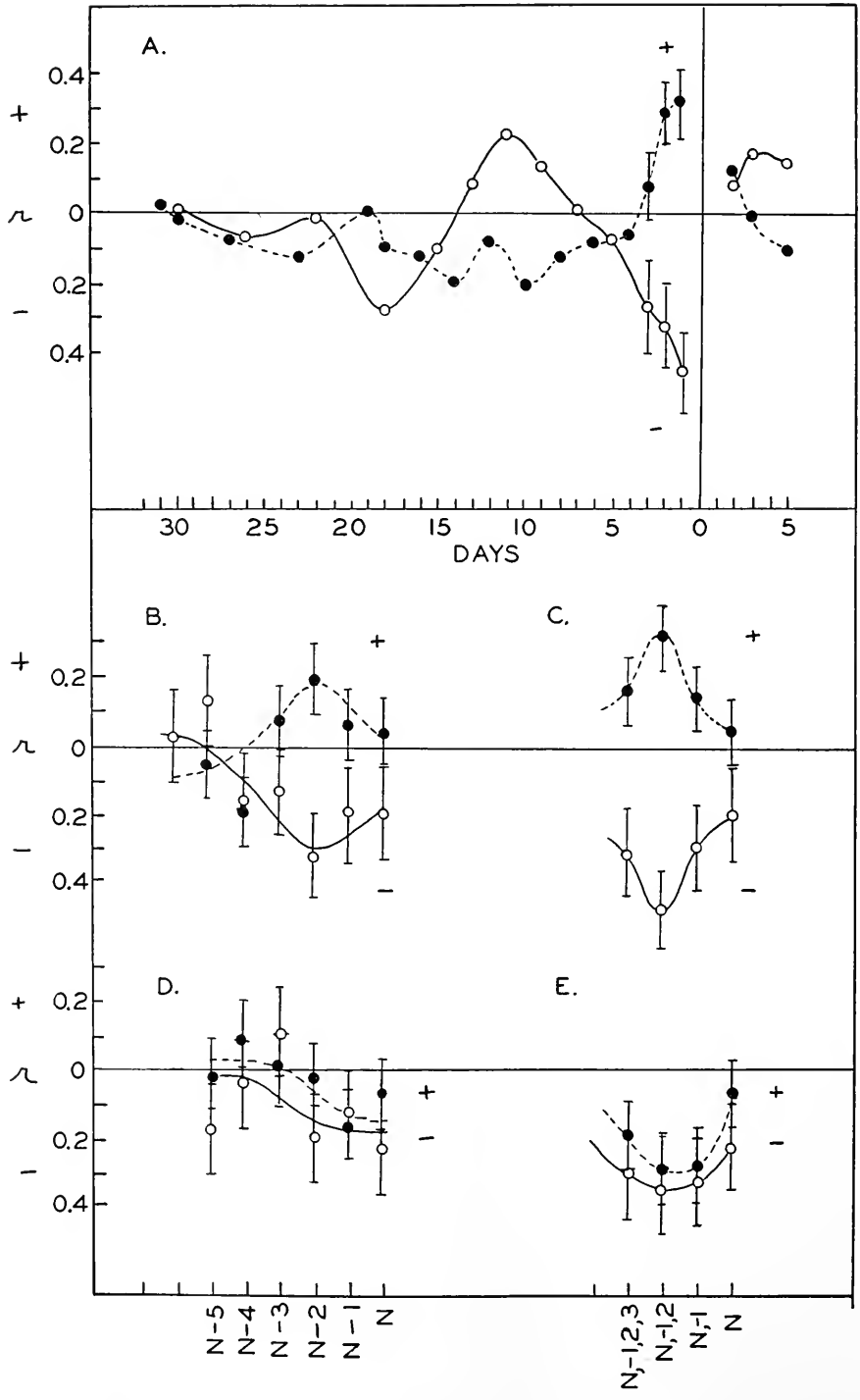
B. The correlations between the mean rate and direction of barometric pressure change during the 2 to 4 A.M. period of a three-day period and the mean rate of O_2-consumption for various three-hour periods on the last of the three days.

C. The correlations between the mean rates of O_2-consumption during various three-hour periods of the day and the mean three-day changes in barometric pressure for the two preceding hours of the day.

D. The correlations between the mean rate of O_2-consumption during the 4 to 7 P.M. period and the mean, three-day changes in barometric pressure during various two-hour times of day, for the negative group.

E. The correlations between the mean rate of O_2-consumption during the period 5 to 8 P.M. and the mean three-day change in barometric pressure for various two-hour periods of the day for the positive group.

The potatoes were maintained in barostats throughout the study. The values of O_2-consumption used are deviations from the daily mean in order to eliminate long-period trends in metabolic rate.


A.
+
0.4
0.2
0
0.2
0.4
−
30 25 20 15 10 5 0 5
DAYS
B.
C.
D.
E.
N-5 N-4 N-3 N-2 N-1 N
N,-1,2,3 N,-1,2 N,-1 N

parently inverted, with a minimum about 6 and a maximum about 9 A.M. The latter were termed the negative group since in the form of the minor fluctuations this was more nearly the form of the 1954 patterns which showed the negative correlation with concurrent barometric pressure changes; the former was termed the positive group.

Barostat No. 1 yielded 14 negative days and 33 positive ones; No. 2 gave 7 negative and 23 positive; No. 3 showed 17 negative and 10 positive; No. 4 had 11 negative and 10 positive; and No. 5 provided 6 negative and 23 positive. Though both positive and negative responses could be found in different barostats on a single calendar-day, there were two or three periods of three or four days, when there seemed to be a high percentage of negative cycles.

In Figure 3D are seen the mean daily patterns for the 99 positive and 55 negative days expressed as mean hourly deviations from the daily means. It is very interesting to note that though these were based exclusively upon selection of an apparent inversion between about 4 and 11 A.M. between the two groups, the remainder of the daily patterns are in extraordinary agreement for the two groups even to most of the minor fluctuations. It was now assumed that, at least for the 4–11 A.M. period, the potatoes were divided into two separate, relatively homogeneous populations with respect to any sign of correlation with any external pressure changes which might be present. On the other hand, it is very important to emphasize here that there was no *a priori* reason why a single value for either of these two groups for any arbitrarily selected single time of day should show any correlation whatsoever with external atmospheric barometric pressure changes.

First, an intensive search was made to learn whether the average rate of O_2-consumption in the 55 days for potatoes of the *negative* group for the 4–7 A.M. period was correlated with external pressure changes in any manner. It was discovered that a correlation, -0.46 ± 0.106, existed between the total respiration at this time on day n, expressed as deviation from the daily mean, and the algebraic sum of the rates of the pressure changes at 2–4 A.M. on days n, $+ (n-1)$, $+ (n-2)$. In Figure 4A it is readily seen that there is a rapid drop to no correlation as one moves away from 2–4 A.M. pressure changes in either direction. In Figure 4A it is also seen that not only did the 99 days of the positive group also show a significant correlation for essentially the same kind of relationship, but with opposite sign, 0.326 ± 0.089. There was similarly a loss of correlation with pressure changes as one moved to other times of day.

FIGURE 5. A. The correlations between the mean deviations in rate of O_2-consumption during the 4 to 7 A.M. period and the mean change in barometric pressure during the 2 to 4 A.M. period during various three-day periods centering on days ranging from 31 days earlier to 5 days later than the day of correlated O_2-consumption, for both positive and negative groups.

B. The correlation between the rate of O_2-consumption during the 4 to 7 A.M. period on day n with the changes in barometric pressure during the 2 to 4 A.M. period on single days ranging from day n to day $n-6$.

C. Correlations between O_2-consumption and barometric pressure for the same hours as in B of the solar day, but now with the means of pressure including increasing numbers of earlier days up to three.

D and E. The same as B and C except that the correlations of pressures at 2 to 4 P.M. with O_2-consumption at 5 to 8 P.M. are used for the positive group and barometric pressure changes between 4 and 6 P.M. are correlated with O_2-consumption for the 4 to 7 P.M. period for the negative group.

Figure 5 demonstrates in two ways that the correlations that have been described are actually the result of a summation by the potato of three or more days of fluctuation in an external factor. First, for both the positive and the negative groups, the correlation not only increases to a maximum as one increases the number of days up to three, but there is a reduction beyond that point (Fig. 5C). Second, it is seen that the best single-day pressure change correlation occurs for both groups with pressure change on day $n - 2$, with lower or no correlation either earlier or later (Fig. 5B). In Figure 5A it is seen that there is a rapid reduction in correlation with summed three-day pressure changes for both positive and negative groups as one attempts the correlations with earlier days or later. The apparent drifts in correlations suggested for three-day periods earlier than $n - 2$, $n - 3$, and $n - 4$, and extending for nearly three weeks probably reflect only auto-correlations of pressures. However, only the correlation with $(n) + (n - 1) + (n - 2)$ is significantly different from 0.

Figure 4B demonstrates that the three-day sums of pressure changes at 2–4 A.M. (days n, $n - 1$, $n - 2$) are not significantly correlated with the rates of respiration (day n) for any time of the day other than the 4–7 A.M. period for either the positive or negative groups. In Figure 4C the rates of O_2-consumption at various periods of day n are correlated with the sums of three-day (day n, $n - 1$ and $n - 2$) pressure changes for the two-hour earlier period. There is a suggestion herein that there may be a second period in the day, in the afternoon, when there is a real correlation for the negative group, though a similar suggestion is lacking for the positive one. When this possibility was explored in detail for the negative group (Fig. 4D), the highest correlation was found between the 4–7 P.M. deviation in O_2-consumption and the 4–6 P.M. (day n, $n - 1$, and $n - 2$) pressure change, -0.347 ± 0.12. There was also found an afternoon correlation for the positive group but not with the same temporal relationship (Fig. 4E). The highest correlation was found between the 2–4 P.M. pressure change and the 5–8 P.M. deviation in respiration, -0.273 ± 0.091. Just as the forms of the mean daily cycles for both groups of potatoes for this part of the days were quite similar, the correlations here also bore the same sign.

It is seen from Figure 5E that the correlation in the afternoon is maximal with the summation of three days (n, $n - 1$, $n - 2$) of pressure changes, with a reduction with fewer or greater number of days. The correlations with single days (Fig. 5D) are relatively small, being about equal for days n, $n - 1$, and $n - 2$.

Evidence supporting the earlier assumption of there being a tendency of the potatoes actually to be reversing from time to time the sign of their response to some external factor became apparent from a study of structure in correlation scatterplots. One of these was the following. When one made no selection whatsoever of the 154 barostat-days of data and determined only the relationship between the extent of the fluctuation, *positive or negative*, from the daily mean for the 5–7 A.M. O_2-consumption on day n, and the algebraic sum of the barometric pressure changes from 2 to 6 A.M. for days n, $n - 1$, and $n - 2$, a coefficient of 0.361 ± 0.070 was obtained. No correlation was obtained unless the sign of the response was ignored.[2]

[2] Further support of this hypothesis of inversions has been obtained since the preparation of this report. With 960 complete potato-days, including hourly data, obtained in barostats during October and November, 1956, the correlation between $3 \times 3 \times 3$-day moving means of the 2 to 6 A.M. barometric pressure change on day n and the three-day moving mean of the 6 A.M. devia-

This highly significant result clearly justified the earlier division of the data into positive and negative cycles. Again, ignoring the sign of deviation of O_2-consumption from daily mean, the correlation between the three-day (n, $n-1$, $n-2$) 5–9 A.M. barometric pressure change and O_2-consumption at 8–11 A.M. yielded a lower value of 0.223 ± 0.077.

There was, further, with no selection of the 154 barostat days of data, found to be a correlation, 0.305 ± 0.073, between the algebraic sum of the 2–6 P.M. barometric changes of day n, $n-1$, and $n-2$ and the deviation in O_2-consumption of the potatoes from the daily mean at the 4–7 P.M. period, if one used simply average rate of pressure change, irrespective of sign. It was evident, however, from examination of a scatterplot that the correlation for those 14 atypical days on which the pressure showed an overall rise at this time of day during the three-day period, was distinctly inferior to that for the 140 days of data of periods for which the pressure was (as typically) falling. The coefficient of correlation between the mean three-day rate of fall in afternoon barometric pressure and 4–7 P.M. deviation in rate of O_2-consumption was 0.355 ± 0.0734.

TABLE II

Correlation between rate of change of barometric pressure and O_2-consumption

Bar. pressure	Potato	Correlation
11–3 A.M.	2–4 A.M.	-0.087 ± 0.08
2–6 A.M.	5–7 A.M.	$\pm 0.361 \pm 0.07$
5–9 A.M.	8–11 A.M.	$\pm 0.223 \pm 0.077$
8–12 A.M.	11–1 noon	$+0.024 \pm 0.08$
11–3 P.M.	2–4 P.M.	-0.056 ± 0.08
2–6 P.M.	4–7 P.M.	-0.305 ± 0.073
5–9 P.M.	8–10 P.M.	-0.084 ± 0.08
8–12 P.M.	11–1 midnight	-0.194 ± 0.078

No other real correlations (except possibly for the 8–12 P.M. pressure change and midnight (11 P.M.–1 A.M.) O_2-consumption) could be found for the potatoes as a whole over the day, even when the structure of correlations was examined for possible reversing responses. The foregoing results are included with some additional ones in Table II.

The potatoes of barostat No. 3 for the 560 hours obtained during May and June showed an hourly correlation of their deviations from the daily means with the concurrent deviations from the daily means of barometric pressure, of $+ 0.238 \pm 0.040$, a value highly significantly different from zero. That this was a correlation with the concurrent hourly values and not based simply upon similarities of the mean forms of two independent average cycles was readily apparent by finding no correlation for the same period with the pressures of day $n + 3$ (0.0923 ± 0.0451), day $n - 1$ (0.100 ± 0.0425), and for half the period with day $n + 1$ (0.0118 ± 0.059), day

tions from the daily means of $5 \times 3 \times 3$-hour moving means of O_2-consumption on day n, yielded a coefficient of 0.58 ± 0.087. This rather high and unquestionably real correlation indicated that during this 60-day period, the potatoes must have been overwhelmingly of one sign in their correlation with the unknown external factor, namely positive, and, furthermore, this unknown effective force must have retained a high correlation with the morning barometric pressure change during this period. In no other lag or lead relationship, except for smaller and obviously explicable real correlations on days $n + 1$ and $n - 1$, did there appear from inspection to be correlations significantly different from zero.

$n - 2$ (0.0437 ± 0.059) and day $n - 4$ (0.0622 ± 0.057) of pressure change. The next highest correlation with hourly barometric pressure, and also highly significantly different from zero, was seen for barostat No. 5 for the same period. Barostats No. 1, 2 and 4 showed no comparable correlation. These results, together, clearly suggested that the failures to obtain correlations at some times with barometric pressure were not due to a lack of capacity of the potatoes to respond to a pressure-correlated external factor, but rather due to a failure to observe the response due to a mutual cancelling of opposite signs of response.

Finally, when all the 2976 available hours of data obtained from May 1 through June 8 were correlated with the hourly differences from the daily means for barometric pressure, a value of -0.0697 ± 0.01825 (Fig. 6) was obtained. This value is obviously not zero though very small chiefly because it must be the residual after cancellation of the frequent changes of sign of response to the barometric-pressure-correlated variable. The calculated regressional relationship between the rate of O_2-consumption, and the pressure, for all data, ignoring inversions, shows the rate of O_2-consumption to increase from about 6.8 to about 8.2 arbitrary units, or about

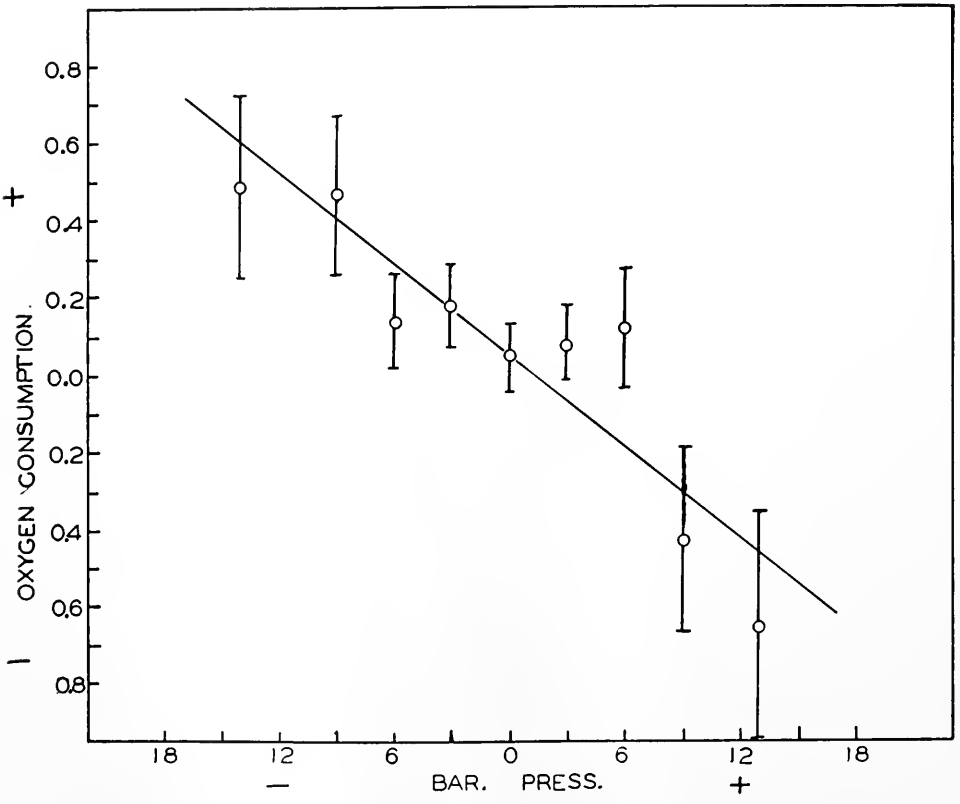


FIGURE 6. The relationship between the deviations from the daily mean of O_2-consumption of five groups of potatoes in barostats between May 1 and June 8, 1955, and the concurrent deviations from the daily mean of barometric pressure ($P < 0.002$).

21%, as the pressure ranges from 0.15 inches Hg above to about 0.15 inch Hg below the daily mean, a moderately large range in the normal pressure changes for a single day.

Discussion

It is quite clear from the preceding account that even when pressure, in addition to light, temperature, humidity and certain other factors are kept constant, there are still substantial fluctuations in O_2-consumption in potatoes. Pressure changes are obviously not the factor responsible. This was strongly suggested in earlier studies (Brown, Freeland and Ralph, 1955) inasmuch as the day-by-day drift in O_2-consumption at 4–7 P.M. appeared to parallel the day-by-day drift in mean barometric pressure, but apparently tending to lead the changes by one or two days.

One might suspect that possibly pressure changes were in some manner responsible for the general inversion of the 1955 cycles relative to the 1954 ones or for the hour-by-hour correlations with pressure change obtained in 1954. That this was not so, has been ascertained through other experiments using the fiddler crab and Fucus in our laboratory during the summer of 1955. These results, to be published elsewhere, show the cycles of these species also to be essentially inverted relative to comparable ones obtained in 1954 and similarly to show no longer the over-all hourly correlation with concurrent pressure changes. With the fiddler crab, however, parallel studies were made under conditions of fluctuating pressure exactly as was done in 1954; the cycles were, like those of the crabs in the barostats, similarly inverted relative to the 1954 ones, and similarly showed no significant over-all hourly correlation with pressure changes. There appears to be only one tenable hypothesis at this time concerning these inversions between the two years, namely, that some significant difference occurred between 1954 and 1955 with whatever fluctuating external factors are responsible for determining the form of the mean biological cycles. All organisms studied in 1955 also appeared to exhibit in some degree, the phenomenon of phase inversions relative to other individuals of the same species being studied concurrently. Whatever the mechanism, it must include to some extent the organism as a biologically responding system. It is possible that various individuals possess different thresholds for some factor which is itself responsible for the changes in sign of response to the external fluctuating factor.

The existence of the inversions which have been described in this report may lead one to question whether these fluctuations in metabolism are strictly rhythmic. Fluctuations in barometric pressure show both solar and lunar tides, but these are, in temperate zones, in good measure obscured by relatively huge climatic fluctuations. The general form of the solar tidal fluctuations may be made evident, however, through the averaging of two to five days of data. This, the potatoes seem also able to accomplish, judging from the results of this study. Hence they are apparently able to exhibit daily and lunar-day cycles of fluctuation, even though these are superimposed upon a much more randomly fluctuating background. But with the more or less erratic sign changes in 1955, the metabolism itself displays a true rhythm only for certain non-inverting components of the solar- and lunar-day cycles. The forms of the cycles are subject to the same fluctuations as are the mean daily pressure cycles. There is always the possibility, however, that the fluctuating factors which produce these responses in organisms, possess some sharper frequency-

determining component than the pressure cycles, from which the organism can obtain an adequate pacemaking signal to regulate their own internal clocks. But just as the large-amplitude random fluctuations in atmospheric pressure make it very difficult to characterize the tides therein, so may externally induced large random fluctuations in the organism tend to obscure a more precise extant organismic metabolic cyclicity.

One should always bear in mind that the metabolic cycles which were the object of this study are not as regularly rhythmic as are numerous overt organismic cycles such as color-change or motor activity in many animals. These latter must be mediated by an internal clock of the same precision as must be postulated to be functioning in these potatoes to integrate the effect of an external stimulus recurring at the same hours of the day over a few days. Such a clock is clearly needed to permit the organism to become an appropriate harmonic analyzer. Such a clock may also act as a buffer to filter out to some extent the large random elements of fluctuation in the external environment. The organism tends to retain for a time and repeat on a 24-hour cyclic basis an environmentally induced pattern of fluctuation.

It is considered highly probable that the responses to the still unknown external factor which are proven to occur by these and earlier experiments in some manner regulate the frequencies of the endogenous clocks.

Summary

1. Fluctuations in O_2-consumption in the potato under constant conditions, including pressure, were observed. The average solar-day cycle was determined and this was found to have in large measure a form which was the mirror image of that obtained during the same months of the preceding year.

2. The average lunar-day cycle was also determined and this, too, was in its principal features an inversion of that found for the same period of the preceding year.

3. A study of the patterns of daily variation for the 154 complete days of data revealed that all the patterns of fluctuation on any given day tended to exhibit a generic similarity to one another, tending to exhibit either parallel, or mirror image fluctuations, and of the same general amplitude.

4. All the daily patterns could be divided in two groups. One group (99 days) called the positive one (since its later discovered correlation with barometric pressure change was positive) possessed a maximum about 6 A.M. and a minimum about 9 A.M.; the negative group (55 days) tended to show the mirror image of this form in the daily period 4 to 11 A.M.

5. The deviation in rate of O_2-consumption from the daily mean for the positive group for the 4–7 A.M. period was found to show a positive correlation with the algebraic sum of the rates of change in barometric pressure during the three preceding 2–4 A.M. periods; the negative group, on the other hand, showed a negative correlation for the comparable relationship.

6. A correlation between the deviation from the daily mean of O_2-consumption at the 4–7 A.M. period and the algebraic sum of the pressure changes for the three preceding 2–4 A.M. periods was found for all 154 barostat-days if one ignored the sign of the deviation in rate of O_2-consumption, proving true an earlier hypothesis

that the sign of the response of the organism to an external pressure-correlated factor changed from time to time.

7. The deviation from the daily mean of O_2-consumption for the 4–7 P.M. period showed a negative correlation with the algebraic sum of the rates of barometric pressure change for the preceding three 2–4 P.M. periods, and the 10–1, midnight deviation, was correlated with the three-day pressure-change from 8–12 P.M.

8. Since it was demonstrated that the correlations were not with single days of an external factor, nor with any averaged three-day periods other than the three immediately preceding daily periods, it was evident that the potatoes were deriving an essential element of the form of their daily fluctuation from a response to an external factor which, since pressure-correlated, clearly possessed average solar-day cycles.

9. The external factor appears to determine in the daily fluctuation of O_2-consumption the amplitude of a morning oscillation (or its mirror image) with about a six-hour period, the height of the late-afternoon maximum, and probably also the extent of the midnight reduction in rate.

10. Possible relationships of the exogenous to endogenous cycles are discussed briefly with reference to the problem of biological clocks.

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THE IMMEDIATE EFFECTS OF LOW DOSES OF X-RADIATION ON THE FREQUENCY OF SEVERAL MITOTIC STAGES IN THE ALLIUM ROOT TIP

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Many studies have been made of the effects of radiations on mitosis in plant cells, but the design of the experiments has been such that they could not be compared readily with effects on animal cells. In the present study an attempt has been made to deal with plant material in the same manner as has been frequently used for animal tissues, namely, (1) to maintain the cells at a constant temperature before and after irradiation, (2) to make accurate dosage measurements at each treatment, (3)

TABLE I

The onion root tip cell mitotic cycle at 32° C.

Stage	Criteria by which the beginning of each stage is distinguished in the fixed, stained cell as observed with 4 mm. objective and 10× ocular	Relative frequency
Interphase	Densely granular appearance of nucleus caused by crowding of chromatin threads; nucleoli visible	.7546 (.7552)
Prophase		
Early	Nucleus enlarged; chromatin threads larger and less crowded; granules larger; nucleoli less distinct	.1672 (.1692)
Late	Chromosomes thicker and better separated; nucleoli absent	.0184 (.0182)
Prometaphase	Nuclear membrane absent	.0105 (.0112)
Metaphase	Chromosomes in equatorial plane	.0133 (.0124)
Anaphase	Proximal ends of chromatids separated	.0154 (.0102)
Telophase	Newly-formed cell plate visible; chromosomes less distinct	.0211 (.0211)

Figures in parentheses from Laughlin (1919).

to subdivide certain of the longer mitotic stages, so that more detailed information on the mitotic effect could be obtained, (4) to define carefully the terminology used to designate the different stages (Table I), and (5) to make regular and frequent counts of both control and treated cells.

[1] This study was submitted in partial fulfillment of the requirements for the degree of Master of Science in Zoology in the Department of Zoology and Entomology of the University of Tennessee. The author wishes to express gratitude to Dr. J. G. Carlson for his help in this study and his critical reading of the manuscript.

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Materials and Methods

Ease in handling and in control of mold as well as bacterial infection of the roots dictated the use of seeds of *Allium cepa* in preference to bulbs. Preliminary experiments showed no significant differences in mitotic frequency between root tips of different seeds.

Ultraviolet radiation was used to inhibit mold growth during germination. Preliminary tests indicated that the dose of ultraviolet used had no effect on the germination time of the seeds or the rapidity of root growth. Seeds were agitated continuously for a period of six minutes in a quartz flask at a distance of about four inches from a four-watt General Electric germicidal lamp. This was done in a closed, sterilized chamber, which was also used to make subsequent transfers. The seeds were then transferred, aseptically, to sterile petri dishes containing filter paper dampened with distilled water. The petri dishes and filter paper had been sterilized by dry heat and the water had been sterilized by ultraviolet radiation.

A darkened incubator maintained at 26° C. was used to assure constant temperature during germination.[3] After twenty-four hours the seeds were removed from the incubator long enough to receive a second ultraviolet treatment for one minute to further retard the growth of any mold that had succeeded in contaminating the dishes. No roots were in evidence at this time.

After sixty hours, the roots had reached a length of 9 to 15 mm. At this time forty germinated seeds were transferred to each of two petri dishes. One of these sets was used as a control. The other was irradiated with either 128 r or 512 r of x-rays at the same dose rate, *i.e.*, 86 r/min.[4] The 128 r and 512 r doses were chosen because they were not large enough to cause death of the cells but sufficiently large to give easily measurable mitotic inhibition. Irradiation was done with a Coolidge tube (122 kv.p., 5 ma., with a 0.28-mm. aluminum filter). The dose rate was determined with a Victoreen dosimeter before each treatment.

Immediately following x-irradiation, roots of two of the irradiated and two of the control seeds were fixed in a solution of three parts absolute alcohol, one part glacial acetic acid and one part chloroform for six hours. The remaining germinated seeds were immediately placed in an incubator maintained at 32° C. and kept there throughout a five-hour period. During this time control and treated root samples were removed and fixed at one-half-hour intervals.

The material was stained with Feulgen's reagent and made into squash preparations according to a method worked out by Dr. Mary Esther Gaulden of the Oak Ridge National Laboratory (personal communication).

The numbers of the different mitotic stages occurring in ten fields at the center of each root tip squash preparation were recorded. A Howard disc was used to avoid any overlapping of these fields. The number of each of the seven mitotic stages recorded in all tips at a given half-hour interval was summated to give obser-

[3] Preliminary experiments, in which the roots were allowed to grow in darkness at a constant temperature, showed that the diurnal mitotic rhythm, as described by Kellicott (1904), only occurs when *Allium* is allowed to grow in an environment where light is present. These experiments confirm that which was inferred by Gray and Scholes (1951).

[4] This was the dose rate as determined with the dosimeter lying on a wooden table at the same level at which the petri dishes were placed during treatment. The scattering effect of the glass, as determined by measurements made with the dosimeter lying on a petri dish, added approximately 1.3% to these doses and dose rates.

vational totals.[5] All observations were made with a 4 mm. objective and 10 × oculars.

A one-tailed chi-square test was run to determine when the experimental percentages differed, at the 5 per cent level of significance, from the control percentages. Using one degree of freedom, $P > 3.84$ indicates this significance. The results of these tests will be found in Tables II and III.

Results

Results of 128 r x-ray treatment

The effects of 128 r of x-rays on the different mitotic stages are shown in Table II. Interphase was the only stage in which the frequencies of cells showed no significant differences from control frequencies within five hours following x-raying.

Table II

Ratios of experimental frequencies to control frequencies for 128 r

Hours after x-raying	Stage						
	Interphase	Early prophase	Late prophase	Prometa-phase	Metaphase	Anaphase	Telophase
0.0	1.01	0.98	0.95	0.81	0.63	1.12	1.11
0.5	1.02	0.97	1.29	*0.53*	0.77	0.84	0.92
1.0	1.02	0.96	1.16	*0.46*	*0.55*	0.84	0.89
1.5	1.00	*1.15*	1.29	*0.47*	0.60	*0.51*	0.54
2.0	1.02	1.12	0.96	0.65	*0.32*	*0.33*	0.68
2.5	1.04	*1.17*	*0.43*	*0.23*	*0.13*	*0.25*	*0.32*
3.0	0.98	*1.39*	*0.59*	*0.33*	*0.14*	*0.29*	*0.29*
3.5	1.00	*1.44*	*0.20*	*0.05*	*0.07*	*0.14*	*0.13*
4.0	1.00	*1.46*	*0.14*	*0.00*	*0.00*	*0.06*	*0.13*
5.0	1.01	*1.36*	*0.17*	*0.00*	*0.00*	*0.00*	*0.00*

The italicized values indicate that the experimental percentage differs from the control percentage at the five per cent level of significance.

In all other stages, a period of "normal" mitotic activity ranging from a half-hour duration in prometaphase to between two and two and one-half hours in late prophase and telophase was followed by a significant change in frequency. The phrase, "normal mitotic activity," is used to denote the period in which no observable changes in mitotic activity in relation to the control cells was evident.

Early prophase cell frequencies increased significantly beginning one and one-half hours after x-raying, and remained significantly higher than the early prophase control cell counts throughout the five-hour sampling period.

X-rayed late prophases showed no significant differences from the controls for two hours, after which they gradually fell to a minimum at four hours.

[5] These totals may be found in the thesis from which this paper is condensed, in the University of Tennessee library. It is from these data that the ratios in Tables II and III were calculated. Totals at each time interval range from 1296–2420 cells for the controls and 1767–2910 cells for the treated root tips in the 128 r experiment, and from 1340–1742 cells for the controls and 1431–2510 cells for the treated in the 512 r experiment.

Prometaphase, metaphase, anaphase, and telophase treated-to-control ratios decrease more or less gradually, virtually reaching zero three to four hours after treatment.

Results of 512 r x-ray treatment

The effects of 512 r of x-rays on the different mitotic stages are shown in Table III. Following one and one-half hours of normal frequency, treated interphase cells increased in number and maintained a level significantly higher than the controls from two to four hours after treatment.

TABLE III

Ratios of experimental frequencies to control frequencies for 512 r

Hours after x-raying	Stage						
	Interphase	Early prophase	Late prophase	Prometa-phase	Metaphase	Anaphase	Telophase
0.0	1.01	0.96	1.29	1.00	0.89	1.19	1.08
0.5	1.02	0.98	0.91	0.67	1.10	0.95	0.98
1.0	0.99	1.04	1.50	0.86	0.99	0.85	0.97
1.5	1.03	1.03	0.99	*0.31*	*0.43*	*0.50*	0.65
2.0	*1.04*	1.12	0.91	*0.05*	*0.12*	*0.23*	*0.48*
2.5	*1.06*	1.08	*0.53*	*0.05*	*0.00*	*0.09*	*0.26*
3.0	*1.05*	*1.20*	*0.38*	*0.00*	*0.00*	*0.03*	*0.09*
3.5	*1.05*	*1.23*	*0.23*	*0.00*	*0.00*	*0.00*	*0.06*
4.0	*1.06*	*1.21*	*0.16*	*0.00*	*0.00*	*0.02*	*0.00*
5.0	1.02	*1.38*	*0.12*	*0.00*	*0.00*	*0.00*	*0.00*

The italicized values indicate that the experimental percentage differs from the control percentage at the five per cent level of significance.

The early prophase frequency does not differ significantly from the control frequency for two and one-half hours. Subsequently it shows a significantly higher frequency, which is maintained through the five-hour sampling period.

Late prophase, prometaphase, metaphase, anaphase, and telophase frequencies, on the other hand, gradually decrease after one to two hours at the normal level. Late prophases have virtually disappeared at the end of five hours; prometaphases, metaphases, anaphases, and telophases at the end of two to three hours after irradiation.

DISCUSSION

It has been found in a variety of materials, both animal and plant, that if tissues are exposed to ionizing radiations, a decrease in the number of prometaphases, metaphases, anaphases, and telophases follows, and if the dose of ionizing radiations is great enough to reduce the number of cells in these stages to zero, the order of disappearance of these stages will be the same as the order in which cells pass through them (Carlson, 1954). Ionizing radiations tend to cause a blockage in one of the mitotic stages. The cells that have passed this stage continue to progress through the mitotic cycle. Hence, it is obvious that the first stage after the blockage point

will be the first stage vacated by the cells in mitosis, and each subsequent stage would be vacated in the order in which they occur. The more or less gradual progression of cell frequencies toward zero in these stages, as determined in this paper, seems to be in complete agreement with Carlson's statement.

Investigators working with *Vicia* root tips have reported different intervals of time between treatment with ionizing radiations and minimal mitotic activity. Deufel (1951) found that if the broad bean root tip was irradiated with 150 r of x-rays at a dose rate of 5 r/minute, and the tips were kept at 18° C. between treatment and fixation, the low point of mitotic activity was reached after 12 hours. Mottram (1936) states that the minimal mitotic activity of the root tip cells was reached nine hours following a 210 r dose of gamma-rays. According to the work of Juengling and Langendorff (1930), the minimal mitotic rates occur (1) fifteen hours following a 175 r dose of x-rays, (2) eighteen hours following treatment with 420 r of x-rays, and (3) thirty-three hours after irradiation with 550 r of x-rays, but there is no change in mitotic rate after treatment with doses of 40 or 80 r of x-radiation. All of Juengling and Langendorff's work was done with a dose rate of about 20 r/minute.

Working with *Allium* root tips, Darlington and La Cour (1945) wrote that minimal mitotic activity occurs (1) about ten hours after treatment with 150 r of x-rays when the root tips are kept at a temperature of 24° C. after treatment, and (2) about twenty-four hours after x-irradiation with the same dosage if the root tips are kept at 16° C. after treatment. Marshak (1937), however, states that minimal mitotic activity occurs in *Allium* root tips three hours after treatment with either 70 or 220 r of x-rays at a dose rate of 20 r/minute. This is confirmed by Gray *et al.* (1940) using a small dosage of gamma-rays and maintaining a temperature of 25° C. following treatment. Gray, in a personal communication to Carlson (1954), however, states that "the true value could easily have been as late as six hours since the minimum tends to be rather flat."

It has been concluded by Carlson (1942) that the low point of mitotic activity in the grasshopper neuroblast, at 26° C., occurs one hundred minutes after treatment with 31 r of x-radiation. Carlson, Snyder and Hollaender (1949) found that the low point of mitotic activity in the grasshopper neuroblasts is reached about sixty-six minutes after treatment with 32 r of gamma-rays when the material is maintained at 38° C. following treatment. It may be the effect of temperature on the time required for irradiated cells to complete mitosis that accounts for the apparent wide discrepancies in the results cited above.

My studies show that minimal mitotic activity of *Allium* root tip cells occurs between three and four hours after treatment with 128 r of x-rays, and between two and one-half and three hours after treatment with 512 r of x-radiation. The material was maintained at a temperature of 32° C. between treatment and fixation.

Concurrence exists among investigators that, if the treatment dosage is sufficiently large, cells in interphase at the time of treatment may be prevented from entering early prophase. These findings are identical with those in the present study (see Tables II and III).

Differences of opinion, however, exist concerning the immediate reaction of prophase cells to irradiation. Koller (1943), working with *Tradescantia* pollen grains, is among those who believe that prophase is not very sensitive to x-radiation since cells in prophase decrease in frequency following irradiation. These prophase cells,

according to him, complete mitosis with little or no delay as do the cells in prometaphase through telophase. Koller, therefore, concludes that cells in interphase are the most sensitive to ionizing radiations.

By dividing the grasshopper neuroblast prophase stage into five sub-stages, Carlson (1940) concluded from statistical calculations that middle prophase is the stage most sensitive to mitotic inhibition. This same publication also indicated that cells treated while in middle and early prophase revert to earlier stages. This reversion of cells in middle and late prophase was later confirmed by direct observation of living cells (Carlson, 1942). St. Amand (1956) also confirmed this from studies of living cells. Deufel (1951) describes the slowing down of prophases in the *Vicia* root tip after irradiation.

As is shown in Table II, *Allium* root tip cells, treated with 128 r of x-rays, exhibit no significant decrease in late prophase frequency until two and one-half hours after treatment. Obviously, they are not progressing into prometaphase because the frequency of cells in this stage has been decreasing, significantly, since the first one-half hour after treatment, and continue to do so until frequency reaches zero. Therefore, it must be concluded that late prophases have begun to revert, two and one-half hours subsequent to treatment, to early prophase. Early prophase has been increasing, significantly, since one and one-half hours after treatment. This was probably due (1) to an accumulation of cells passing into early prophase from interphase and being, at least partially, blocked from continuing to subsequent stages, and (2) to reversion of late prophases after two hours.

Treatment of the root tip cells with 512 r of x-rays causes this same effect (see Table III) but to a greater degree. Late prophase cells could not be progressing to prometaphase after the third hour, at the latest, as prometaphase frequency is zero after that time. Therefore, as their count is dwindling, late prophase cells must be reverting to early prophase. Some early prophase cells are reverting to interphase as witnessed by a significant experimental increase in interphase cell counts at, and following, the second hour after treatment. The reversion of early prophase cells is indicated by the fact that late prophase is decreasing at the hours when early prophase is showing no significant change. Hence, reversion of early prophase cells would counterbalance the influx of cells into that stage from late prophase. Interphase is increasing significantly at this time. Of course, some of the interphase increase is due to the cells passing from telophase into interphase, but this influx ceases before interphase increase stops.

If these hypotheses and conclusions are justified, they would indicate that late prophase is the most sensitive stage and early prophase is the second most sensitive stage to the effects of x-radiation. Since the criteria I used to designate late prophase seem to include the late and middle prophases of Carlson (1940, 1941, 1942) and St. Amand (1956), the stage sensitivities shown in this paper closely parallel the results reported by these two investigators. Reversion is not a limited phenomenon by any means. Beatty and Beatty (1954a and 1954b) have reported cases of it in *Tradescantia,* while Darlington and La Cour (1945) have noted evidence of reversion in *Trillium.* Reversion of early prophase to interphase may explain the long period in which no change in frequency is observed in early prophase.

The period in which no significant difference between experimental and control frequencies occurred, demonstrated by cells in late prophase, both after 128 r and 512 r doses, could be explained, possibly, in this manner. The critical period must

be in late prophase if reversion occurs as has been previously stated. This period could not be too far into late prophase, however, as no piling-up of cells occurs in this stage. Some cells may pass through this block following treatment to give the "normal" mitotic period in late prophase. This passage will also contribute to the "normal" mitotic periods in subsequent stages for a short time following treatment.

Piling-up in late prophase need not occur, even if the critical period is in late prophase, if all the cells very close to the critical period at the time of irradiation are so sensitive as to revert.

Summary

1. Root tip cells of germinated *Allium* seeds were given doses of 128 or 512 r of x-rays. The mitotic effects of this treatment were determined by making counts, in fixed preparations, of cells in several mitotic stages at regular intervals following x-raying.

2. After treatment with 128 r of x-rays virtual disappearance of cells in late prophase, prometaphase, metaphase, anaphase and telophase was observed between three and four hours. No observable change occurred in the interphase frequencies, but an increase was observed in early prophase one and one-half hours subsequent to treatment.

3. After receiving 512 r of x-radiation, late prophase, prometaphase, metaphase, anaphase and telophase cells fell to minimal frequency between one and one-half hours and three hours. The fall in frequencies after this dose was noticeably more rapid than following the 128 r dose. An increase was noted in interphase frequency two hours following treatment and in early prophase three hours following treatment. Again, late prophase frequency did not reach zero, but was at its lowest point five hours subsequent to treatment.

4. Minimal mitotic activity in the root tip cells of *Allium cepa* is reached between three and four hours subsequent to treatment with 128 r of x-rays, and between two and one-half and three hours following treatment with 512 r of x-rays. Between treatment and fixation all root tips were maintained at 32° C.

5. Reversion of cells from late prophase to early prophase is indicated when the cells are treated with 128 or 512 r of x-radiation and from early prophase to interphase when the cells are treated with 512 r of x-rays. It is probably due to this latter reversion that the minimal mitotic rate was reached in less time by the cells treated with the larger dose of x-rays.

6. Late prophase, which includes the latter portion of middle prophase of certain other investigators, seems to be the most sensitive and early prophase the second most sensitive stage to the mitosis-inhibiting effect of x-radiation.

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LARVAL DEVELOPMENT OF BALANUS EBURNEUS IN THE LABORATORY [1]

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Most of the descriptions of larval development of barnacles have been based upon material obtained from the plankton. By this method Willemoes-Suhm (1876) found that *Lepas fascicularis* passed through six free-swimming naupliar stages and one cyprid stage. Since his publication others have made similar studies on different species of acorn barnacles and reported that they may pass through from six to eight naupliar stages and one cyprid stage. Because of the similarity of naupliar and cyprid stages of different species in the plankton the sequence of stages in reconstructions has been questioned. Hence many investigators have attempted to verify reconstructed life cycles by rearing barnacles from the egg to the sessile stage in the laboratory.

Attempts to rear nauplii from unhatched eggs or from naupliar stages in the plankton have met with limited success. Groom (1894a), for example, only maintained *Balanus perforatus* nauplii through the second stage and Treat (1937) was unable to rear *Balanus balanoides* beyond the third stage. Sandison (1954) was also unsuccessful in maintaining several South African barnacles (*Balanus algicola, Balanus amphitrite denticulata, Balanus maxillaris, Balanus trigonus, Chthamalus dentatus, Octomeris angulosa* and *Tetraclita serrata*) beyond the third naupliar stage. Bassindale (1936) was able to rear *Verruca stroemia* to the cyprid stage by feeding the nauplii on *Nitzschia* sp. but the cyprids failed to settle. By using the same methods he could raise *Balanus balanoides* to the fifth naupliar stage only. Batham (1945) maintained the non-feeding nauplii of the goose-barnacle, *Pollicipes spinosus,* from the egg to a stage described as "post-cypris," but no attachment occurred. There have been two definite reports of barnacles being successfully reared from the egg to the settled pin-head: that of Herz (1933) for *Balanus crenatus* and Hudinaga and Kasahara (1941) for *Balanus amphitrite hawaiiensis.* Hudinaga and Kasahara also reared *Tetraclita squamosa* on *Skeletonema costatum* and *Nitzschia closterium* but they all died in the cyprid stage. Pyefinch (1948b, 1949a) refers to culturing and describes the larval stages of *Balanus crenatus,* giving the time of appearance after hatching for the individual stages in the laboratory. He does not discuss the duration of the stages or mention successful attachment and metamorphosis of the cyprid. All investigators to date have reared larvae in mass culture and have determined time and stage of molting by daily sampling. While this method may indicate the number of larval stages it does not give accurately the molting frequency or the variations in intermolt periods within the population nor does it take into account the per cent mortality.

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The barnacle selected for this study was *Balanus eburneus,* for neither has it been reared in the laboratory nor have its larval stages been described, even though it has a range from Massachusetts to South America (Pilsbury, 1916). Its larval history is known only through the report of Grave (1933) who merely stated that it passes through naupliar, metanaupliar, and cyprid stages in 7 to 10 days at Woods Hole, Mass. Therefore, the present study was undertaken to determine the food which would support complete development, the number and description of the larval stages, the frequency of molting, duration and mortality of each intermolt and the length of time required for complete development.

Methods

Intact adult *Balanus eburneus* were removed from pilings and cleaned of attached organisms. In the laboratory the basis of the barnacle was chipped away and the egg lamellae removed. The developing ova obtained and successfully reared had attained the distinct median eye spot and gray color which corresponds to the "H" stage of Groom (1894a). The lamellae were placed in finger bowls containing filtered sea water with a salinity of 28.5 per thousand, *Chlamydomonas* sp., and 200,000 to 400,000 units of penicillin per liter. The bowls were then covered and maintained at 26° C., the average outside water temperature, in a constant-temperature culture cabinet lighted by daylight fluorescent lamps. In order to obtain nauplii of known age only those which were observed to hatch were used. At the time of removal each nauplius was placed in a separate compartment of the rearing assembly. The assembly was made by drilling 100 holes in a piece of ⅜″ lucite with a second solid piece of lucite forming the bottom. Each well had a capacity of 1.2 cc. The assembly was then placed in a glass dish, covered, and maintained in the culture cabinet. The contents of the wells were checked two to three times daily with a binocular dissecting microscope. When an exuvium was found it was removed, placed in 70 per cent alcohol, and the time, number of the molt, and mortality recorded for each nauplius. After the second molt freshly fertilized *Arbacia punctulata* eggs were introduced daily into the compartments in addition to the *Chlamydomonas* sp. At this time the larval plutei, developed from the eggs of the previous day, were removed.

The naupliar stages were determined from the number of molts under segregated conditions. These were drawn to scale on graph paper, with the aid of a Whipple disc, from the exuviae, fixed specimens, and, in a few cases, from the living nauplii. The setation formulae were obtained from the exuviae and dissected appendages of known stages. Measurements were made with an ocular micrometer mounted in a compound microscope. In addition to the 121 nauplii raised in individual compartments, hundreds of newly hatched larvae were maintained in finger bowls and plastic compartmented boxes. From these sources specimens were fixed daily in 70 per cent alcohol and 60°-C. Bouin's. After the stages had been determined from exuviae of segregated barnacles the fixed specimens were staged and studied to determine the consistency in appendage setation.

Results and Discussion

The nauplii of *Balanus eburneus,* reared individually, pass through six stages and one cyprid stage, a conclusion which is consistent with Bassindale's (1936) rear-

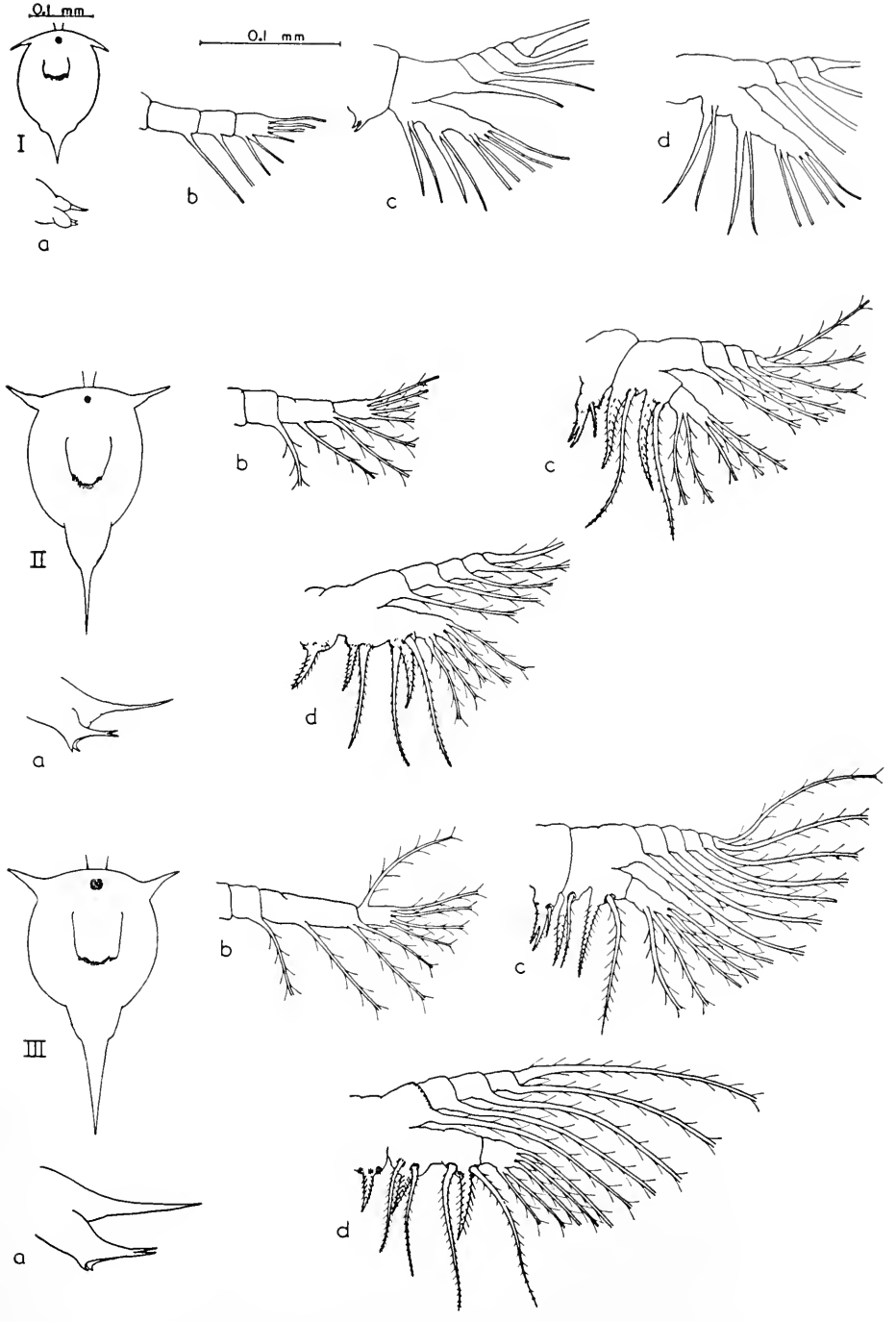

FIGURE 1. Carapace, caudal and abdominal processes, and appendages of naupliar stages I, II, and III of *Balanus eburneus* reared in the laboratory. All swimming setae are cut short. **a,** lateral view of abdominal and caudal processes; b, antennule; c, antenna; d, mandible.

ing of *Verruca stroemia,* and *Chthamalus stellatus* and the reconstructions of larval stages obtained from the plankton on *Balanus perforatus* (Groom, 1894b), *Balanus balanoides, Balanus crenatus, Verruca stroemia* (Pyefinch, 1948a), *Elminius modestus, Balanus improvisus, Balanus crenatus* (Knight-Jones and Waugh, 1949), and *Balanus algicola, Balanus trigonus, Octomeris angulosa* (Sandison, 1954). However, our results, Bassindale's (1936), and those based on reconstructions from the plankton do not agree with those obtained from culture methods reported by Herz (1933) and Hudinaga and Kasahara (1941). Herz (1933) found that *Balanus crenatus* passed through eight naupliar stages and one cyprid stage and Hudinaga and Kasahara (1941) reported seven naupliar stages and one cyprid stage for *Balanus amphitrite hawaiiensis.* The discrepancy between staged barnacle larvae obtained from the plankton and those taken from mass culture by Herz (1933) and Hudinaga and Kasahara (1941) may be due to the use of appendage setation and other morphological characteristics as the main criteria for staging rather than the use of the exact number of observed naupliar exuviae from the egg to the cyprid stage. As is shown below, setation and spine structure were considered in this study but the number of naupliar stages was based solely on the exact number of molts through which each individual passed.

Nauplii. The most significant characters for each naupliar stage are given below.

Stage I. (Fig. 1, I.) The small frontolateral horns appear slightly recessed and project caudally. The horns are frequently hidden on the ventral side by the antennules and antennae. The caudal process is short and blunt and the abdominal process terminates in two short spines (Fig. 1, Ia). All setae are devoid of setules.

Stage II. (Fig. 1, II.) The frontolateral horns project from the carapace at approximately right angles. The bases of the horns appear slightly swollen. The abdominal process now bears one spine on each side of the base and is approximately half the length of the caudal process (Fig. 1, IIa). All setae bear setules.

Stage III. (Fig. 1, III.) The frontolateral horns are tapered gradually from the junction with the carapace. The abdominal process bears the same spines as in stage II but is now greater than half the length of the caudal process. While larger and slightly heavier than stage II the primary distinguishing features are differences in setation of the antennules and antennae.

Stage IV. (Fig. 2, IV.) The posterior edge of the carapace is delimited from the caudal process for the first time and bears a pair of carapace spines. The abdominal process bears two spines on each side. One pair is located at the base and the second pair in line with the division between the caudal process and the abdominal process (Fig. 2, IVa).

Stage V. (Fig. 2, V.) There are two pairs of spines near the terminal portion of the abdominal process, a large lateral pair and a smaller median pair. Anterior to these is a pair of spines between which is a small, mid-ventral spine. The base is quite swollen and shows partial segmentation. The maxillule first appear (Fig. 2, Va).

Stage VI. (Fig. 2, VI.) The abdominal process is considerably enlarged over the fifth stage and six pairs of small spines mark the developing cirriform appendages beneath the exoskeleton. The spines on each side of the mid-ventral

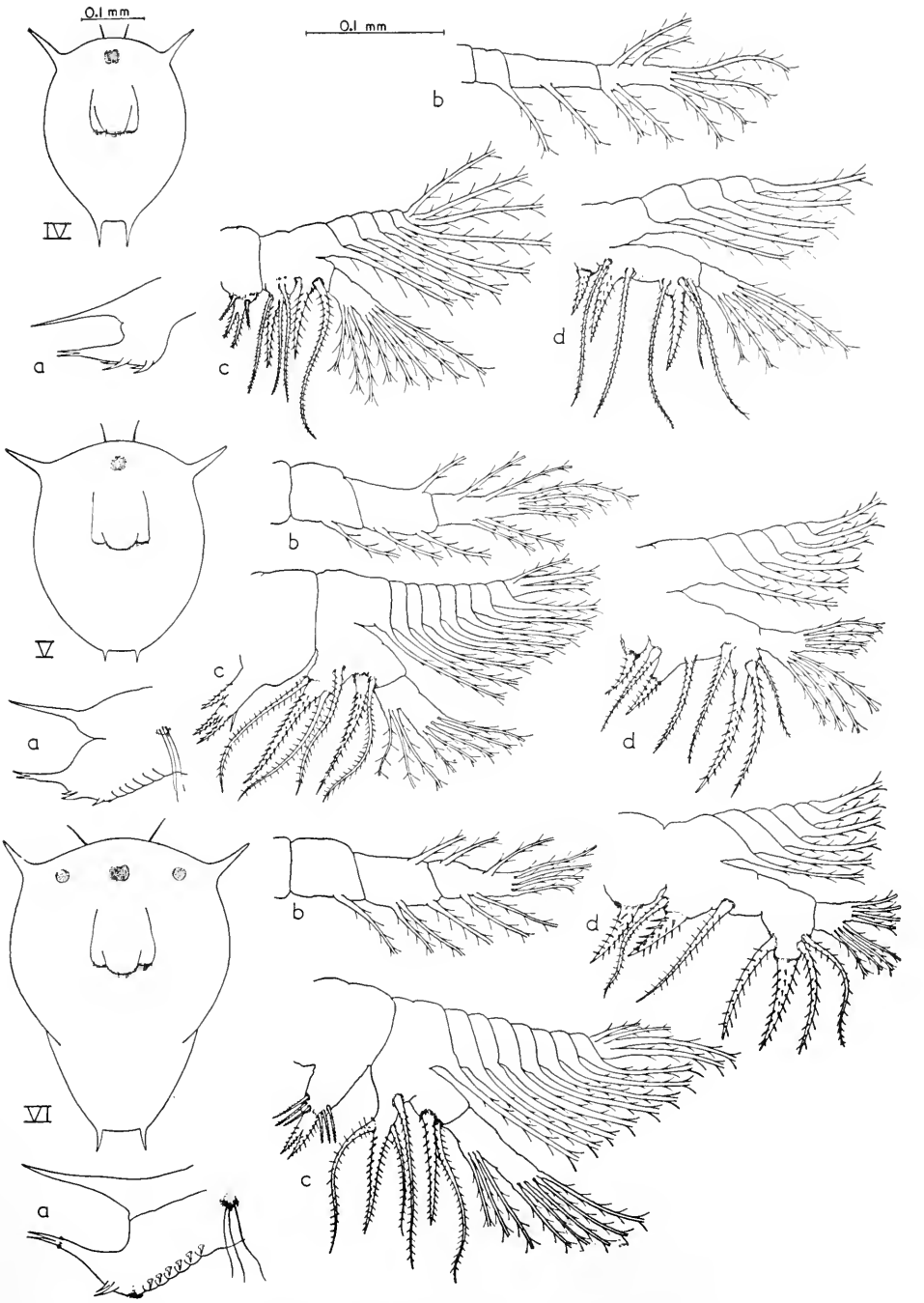

FIGURE 2. Carapace, caudal and abdominal processes, and appendages of naupliar stages IV, V, and VI of *Balanus eburneus* reared in the laboratory. All swimming setae are cut short. a, lateral view of abdominal and caudal processes; b, antennule; c, antenna; d, mandible.

spine, found in the fifth stage, are no longer present. The paired eyespots become quite distinct in the later sixth stage nauplii.

Cyprid. No significant characteristics were observed which distinguish *Balanus eburneus* cyprids from those of *Balanus amphitrite denticulata* (unpublished data).

Table I gives the setation formulae for each naupliar stage of *Balanus eburneus.* Since Bassindale's (1936) introduction of this system it has been applied to nauplii of many species of barnacles. Unfortunately, as asserted by Bassindale, the setation formulae alone do not give a definite indication of stage or species. Knight-Jones and Waugh (1949) point out the extreme differences between types of setae and believe it to be misleading. They note a remarkable similarity between the setation of earlier stages of several species. Stage I of *Elminius modestus, Balanus improvisus* and *Balanus crenatus* were found to be identical and stage II of *Elminius modestus, Chthalamus stellatus,* and *Verruca stroemia* had corresponding setation. San-

TABLE I

Setation formulae of the six naupliar stages of Balanus eburneus

Stage	Antennule	Antenna	Mandible
I	0.4.2.1.1.	0.1.4.–0.3.2.2.2.G.	0.1.3.–0.3.2.2.2.G.
II	0.4.2.1.1.	0.2.4.–0.3.2.2.2.G.	0.1.4.–0.3.2.3.2.G.
III	1.4.2.1.1.	0.2.5.–0.3.2.2.2.G.	0.1.4.–0.3.2.3.2.G.
IV	1.1.4.2.1.1.	0.3.5.–0.5.3.2.4.G.	0.1.4.–0.4.2.4.3.G.
V	1.1.1.4.2.1.1.1.	0.3.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.
VI	1.1.1.4.2.1.2.1.	0.4.8.–0.5.3.2.4.G.	0.1.5.–0.4.4.4.3.G.

dison (1954) gives the setation formulae for *Balanus algicola, Octomeris angulosa,* and *Tetraclita serrata.* The first stage of *B. eburneus* corresponds in setation to those forms listed by Sandison (1954) and also *B. crenatus* which in turn makes the first stage of *B. eburneus* identical to *E. modestus* and *B. improvisus.* The setation of the second to sixth stages of *B. eburneus,* to our knowledge, is different from that described for any other species. Norris, Jones, Lovegrove and Crisp (1951) found the setation formulae to be of very limited value in studies on *Balanus perforatus, B. improvisus,* and *B. amphitrite denticulata* and suggest setation to be a developmental feature rather than a specific one for the separation of barnacle larvae. In both *B. improvisus* and *B. amphitrite denticulata* Norris *et al.* (1951) found some variation in the setation of the mandibular exopodite of stage II. They observed that it may "sometimes" resemble the third stage by bearing five setae instead of four and suggest it as a possible explanation for the eight stages found by Herz (1933). Pyefinch (1949a), however, examined thousands of staged nauplii of *Balanus crenatus* and found setation to be uniform within each stage. Norris *et al.* (1951) propose that the rate of morphological development of eyes, limbs, and setae is more strongly influenced by temperature than the rate of growth and onset of ecdysis and consequently the latter do not always occur in step with the former. Hudinaga and Kasahara (1941) note that there is no change in setation of the three appendages between the sixth and seventh stages of *B. amphitrite hawaiiensis.* They discriminate between the two stages primarily on the presence of completely developed paired eyes in the seventh stage as compared to the rudimentary nature in the sixth stage. The present study of *B. eburneus,* in addition to the accounts of other species

by Pyefinch (1949a), Bassindale (1936), and Sandison (1954), shows that the change from rudimentary to completely developed paired eyes takes place in the sixth stage. The development of *B. eburneus*, under controlled temperature conditions, does not reveal any variation in setation within any one stage. Exuviae collected from nauplii undergoing a known molt showed consistency in the setation for that particular stage.

Pyefinch (1949a) describes the third stage of *B. crenatus* as the first one with a delimited carapace bearing a pair of carapace spines. This feature is seen first in the fourth stage of *B. eburneus* and also in *Balanus algicola, Balanus trigonus, Octomeris angulosa* (Sandison, 1954) and *Pollicipes spinosus* (Batham, 1945). The consistency of the mandibular exopodite setation in all 6 naupliar stages of *B. crenatus* appears to be an unusual feature when compared with the increase in setation described for other species.

TABLE II

Measurements of larval stages of Balanus eburneus reared under laboratory conditions

Stage	Carapace		Total length (mm.)
	Width (mm.)	Length (mm.)	
I	0.16–0.18	—	0.19–0.23
II	0.21–0.23	—	0.32–0.34
III	0.23–0.27	—	0.35–0.38
IV	0.27–0.32	0.30–0.33	0.40–0.42
V	0.29–0.34	0.35–0.38	0.44–0.48
VI	0.36–0.39	0.42–0.50	0.54–0.60
Cyprid	—	—	0.46

Table II gives the range in size of the six naupliar stages and one cyprid stage of *B. eburneus*. Knight-Jones and Waugh (1949), using size distribution followed by setation formulae for staging planktonic material, found greater variation in size in the later stages. They also note that "total length" of nauplii is affected by flexing of the abdomen and thus this measurement may be erroneus. Greater variation in size of the later stages was also observed in this study of *B. eburneus* in the laboratory. With setation and observable internal development, such as paired eyes and cirriform appendages, the carapace width and length would be more reliable than total length for staging laboratory reared larvae and presumably planktonic material. The latter study, however, must be made before definite conclusions can be drawn.

Figure 3 gives the variation in duration of intermolt period for the individual stages. Grave (1933) postulated 7 to 10 days as the time required for over-all development of *B. eburneus* at Woods Hole but did not include periods for each stage. Hudinaga and Kasahara (1941) estimated that the individual naupliar stages of *B. amphitrite hawaiiensis* lasted one day each and Pyefinch (1948b), using plankton samples taken at three-day intervals, postulated that each stage of *Balanus balanoides* was three days, or less, in duration. In the present study the duration of the first stage of *B. eburneus* ranged from 15 minutes to 4 hours. Many authors have reported that it is quite short in other species and Hudinaga and Kasahara (1941)

noted that it lasted for 15 minutes to 2 hours for *B. amphitrite hawaiiensis*. The second stage of *B. eburneus* lasts approximately 24 hours and the third 36 hours but with greater variation (Fig. 3). Stage IV averages 2 days, stage V, 2.6 days, and stage VI, 2.5 days but with less uniformity than the earlier stages.

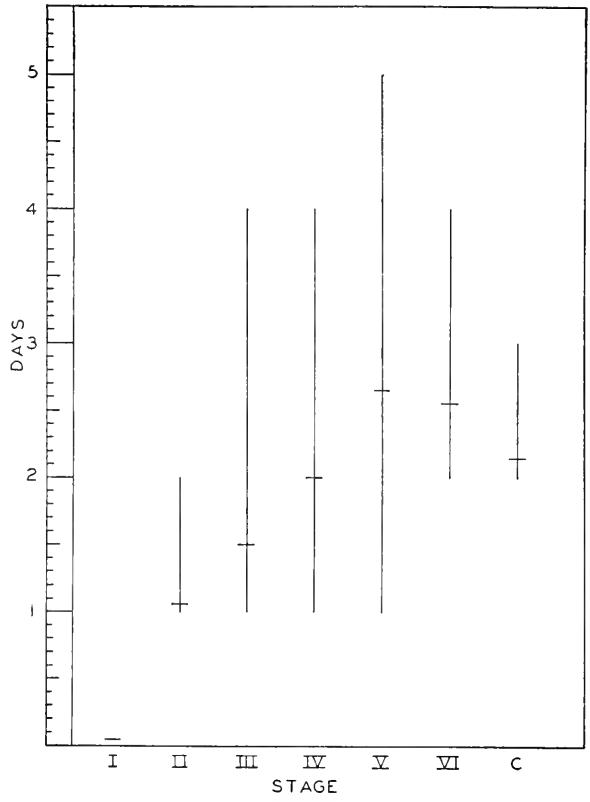

FIGURE 3. Duration of intermolt of naupliar stages (I–VI) and cyprid stage (c) of 121 *Balanus eburneus* reared under segregated conditions. The vertical line indicates the range. The horizontal line indicates the mean.

One hundred per cent of the first stage nauplii underwent ecdyses from 15 minutes to 4 hours after hatching. This was true whether food was available or not. The second stage nauplii molt from 10 to 35 hours after hatching. During this stage the gut appeared green with *Chlamydomonas* sp. The third stage nauplii may molt from the second to fifth days (Fig. 4). As indicated by Sandison (1954) this is a period when many nauplii die if proper conditions are not present. Therefore, it was at this stage that we added to the diet of *Chlamydomonas* sp. fertilized *Arbacia* eggs to furnish food of animal origin. The latter were ingested and the gut took on the echinochrome color of the *Arbacia* eggs. Even so, there was a mortality of 6 per cent (Fig. 5). The fourth naupliar stage shows a greater degree of variation in molting and ecdysis may occur from the third to eighth day with a majority molting on day 4 (Fig. 4). This is accompanied by an increase in mortality (Fig.

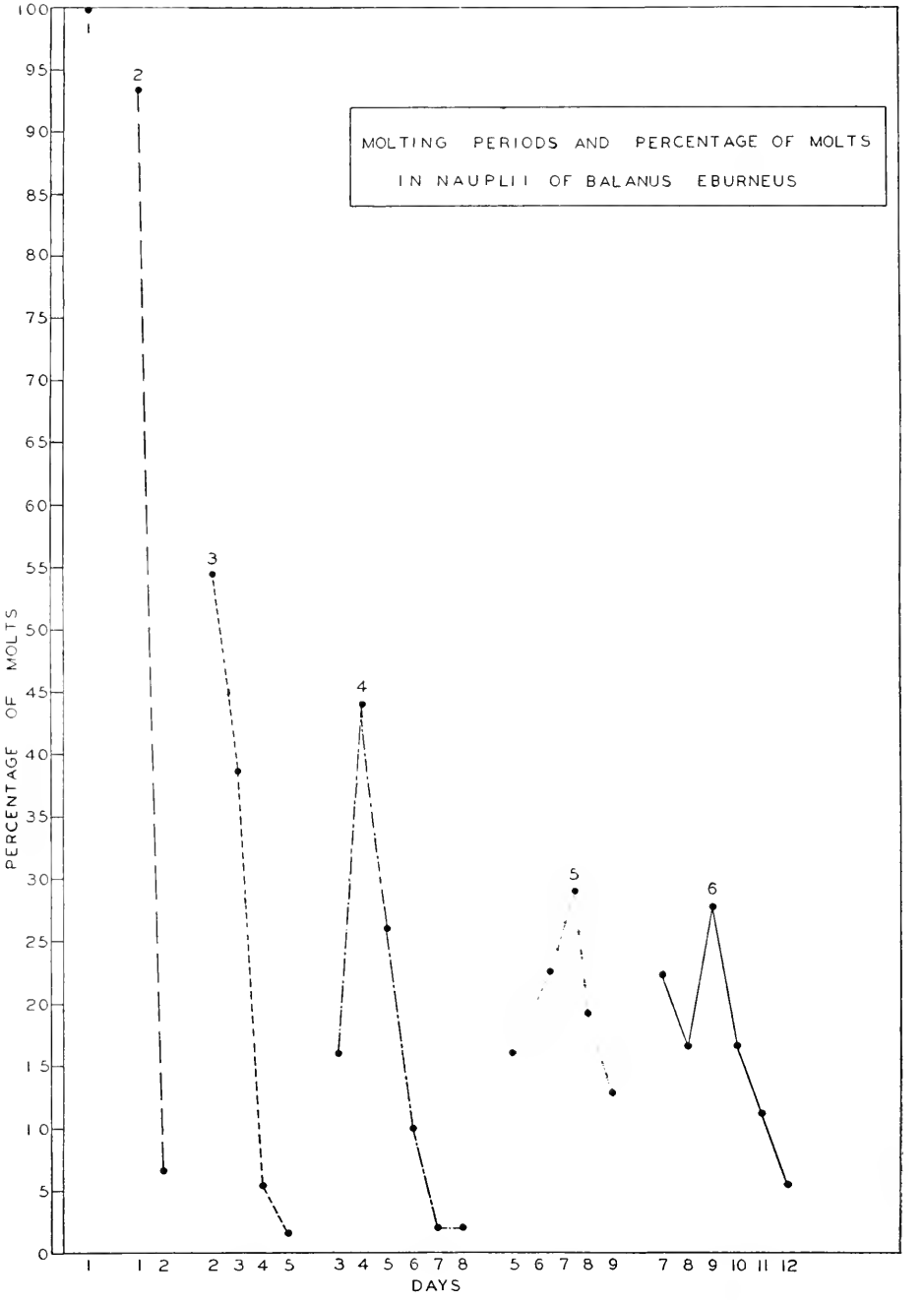

FIGURE 4. Molting frequency of naupliar stages of 121 *Balanus eburneus* reared under segregated conditions. Molts are indicated by numbers.

5). Molting of the fifth naupliar stage occurs from day 5 to 9, with a majority on day 7. This stage showed greater mortality than any other stage, it being approximately 33 per cent. The sixth and final naupliar stage molts into a cyprid on the seventh to twelfth day, with a mortality of 22 per cent.

There is apparently considerable variation in the duration of the cyprid stage. Pyefinch (1948b) reports that the cyprid of *Balanus balanoides* remains free-swimming in laboratory tanks for five days at 4–5° C. but estimated a shorter period in nature. Under laboratory conditions we found that those which settled and underwent metamorphosis to the pinhead did so within one to three days, whereas

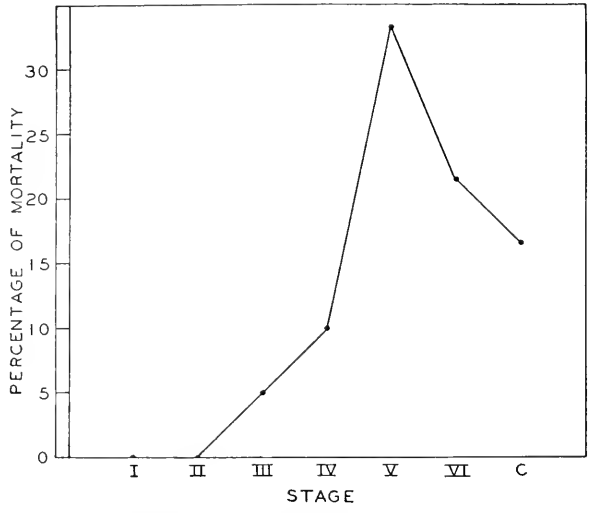

FIGURE 5. Mortality in relation to naupliar stages (I–VI) and cyprid stage (c) of 121 *Balanus eburneus* reared under segregated conditions.

those which persisted as cyprids for longer periods, up to 14 days, failed to settle and died. Mortality in the cyprid stage was approximately 16 per cent. In some cases the mortality was due to incomplete closing of the carapace following the sixth naupliar molt. These abnormal cyprids could live as swimming larvae for two to three days but they failed to settle.

The over-all time of development for *B. eburneus* is quite short when compared with the times given for most other species. Bassindale (1936), although not giving water temperatures, reported 13 days as the minimum time for completion of the sixth stage of *Chthamalus stellatus* and a 22-day minimum for the sixth stage of *Verruca stroemia*. Unfortunately, comparison between species is not too reliable, for the effect of temperature on individual species has not been determined and can only be inferred from studies on other species at different temperatures. Hudinaga and Kasahara (1941) found that the minimum time for development of *B. amphitrite hawaiiensis,* from the first naupliar stage to settling, was 7 days at 23–28° C. This falls within the range found by us for *B. eburneus* at 26° C. Grave (1933) postulated from planktonic material that *B. eburneus* takes 7–10 days for complete development. This figure corresponds with ours even though the temperature at Woods Hole is normally 4 to 8° C. lower than at Beaufort.

The best evidence to date that reduced temperatures increase the time of over-all development of barnacles is shown in the work of Pyefinch (1948b) and Batham (1945). Pyefinch (1948b) found that the over-all development of *B. crenatus* took approximately 30 days at 4–5° C. whereas at 15° C., "or more," the time was reduced to 16 days. Batham (1945) found that the goose-barnacle, *Pollicipes spinosus*, takes 12 days to pass through all larval stages at 18° C. while in the "cold room" (temperature not given) it required 20 to 21 days. The times given by Batham (1945), however, did not include normal completion of the cyprid stage or settling.

The relationships of the substratum and physical factors to settling are outside the scope of this paper. In the present study the only substratum offered was lucite and the physical factors of temperature and light were similar throughout the experiment, in that the temperature was maintained at 26° C. and the rearing assembly received constant illumination from below.

There is always the question of normality when organisms are reared in the laboratory and the query of how survival compares with that in nature. In this experiment if a barnacle completed development, settled, and metamorphosed it was considered normal. Pyefinch (1949b) estimated the survival of barnacle larvae to be between 1 and 9 per cent, depending on the species with which he was working. Bousfield (1955) questions his estimate because of the methods used and was of the opinion that natural survival was approximately 10 per cent. Therefore, our observed survival of 16.3 per cent, under laboratory conditions, is higher than that estimated in nature. Whereas it is undoubtedly true that the chief sources of mortality in nature are dispersal seaward and predation, improper food and bacteria are the chief causes in the laboratory. In preliminary experiments we found that *B. eburneus, B. amphitrite denticulata*, and *Chthamalus fragilis* larvae could not be reared beyond the third stage on a diet of *Chlamydomonas* sp. alone. When developing *Arbacia* eggs and penicillin were included, complete development occurred. The actual value of the penicillin is not known but it was observed that the tendency of nauplii to stick to surfaces of the rearing assembly was considerably reduced.

Summary and Conclusions

A technique has been devised for rearing segregated barnacle nauplii, under controlled laboratory conditions, which permits daily observations on the frequency of molting, the number of stages, and the specific characteristics of each stage. From a study of 121 segregated *Balanus eburneus*, plus hundreds in mass culture, reared on *Chlamydomonas* sp. and *Arbacia* larvae at 26° C. the following conclusions may be drawn:

1. Ecdyses provide a definitive method for staging nauplii. The larval phase of *B. eburneus* consists of six naupliar stages and one cyprid stage. Secondary criteria, such as body size, spine structure, and appendage setation, are given for the larval stages.

2. The duration of the six naupliar stages is as follows: first stage, 15 minutes to 4 hours; second stage, one to two days with an average of one day; third stage, one to four days with an average of 1.5 days; fourth stage, one to four days with an average of two days; fifth stage, one to five days with an average of 2.6 days; and the sixth stage, two to four days with an average of 2.5 days.

3. The cyprid stage ranges from one to fourteen days but successful attachment was observed only in those which settled one to three days following the final naupliar molt.

4. The over-all larval development in the laboratory ranges from 7 to 13 days.

5. The first ecdysis occurs from 15 minutes to 4 hours after hatching and is usually followed by the second molt during the first day. The third molt occurs from the second to the fifth days and the fourth molt takes place from the third to the eighth day. The fifth ecdysis occurs from the fifth to the ninth day with the sixth molt ranging from the seventh to the twelfth day.

6. Tables are given for the size of the nauplii and the setation of the appendages.

7. Successful metamorphosis and attachment was observed in 16.3 per cent of the 121 barnacle nauplii studied under segregated laboratory conditions.

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AMINO ACID CONTENT OF MARINE BORERS

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Although a great amount of study on the prevention of marine borer attack on wooden structures has been made in the last century, relatively little is known of the biochemical make-up of these organisms. The only published investigation of the amino acid content seems to be that of Lasker and Lane (1953) who reported eight amino acids present in *Teredo bartschi* Clapp. In view of the current idea (Fox, 1956) that primitive organisms have proteinaceous matter similar to that found in higher forms of life, it does not seem likely that several commonly occurring amino acids would be absent from the protein structure of *Teredo*.

In the present investigation *Teredo diegensis*, *Bankia setacea* (another member of the Teredinidae family) and two species of the crustacean borer *Limnoria* were investigated to determine their amino acid content. In all cases considerably more than the eight amino acids reported by Lasker and Lane were found to be present. In addition, southern yellow pine, redwood, and greenheart (*Ocotea rodiaei*) were hydrolyzed for identification of the amino acids present. The latter species of wood has been reported to possess a high protein content (Marchán, 1946) and to be highly resistant to marine borer attack (Baldwin, 1938).

Materials and Methods

The marine borers investigated were *Teredo diegensis, Bankia setacea,* and *Limnoria tripunctata* and *quadripunctata*. The first two were removed from both pine and redwood blocks, the *Limnoria tripunctata* from a creosoted log, and the *quadripunctata* from a redwood block in the same waters. Whole adult animals taken from the harbor and *Teredo* larvae produced in the laboratory were hydrolyzed. The larvae were collected by screening the overflow sea water from a tank containing pine blocks infested with *Teredo*. In addition to analyzing whole animals, *Bankia* was dissected into viscera, mantle, and gill, and *Teredo* into viscera, mantle, and reproductive organ containing larvae, and each section was analyzed individually. These dissected parts were extracted with 80% alcohol prior to hydrolysis in order to separate the free amino acids from those bound in the protein structure of the animals. The extracts were concentrated, the fatty substances extracted with chloroform, and the residues spotted for chromatography without further treatment.

Acid hydrolysis was in all cases effected by heating with 6 N HCl in a boiling water bath for twenty hours. Excess of HCl was removed by repeated evaporation to dryness under reduced pressure. Two-dimensional chromatograms using Whatman No. 1 filter paper with phenol solvent (Block, 1950) in the first direction and lutidine-collidine (Dent *et al.*, 1947) in the second were routinely run by the capil-

lary ascent method of Williams and Kirby (1948). The leucines were separated on a one-dimensional descending chromatogram using water saturated *tert*-amyl alcohol in an atmosphere of diethylamine. Confirmation of the identity of each spot was made by running suitable quantities of authentic amino acids simultaneously. The amino acids were revealed with the ninhydrin reagent of Levy and Chung (1953). Histidine and tyrosine were further identified by Block's (Block *et al.*, 1952) modification of the Pauly reaction; arginine, by one-dimensional chromatography using the solvent system of 77% alcohol and diethylamine (Block and Bolling, 1951); and the sulfur-containing amino acids, with the platinic iodide reagent (Toennies and Kolb, 1951). Dent's (1948) hydrogen peroxide treatment likewise proved to be useful in identifying the sulfur-containing amino acids.

A spot corresponding to lanthionine was eluted by the method of Dent (1947) from an unsprayed two-dimensional chromatogram. It was then spotted on a one-dimensional descending paper and run for five days using *n*-butyl alcohol-acetic acid (Partridge, 1948) as the solvent.

Glutamine, which was found to occur as a free amino acid in the Teredinidae, was further identified by elution (Dent, 1947) from an unsprayed two-dimensional chromatogram, by hydrolysis with acid, and by rechromatography. Glutamic acid was the only amino acid found on this latter chromatogram.

The barium hydroxide hydrolysis method of Levy and Chung (1953) was used to detect tryptophan which is unstable to acid hydrolysis.

The three woods investigated were hydrolyzed with 6 N HCl in a boiling water bath for twenty hours. Before spotting, each hydrolyzate was partially purified by adsorption on a column of the hydrogen form of Dowex 50-X8 (200 to 400 mesh), washing with water, and elution with 4 N ammonia. A synthetic mixture of amino acids was similarly treated.

Results

The amino acids found in the acid hydrolyzates of whole living organisms are summarized in Table I for each of the species studied. Taurine is also listed in this Table, as its spot was readily detected in these hydrolyzates. When the freshly-ground tissues of the Teredinidae were treated with 80% alcohol, taurine and β-alanine were extracted into the alcohol. All other amino acids listed in Table I were found in the hydrolyzates of the 80% alcohol-insoluble residues.

The alcoholic extracts of the dissected Teredinidae fragments all gave readily identifiable spots for α-alanine, β-alanine, glutamic acid, glycine, and taurine. In addition, some of the fragments, notably the mantle, gave very faint tests for some of the other amino acids in Table I, but these were not further investigated. The *Bankia* gill, the *Teredo* reproductive organ, and the mantles from both organisms were found to contain glutamine.

The acid hydrolyzates of both species of Teredinidae removed from redwood gave a spot corresponding to lanthionine, while those from pine did not. The spot gave a positive sulfur test with the platinic iodide reagent and proved to be chromatographically indistinguishable from lanthionine in the solvent systems used.

An unidentified spot was found in the acid hydrolyzates of both species of *Limnoria*. It is designated "A" in Table I. The spot persisted even when the tissues were hydrolyzed for an additional twenty-four hours. It was located on a

TABLE I

Amino acids in acid hydrolyzates of whole organisms

Amino acid	TP	TR	TL	BP	BR	LT	LQ
α-Alanine*	X	X	X	X	X	X	X
β-Alanine	X	X	X	X	X	O	O
Arginine*	X	X	X	X	X	X	X
Aspartic acid	X	X	X	X	X	X	X
Cystine-cysteine	X	X	X	X	X	X	X
Glutamic acid	X	X	X	X	X	X	X
Glycine	X	X	X	X	X	X	X
Histidine	X	X	X	X	X	X	X
Isoleucine	X	X	X	X	X	X	X
"Lanthionine"	O	X	O	O	X	O	O
Leucine*	X	X	X	X	X	X	X
Lysine	X	X	X	X	X	X	X
Methionine*	X	X	X	X	X	X	X
Phenylalanine*	X	X	X	X	X	X	X
Proline*	X	X	X	X	X	X	X
Serine	X	X	X	X	X	X	X
Taurine	X	X	X	X	X	X	X
Threonine	X	X	X	X	X	X	X
Tyrosine*	X	X	X	X	X	X	X
Valine*	X	X	X	X	X	X	X
Spot "A"	O	O	O	O	O	X	X

* Reported by Lasker and Lane to be present in *Teredo bartschi* Clapp.

X Present in readily detectable amounts.

O Not present in readily detectable amounts.

TP—*Teredo* from pine; TR—*Teredo* from redwood; TL—*Teredo* larvae; BP—*Bankia* from pine; BR—*Bankia* from redwood; LT—*Limnoria tripunctata*; LQ—*Limnoria quadripunctata*.

two-dimensional phenol and collidine paper between the spots for threonine and tyrosine. The R_f value in phenol was considerably lower when run in an atmosphere of acetic acid (Dent, 1948) than in an atmosphere of ammonia.

Tryptophan was not found in any of the alkaline hydrolyzates. This may have been because of its relative insensitivity to the ninhydrin reagent as compared to other commonly occurring amino acids.

The amino acids found in the three species of wood are listed in Table II. The greenheart hydrolyzate gave a much stronger phenylalanine spot than did the hydrolyzates of either pine or redwood. When a synthetic mixture of amino acids was treated with Dowex-50, all but lanthionine were eluted with ammonia.

DISCUSSION

These data indicate that there are at least eleven amino acids present in *Teredo diegensis* in addition to the eight reported present in *Teredo bartschi* Clapp by Lasker and Lane. It is considered unlikely that so many additional amino acids would occur in the one species and not the other.

The fact that taurine and β-alanine are not present in any of the hydrolyzates of tissues that had been extracted with 80% alcohol indicates that these compounds are

not present in the protein of the borers investigated. Seventeen amino acids are common to each species. All the amino acids seem to be distributed throughout all the Teredinidae sections. In addition, spot "A" was detected in the hydrolyzates of both species of *Limnoria,* and a spot corresponding to lanthionine was found in the hydrolyzates of both species of Teredinidae which had been removed from redwood. A corresponding spot was not found in the hydrolyzates of animals removed from pine. Cystathionine is reported (Dent, 1948) to have chromatographic properties similar to those of lanthionine. Neither of these two amino acids has been found in nature (Dent, 1948).

TABLE II

Amino acids readily detected in acid hydrolyzates of woods studied

α-Alanine	Lysine
Aspartic acid	Phenylalanine
Glutamic acid	Proline
Glycine	Serine
Hydroxyproline	Threonine
Isoleucine	Valine
Leucine	

The three species of wood studied contain thirteen amino acids in common. Hydroxyproline was found in all three woods but in none of the marine borers. A spot corresponding to lanthionine was not found in the chromatograms of the acid hydrolyzed redwood, but it was shown that lanthionine is not eluted with the other amino acids when a synthetic mixture is similarly treated.

SUMMARY

1. Eighteen naturally-occurring amino acids and taurine have been identified chromatographically in the acid hydrolyzate of *Teredo diegensis* and *Bankia setacea,* and seventeen naturally-occurring amino acids and taurine have been identified chromatographically in *Limnoria tripunctata* and *quadripunctata.* A spot corresponding to lanthionine has been found in the above Teredinidae living in redwood blocks but not in those living in pine blocks. Glutamine has been found in the alcoholic extract of freshly-ground Teredinidae sections.

2. Three species of wood have been hydrolyzed with acid, and all were found to contain the same thirteen amino acids.

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THE RATE OF FEEDING OF THE COMMON OYSTER DRILL, UROSALPINX CINEREA (SAY), AT CONTROLLED WATER TEMPERATURES

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Information on the voracity of the oyster drill, *Urosalpinx cinerea* (Say), within certain ranges of water temperature has been reported by a number of investigators, but evaluation of their destructiveness at specific temperatures maintained within close limits is virtually non-existent. In addition, the conclusions regarding the destructiveness of drills within the different temperature ranges are drawn from observations made at widely separated geographical areas (Carriker, 1955).

Observations by Stauber (1950) and Loosanoff and Davis (1950–51) on certain activities of drills from different areas of the North Atlantic Coast suggest the existence of distinct physiological races. If such races exist, a temperature-dependent activity, such as feeding, determined for drills from one area would not necessarily be applicable to drills of another habitat. Therefore, we have attempted to establish the feeding rate of a single geographical population of oyster drills at several constant temperatures. It was felt that such a study would not only provide more detailed information about the predation of drills, but also present a basis of comparison for investigators interested in the problem of physiological races.

Methods

A bank of wooden frames, constructed to hold a number of enamel trays (18″ × 20″ × 3″), was arranged on a laboratory water table. Each tray was supplied with a separate, continuous flow of sea water. Temperatures were adjusted by mixing cold and heated sea water in glass cylinders, just above the frames, giving each bank of trays a separate supply of water at a constant temperature (Loosanoff, 1949). Water in the trays was maintained at approximately ± 1.0° C. of the temperature desired.

Since the drills had a tendency to move up the sides of the tray and leave the water, covers were made to fit into each tray to keep the drills below the water line. They were constructed of Lucite plastic frames with a covering of ⅛″-mesh Saran plastic screening.

The salinity of the water deviated only slightly from 25‰ throughout the experiments and is, therefore, considered to have exerted no influence on differential feeding at the various temperatures.

To obtain a more comprehensive evaluation of feeding by drills at different temperatures, two species of bivalves, the common oyster, *Crassostrea virginica* Gmelin, and the mussel, *Mytilus edulis* Linné, were used as foods in separate experiments.

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Each tray contained 20 adult, Long Island Sound drills measuring from 20.0 to 25.0 mm. in height, and either 30 to 40 oyster spat, ranging in size from 10.0 to 30.0 mm., or 40 mussels, ranging in size from 20.0 to 30.0 mm. Spat were growing on cultch (old oyster shells) in such numbers that four to five shells supplied the total needed. The oyster spat clusters or mussels were so distributed about the tray that no large concentration of food occurred at any one point. The drills were dispersed throughout the tray to decrease the opportunity for feeding by two or more drills on the same bivalve. At no time during the experiments did the drills consume all of the bivalves available in any tray, thereby indicating that feeding was maximal for each feeding period.

The drills were brought to the experimental temperatures by keeping them for two to three hours at each five-degree level until the desired temperature was reached. After the experiment was begun the number of bored bivalves, as well as the number and condition of drills present, was ascertained at regular intervals. Following each such examination, oyster spat or mussels were added to replace those destroyed by the drills. Mortality of drills was low at all temperatures, except 30.0° C. All dead drills were replaced by drills from stocks kept at the same water temperature as the experimental groups to assure that their prior conditioning was the same.

Results

The feeding rates are expressed as the number of bivalves destroyed per drill during one week of feeding. Since low temperatures could not be maintained during the spring and summer, observations on the groups at 5.0° C. were discontinued after 52 days, on the 10.0° C. groups, at 69 days, and on the 15.0° C. groups, at 90 days; while the groups at 20.0°, 25.0° and 30.0° C. were under observation for 102 days (Table I).

At 5.0° C. the drills did not feed, nor did they show any tendency to attack the spat during the 52 days of the experiment. They usually remained in groups, each

Table I

Feeding rate of U. cinerea on oyster spat, size range 10–30 mm., at controlled water temperatures

Temperature ° C.....	5.0		10.0		15.0		20.0		25.0		30.0	
Tray.....	1	2	1	2	1	2	1	2	1	2	1	2
No. of drills	20	20	20	20	20	20	20	20	20	20	20	20
Feeding period (days)	52	52	69	69	90	90	102	102	102	102	102	102
No. of spat consumed	0	0	49	41	124	133	313	355	429	398	285	327
Spat consumed per drill per week	0	0	0.25	0.21	0.48	0.52	1.07	1.21	1.47	1.37	0.98	1.12

drill with the foot extended and attached firmly to the tray. Movement of a few inches by some drills, as shown by mucus paths, was noted on several occasions.

Feeding at 10.0° C. appeared to be limited to occasional attacks by individual drills since the rate of feeding was only about one spat per drill every four to five weeks. The rate of feeding on oyster spat increased as the temperature increased from 10.0° to 25.0° C. The rate approximately doubled for each five-degree increase in water temperature from 10.0° to 20.0° C. However, the next five-degree rise, to 25.0° C., increased the rate of feeding only about 25 per cent over that at 20.0° C., and a distinct decrease in the rate of feeding occurred at 30.0° C. Thus, the optimum temperature for feeding of drills on oyster spat was at or near 25.0° C.

The drills maintained at 30.0° C. were also slower in turning over and attaching to the tray than those kept at 25.0° C. On two occasions, when the temperature rose to about 34.0° C., 70 to 80 per cent of the drills were killed, although in neither

TABLE II

Feeding rate of U. cinerea on mussels, size range 20–30 mm., at controlled water temperatures

Temperature ° C.	10.0		15.0		20.0		25.0		30.0	
Tray	1	2	1	2	1	2	1	2	1	2
No. of drills	20	20	20	20	20	20	20	20	20	20
Feeding period (days)	34	34	57	57	57	57	44	44	44	44
No. of mussels consumed	1	4	39	32	88	73	101	109	89	105
Mussels consumed per drill per week	0.01	0.04	0.24	0.20	0.54	0.45	0.80	0.87	0.71	0.82

instance were they exposed to this temperature for more than 16 hours. The surviving drills were not attached to the tray or to the oyster spat, as they usually were at 30.0° C. These observations suggest that while drills do feed at 30.0° C., this approaches the lethal temperature level.

Comparable data for oyster drills feeding on mussels show that the optimum feeding temperature again lies near 25.0° C. (Table II). Apparently, only a few of all the drills kept at 10.0° C. fed during the 34 days of the experiment. The rate of feeding at 15.0° C. approximated one mussel per drill every five weeks. This rate was about doubled for each five-degree rise in temperature from 15.0° to 25.0° C. Although the rate of feeding at 30.0° C. was lower than that at 25.0° C., it was not as low as would be expected from the results when oyster spat were used as food. Probably, the rate of drill feeding at 30.0° C., in the mussel-fed experiment, was somewhat higher due to differences in handling. It was necessary to examine each tray and change the mussels daily to avoid a high mortality and resultant bacterial decomposition of mussels at 30.0° C. Thus, the drills may have been induced to attack more mussels because they were unable to feed without interruption.

Statistical treatment of the data by analysis of variance indicates that differences in feeding rate between temperature levels are highly significant ($F = 85.96$ with $df = 4,5$ for mussels as food and $F = 143.84$ with $df = 5,6$ for oyster spat as food; both values being significant beyond the 0.001 level). In order to determine which of the differences between temperatures were significant, a test for significance of gaps between means was applied (Bliss and Calhoun, 1954). Results of this test

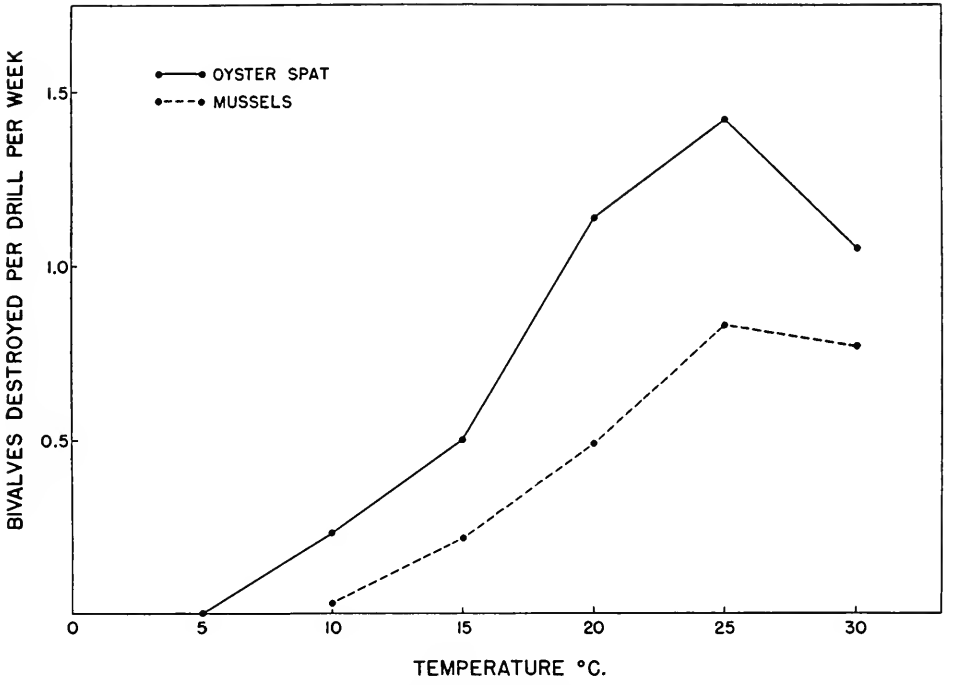

FIGURE 1. Feeding rates of *U. cinerea* at constant temperatures on young oysters or mussels. (Points are based on combined data of replicates at each temperature level.)

indicated a minimum difference between means of 0.162 for oyster spat as food. Thus, all gaps between means are significant except between 20.0° and 30.0° C. (Fig. 1). With mussels as the food organism, the minimum difference was 0.136. Thus, all gaps between means are significant except between 25.0° and 30.0° C.

DISCUSSION

The rate at which the drills destroyed oyster spat was higher than the rate at which they destroyed mussels at each of the temperatures used in these experiments (Fig. 1). This may indicate a preference of the drills for oyster spat, rather than mussels, as food. However, it is more probable that much of this difference was due to the spatial arrangement of the mussels in the tray, which necessitated more movement by the drills between feedings than when oyster spat, growing in clusters, was used for food. In addition the slightly larger average size of the mussels gave the drills somewhat more food per mussel killed than per oyster spat.

This comparison of feeding by drills on two species of bivalves shows a marked increase in destruction for each five-degree rise in water temperature within the range from 10.0° to 25.0° C. With either food, the peak of feeding occurred at 25.0° C. Since this temperature is seldom reached over the oyster beds in Long Island Sound, it is doubtful that drills in our waters are often feeding at their maximum rate.

As the drills did not feed at 5.0° C. and their rate of feeding on oyster spat was lowest at 10.0° C., a similar experiment was run at 7.5° C. to determine whether this temperature was above or below the threshold at which feeding on spat could occur. As with drills at 10.0° C., feeding at 7.5° C. was sporadic. In one experiment when 20 drills were used, one spat was killed after 27 days and two more during the next 37 days. Another group of 20 drills destroyed two to three spat per week during the first two weeks, did not feed for the next two weeks and, again, killed two to three spat per week for the final four weeks of the experiment.

The minimum temperature for feeding of drills has been reported as 9.5° C. (Galtsoff *et al.*, 1937), 9.0° C. (Loosanoff and Davis, 1950–51), 8.0° C. (Engle, 1953), and 6.5° C. by Andrews and McHugh (Carriker, 1955). The last figure was an average over a period in which the maximum temperature reached 9.5° C. and, therefore, cannot be regarded as the actual temperature at which feeding occurred. The lower temperature limit for feeding of 7.5° C. established in this study is in general agreement with these figures. However, it is apparent that short-term observations will not suffice to determine the lower temperature limit for feeding. Drills went as long as 27 days at 7.5° C. before any feeding occurred, and thus, it is possible that drills will feed at even lower temperatures, if kept for many months. In this study no feeding on oyster spat was observed at 5.0° C. during 52 days of exposure.

The author wishes to express thanks to Dr. V. L. Loosanoff for his many helpful suggestions and guidance throughout the experimental work, to Mr. C. A. Nomejko for preparation of the graph, and to Mrs. Barbara Myers for statistical treatment of the data.

Summary

1. Feeding by drills, *Urosalpinx cinerea* (Say), of Long Island Sound did not occur at 5.0° C.

2. The lower temperature limit for feeding was about 7.5° C., although feeding at this temperature was intermittent.

3. The feeding rate increased steadily as the water temperature was raised from 10.0° to 25.0° C., and decreased as the temperature was increased from 25.0° to 30.0° C.

4. The optimum temperature for feeding, when either *Crassostrea virginica* (Gmelin) or *Mytilus edulis* Linné was used as food, was 25.0° C.

5. The upper temperature limit for feeding was about 30.0° C.

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ORIENTATION IN BOX TURTLES, TERRAPENE c. CAROLINA (LINNAEUS)

EDWIN GOULD [1]

Homing ability of the box turtle, *Terrapene carolina*, was reported by Breder (1927) and by Nichols (1939), who recorded several returns to the home territory after transportation to distances as great as 4200 feet. Stickel (1950) estimated the average diameter of the range of this species to be about 350 feet, basing this estimate on 440 recoveries of 55 turtles. Home ranges of individual turtles overlap, and most recorded territories were quite similar in succeeding years. Longer journeys may be made to lay eggs or for other reasons, and some turtles had two home ranges. The longest spontaneous movement recorded was 2540 feet. But these important observations give no indication of the means of orientation employed by these turtles in finding their way home.

During the summer and fall of 1956, while stationed at the Army Chemical Center, Edgewood, Maryland, the author had the opportunity to experiment with over 100 of these turtles in an area only thirty-five miles from the location of Stickel's studies. The results demonstrate that many turtles of this species start to walk in the homeward direction within a few minutes after being released in unfamiliar territory, and furthermore, that the sun is one of the cues on which this orientation is based. These preliminary findings are of considerable interest, since they demonstrate that box turtles are capable of at least the same order of precision in celestial navigation as the birds studied by Matthews (1955) and Kramer (1953).

I am greatly indebted to the members of Boy Scout Troop 369, who brought many turtles and helped with certain field experiments which would have been impossible without them. Special thanks go to James Laney, who was particularly helpful in actively searching for turtles when they were most needed. My appreciation is also extended to Kile R. Barbehenn, Kenneth S. Rawson, and Lucille F. and William H. Stickel for their advice, to John R. Audett for drawing the figures, and to Jason I. Adleman for statistical advice. To Donald R. Griffin I am particularly indebted for his encouragement and numerous important suggestions concerning the planning of the experiments and the analysis of the resulting data.

Methods

Turtles were picked up in the fields and woodlands about Edgewood. The source of each turtle was carefully noted on a topographic map and it was given a permanent number denoted by combinations of notches filed into the edges of the posterior carapace (Cagle, 1939). In order to facilitate visual recognition in the field, its number was also painted on the shell or written with a wax pencil and covered with balsam.

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When observing a turtle for a short period it was of the utmost importance that it continue its heading as long as possible rather than stopping to make a form—a well-shaped cavity made by digging with the front feet and pushing and moving about from side to side in high grass or soft soil (Stickel, 1950). Level fields having closely cut grass were found to be most suitable for release areas. In hot weather the turtles would often head for a shady spot, and release points were therefore located insofar as possible near the center of large open areas such as a parade ground or golf course. The three areas used for most of these observations are described below.

Release Point G. Part of a golf course, this area is 350 yards long running east and west and about 125 yards wide, with a northern border formed by a vine-covered fence and high trees several hundred feet in the background. Along the southern edge stands an almost unbroken line of buildings. To the east is a well-traveled road bordered by large shade trees spaced about 80 feet apart. The western portion narrows to a blunt point of a few buildings and some low shrubs.

Release Point P. A parade field, 250 by 400 yards, was found to be the most suitable release point available. Its length runs north and south; the southern end was not visible to the turtles, due to the slight elevations of the ground. A line of buildings and shrubs are to the north. Along the west side is a broken line of mixed coniferous and deciduous trees, and to the east is a road beyond which lies another group of buildings.

Release Point B is located 0.35 mile south of G, and P is 1.55 miles south-southwest of B. For the various turtles used, the distances from the release points to the home ranges varied between 0.28 and 5.80 miles; and since all release points are located within an active military post and surrounded by roads, fences, ditches, and numerous buildings irregularly situated, there was little chance that they were within territory previously visited by these turtles.

Methods of observation

Forty-three box turtles were released and observed at least once, and 22 proved to be suitable for these experiments. Selection was based on whether the turtle headed in approximately the homeward direction, and whether it moved any appreciable distance during the first period of observation, which lasted for from ten minutes to two hours. Carr (1952) mentions the various differences in the personality of turtles, and I found, for example, that some would never even open the hinges of their plastrons during a 60-minute period of observation; others would not pull themselves in unless touched, and were quite active when let alone; still others were completely at ease even when approached or picked up.

Groups of two to twelve turtles were placed at numbered stakes on the same part of one of the three fields in each release. The head of the turtle was faced opposite to the homeward direction so that a heading in the direction faced would not be confused with an accurate heading. As each turtle walked away from its stake, the compass bearing of its course was read by a compass accurate to two degrees; then the distance traveled was paced off and a piece of cloth dropped just behind the turtle. After recording one I then went to another turtle and checked its progress so that the first was not disturbed in my absence. Unless witnessed, it is difficult to imagine how little effect the observer has upon the heading of the turtle under

these conditions. It is true that some will often stop suddenly and pull into their shells when closely approached, but this seemed to have no effect whatever upon the direction in which they continued to travel. Movements over distances from 65 to 600 feet were measured, the distance depending on the time available and the activity of the individual turtle.

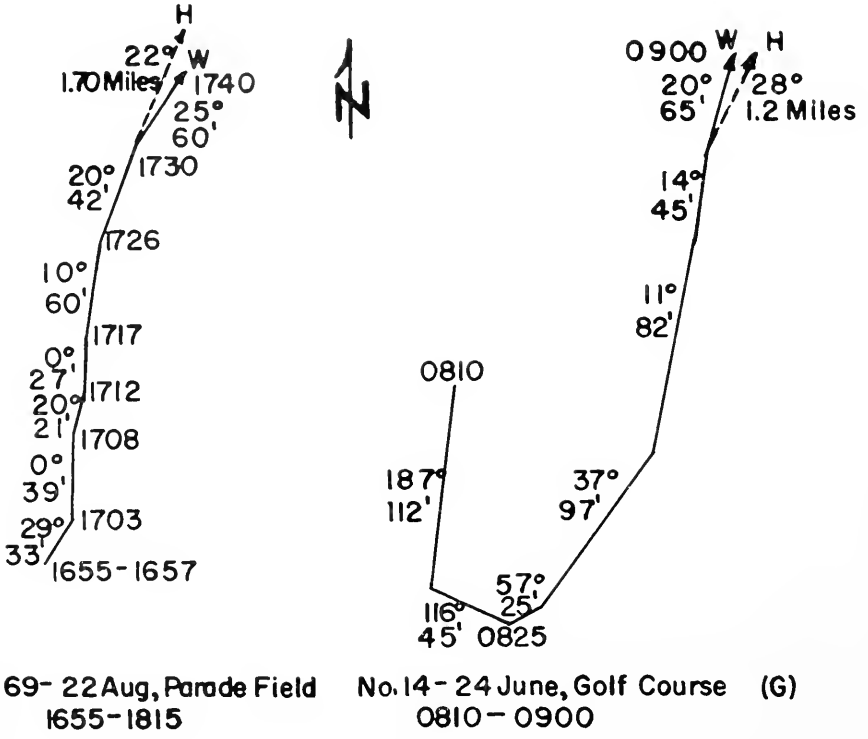


FIGURE 1. Two typical headings. H is home direction; W is direction in which the turtle went. Each straight portion of a turtle's course indicates the direction and distance traveled between compass readings. Time of day (EST) is also noted beside most of the bearings.

Because it was sometimes impossible to spend an hour watching the turtles, they were often left alone and their course recorded by attaching a spool of thread to the top of the carapace, a modification of the procedures described by Breder and Stickel. The end of the thread was fastened to a stake and unwound as the turtle walked off. Even in the short grass the slack thread would catch on small uncut weeds and the like, and would leave an accurately plotted course. A total of forty-five observations out of approximately 200 were made in this way. There were never any apparent differences noted in the headings between those recorded with and those without the use of thread. Both Breder and Stickel agree that turtles carrying the spools of thread move and behave quite normally. Stickel compared recorded movements of both and could find no discernible difference.

The turtles were transported in cloth bags or in closed boxes so that they could see nothing of their surroundings, and they were taken to the release areas by auto-

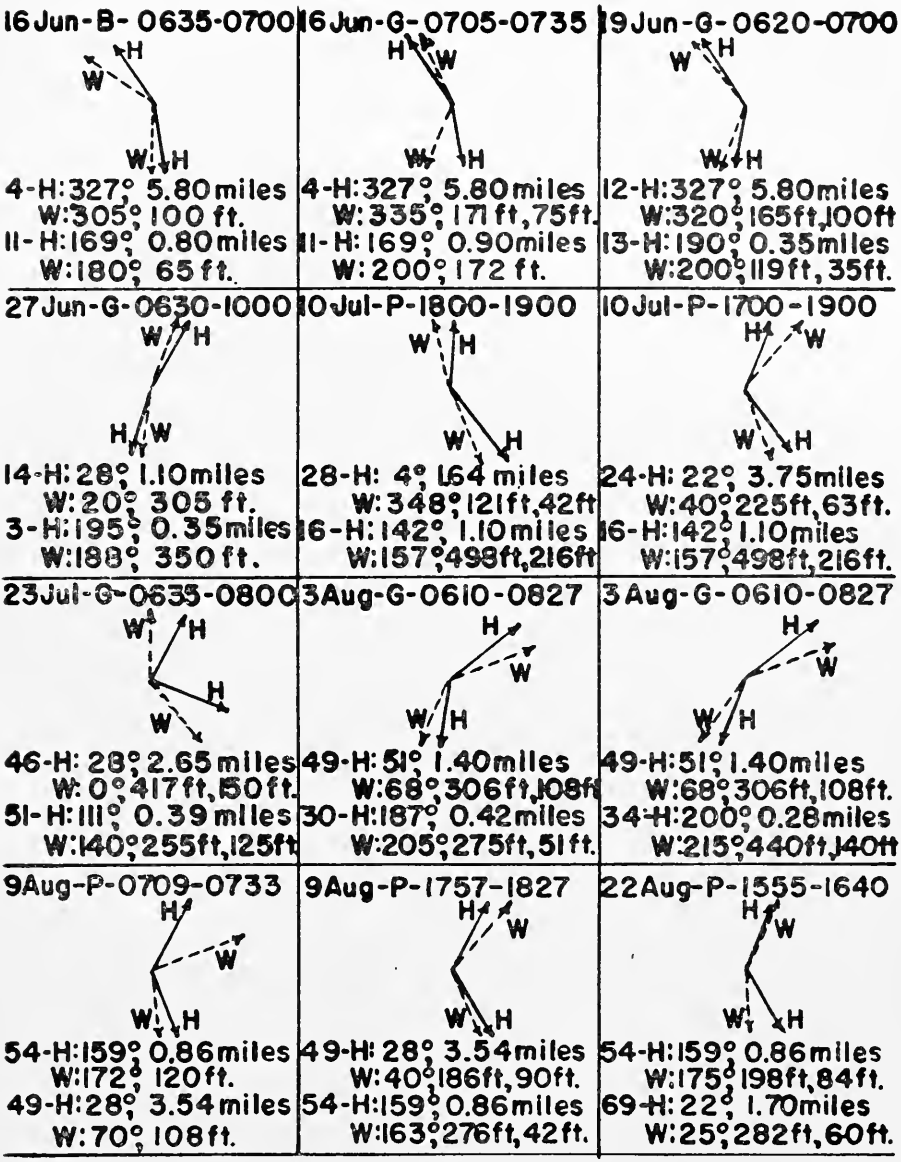
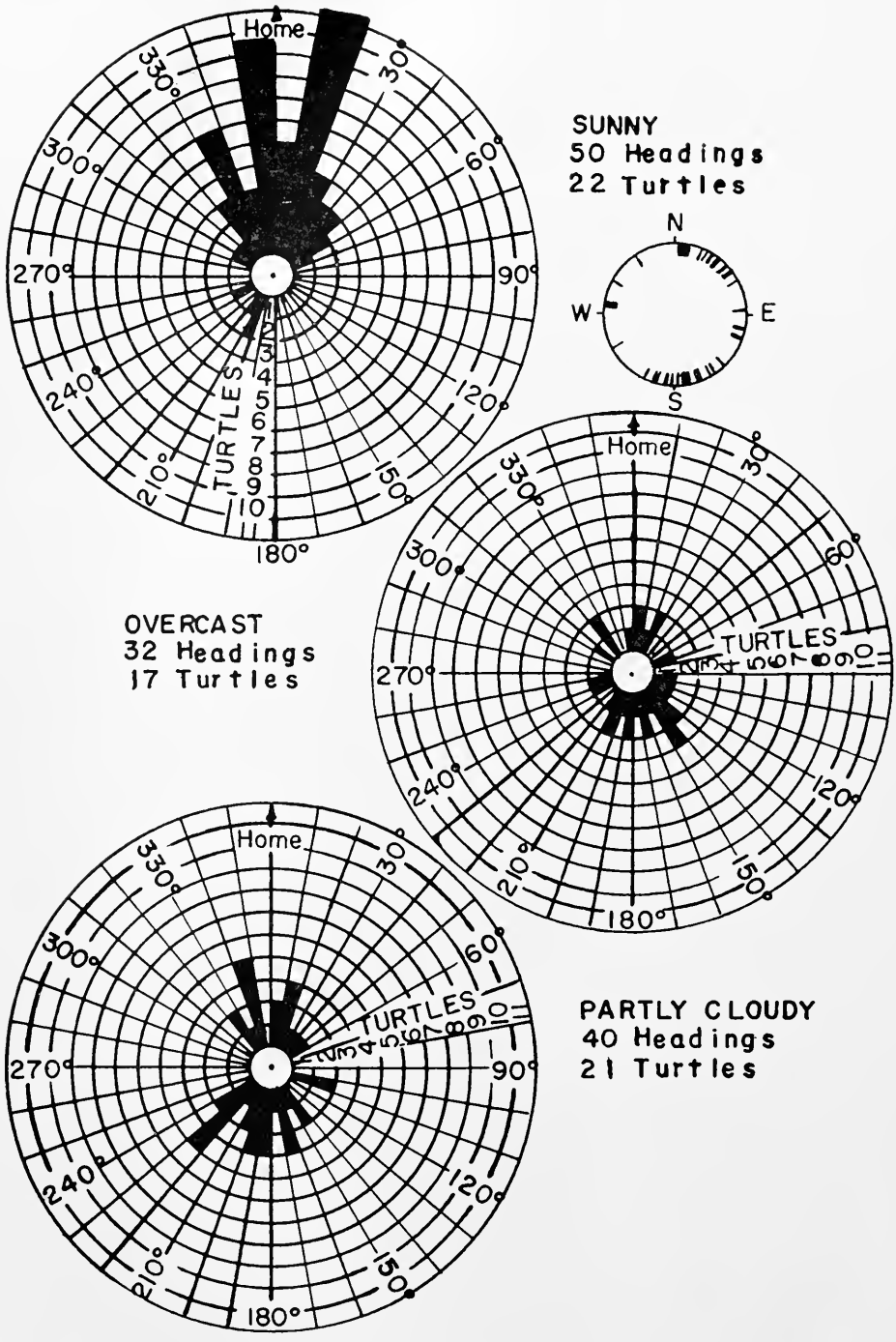


FIGURE 2. Twelve cases in which two turtles with opposite homeward directions were released from the same point at the same time. Sixteen different turtles were used. Symbols used: Release Points B, G, P. Time is EST. Turtle numbers at lower left. H is home direction and distance to home. W is direction and total distance turtle went before being picked up. Second distance indicates the length of the last direction headed if there was a variation. If only one distance is shown the course was a straight line.


Home
30°
60°
90°
120°
150°
180°
210°
240°
270°
300°
330°
TURTLES
1
2
3
4
5
6
7
8
9
10
SUNNY
50 Headings
22 Turtles
N
W
E
S
OVERCAST
32 Headings
17 Turtles
PARTLY CLOUDY
40 Headings
21 Turtles

mobile or bicycle. Between experiments they were kept in a small storage shed or in a pit dug in a woodlot. While in captivity they were well fed with fruits, vegetables and cockroaches, and allowed to wallow in muddy water when they pleased. Observations were usually made during the morning and late afternoon because of the objectionable effect of heat. On very hot days the turtles would immediately head for the nearest shade or would burrow into a form.

Observations

Typical behavior after release

In Figure 1 are shown two typical cases in which the headings of two turtles in clear weather were measured several times for about three-quarters of an hour after release. No. 14 started walking immediately after being set free, and No. 69 moved off after only two minutes' delay. No. 69 was returned to the same release point immediately after the heading shown in the figure, and it was observed to walk over a very similar course. No. 14 had been faced away from home, but it turned within 15 minutes and then continued in an essentially correct course until the observation was terminated. The heading chosen by a particular turtle was thus consistent from moment to moment, and was also very similar in most cases when the experiment was repeated on the same or on different days. On seven occasions a turtle was moved about 300 feet after it had indicated a definite heading, and in no case was there a significant change in the direction the turtle continued to head.

To determine whether local factors unrelated to the home direction were guiding the turtles, a total of twelve experiments were performd in which two animals from quite different home ranges were released at the same time and at the same release point (often within ten feet of each other). The turtles were so selected that the homeward direction would be 90 to 180 degrees different for the members of each such pair. If some local factor were responsible for their initial headings, both should be more or less similarly affected; if the direction of home was the important factor, they should separate. The results of these experiments are shown in Figure 2. While not all headings were perfectly correct, there is no doubt that each animal selected the approximate direction of its own home independently of the direction chosen by the other member of the pair.

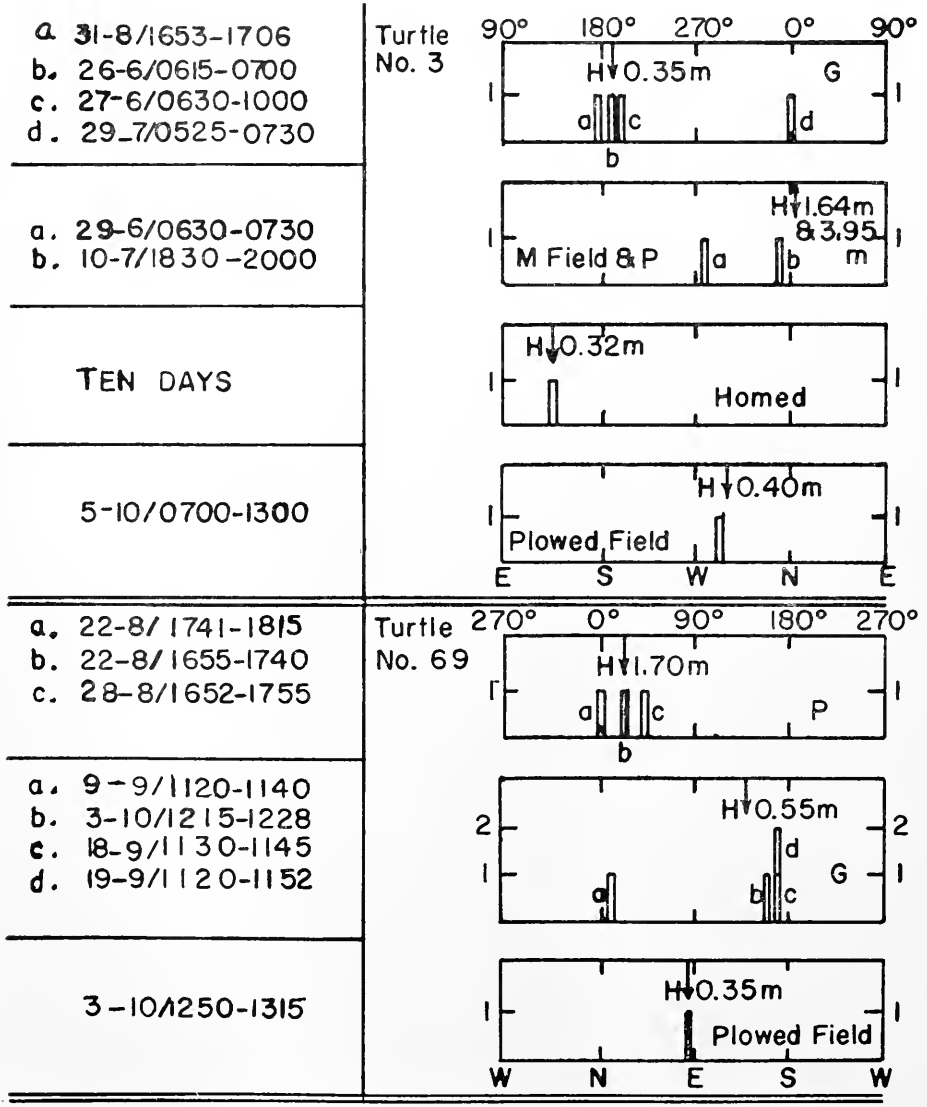
The importance of clear skies

As soon as these observations of homeward headings of box turtles had been repeated on several different days it became obvious that when the sun was clearly visible the headings were far better than when the sky was partly or wholly overcast. The results of the whole season's experiments under varying weather conditions are presented in Figure 3, which shows that the initial headings of these turtles are at least as accurately directed toward their homes as were those of pigeons and Manx shearwaters studied by Kramer and Matthews. The upper graph of Figure 3 con-

Figure 3. Initial headings of box turtles (plotted as deviations to the right and left of the homeward direction) under sunny, overcast, and partly cloudy skies. Most of the seventeen turtles in the middle graph were released at two different release points with similar homeward directions under sunny skies. The small circle to the right of the upper graph indicates the distribution of the forty-one different homeward directions which were used in sunny-weather releases.

tains the observations of twenty-two turtles which in most cases were tested at two or more release points as described above. The actual homeward directions were widely distributed around the compass. As has already been made clear in Figure 2, these headings represent in most cases a definite choice of the homeward direction.

It is of great interest that these accurate homeward headings largely disappeared under partly or completely overcast skies, for this at once indicates that the turtles were choosing the home direction by some form of celestial navigation similar to that studied in birds by Matthews, Kramer, and others. These releases were made at distances varying from 0.28 to 5.6 miles away from the place where the turtles



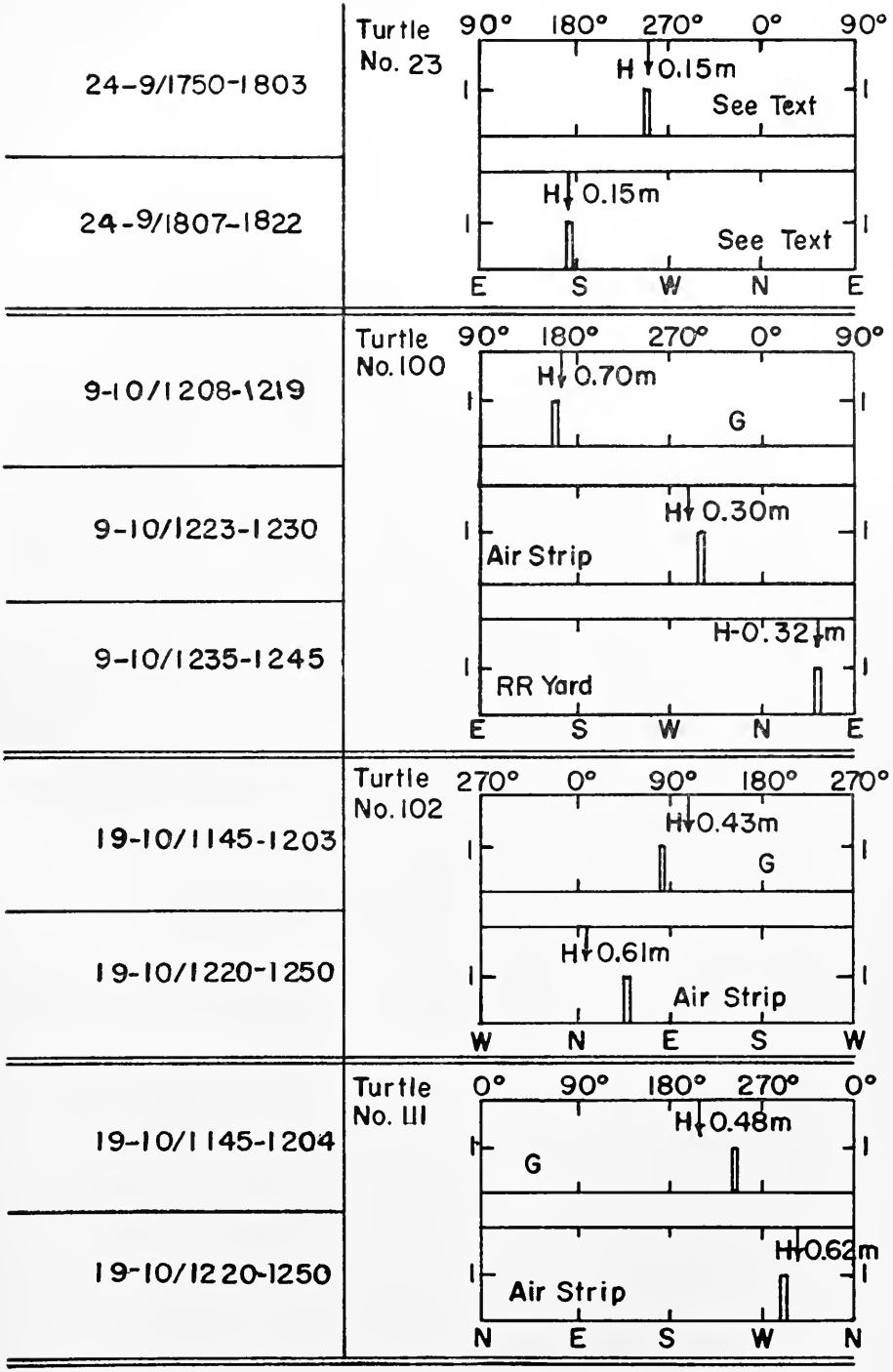
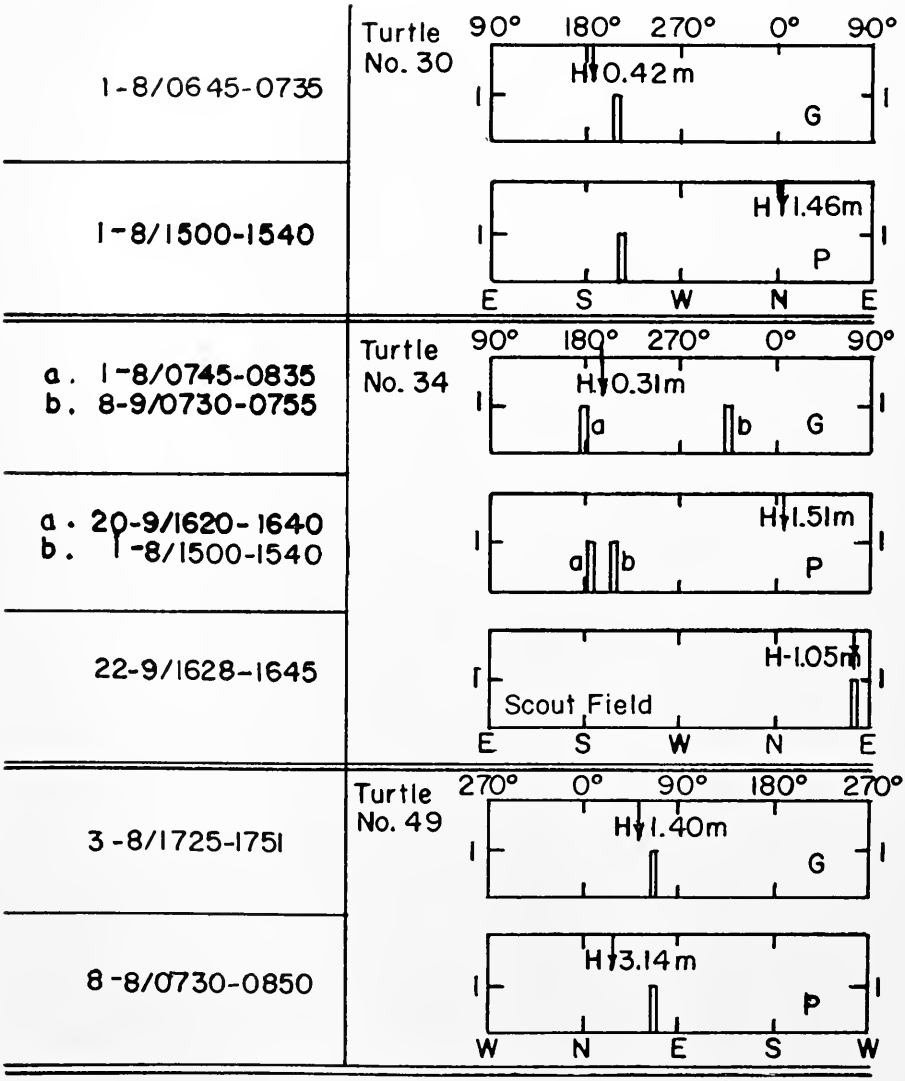


FIGURE 4. Type III orientation illustrated by graphic comparisons of turtles released from areas having different homeward directions. Release points: G, P, M field, Plowed field, RR yard and air strip. H is homeward direction. Distance from home in miles. Date and time in left column.

had been picked up. In cases where turtles were released at short distances from home, the inability to orient in cloudy weather supported other indications that the turtles did not recognize terrain features from past experience.

Alternating sunny- and cloudy-weather releases at irregular intervals resulted in the expected correct orientation in sunny weather and in poor orientation in cloudy weather. However, one turtle (No. 3) was released five times from the same place in sunny weather before it was able to head correctly in cloudy weather on the fifth release. The same result was achieved with another turtle (No. 30) under similar conditions.

It may be noticed in Figure 3 that turtles released in cloudy weather headed in the direction opposite from home more frequently than would be expected by chance.



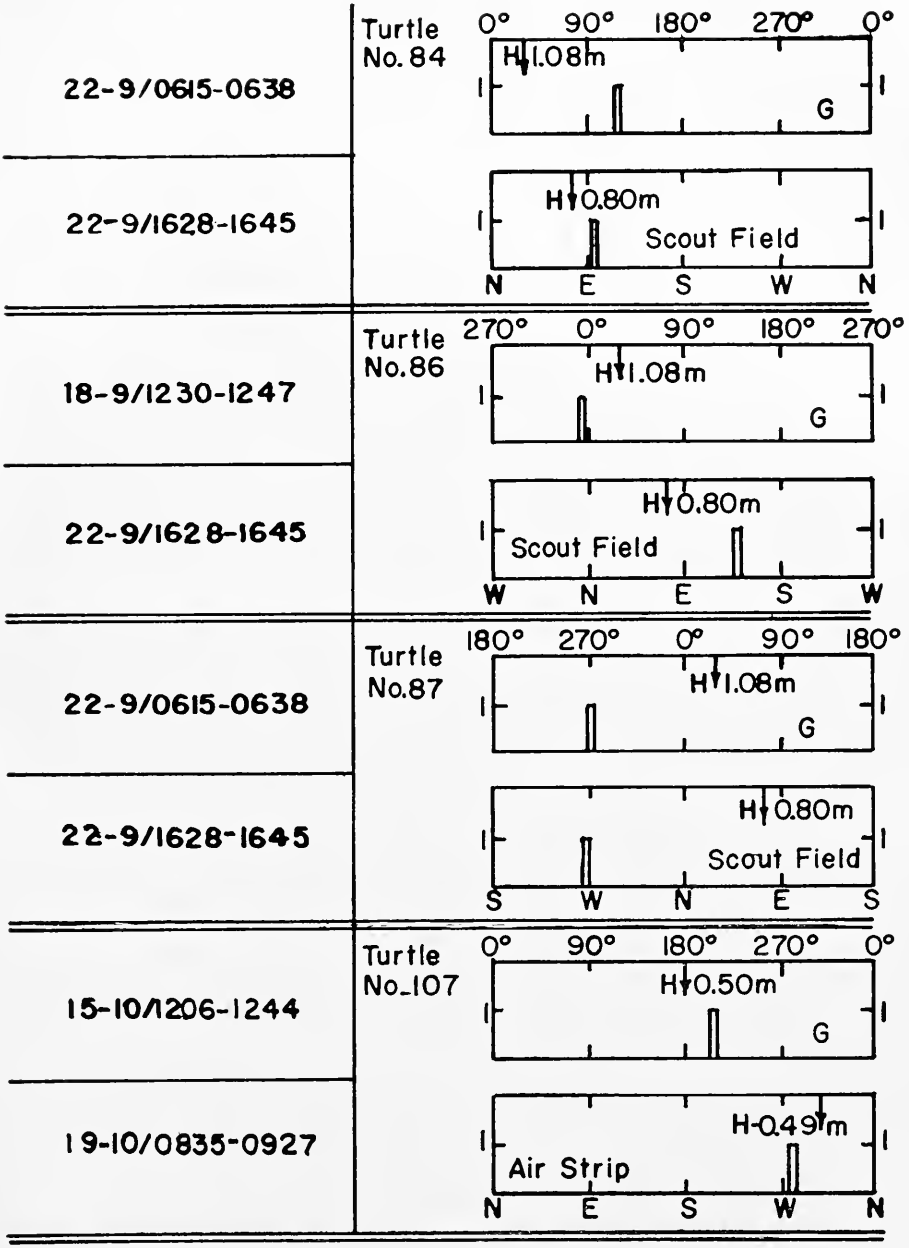


FIGURE 5. Type II orientation illustrated by graphic comparisons of turtles released from areas having different homeward directions. Release points: G, P, Scout field and air strip. H is homeward direction. Distance from home in miles. Date and time in left column.

Others had a strong tendency to go in the direction they were faced when first released, and in most cases this was opposite to the homeward direction. Facing the turtle in different directions in sunny weather had no effect, although very active turtles which move off immediately upon being released will often start in the direction faced and then veer off to the homeward direction after a short distance.

Turtles released under overcast skies showed other differences in behavior from those released in sunny weather. Eight turtles released in cloudy weather walked in circles ranging from seven to thirty feet in diameter. In a single case one turtle actually retraced its track three times in a circle seven feet across. One very obvious difference was the hesitancy of the turtle immediately after release in cloudy weather as well as the shorter distance traveled and the general inactivity. The rate of movement was computed for sixteen turtles released twenty-four times in cloudy weather and twenty-eight times in sunny weather. The mean for cloudy weather was 3.2 feet per minute and the mean for sunny weather, 5.8 feet per minute; standard deviations were 1.6 and 2.5; variations ($sd/\bar{x}$) were 50% and 43%, respectively.

Evidence for Type II and Type III orientation

Griffin (1952) distinguishes three types of orientation used by homing birds. Type I is a reliance on visual landmarks within familiar territory and the use of exploration or some form of undirected wandering when released in unfamiliar territory. Type II is homing in which an animal goes in a certain direction even when it is carried into unfamiliar territory in a new and unaccustomed direction. Type III is the ability to choose the approximately correct direction of its home even when it is carried into unfamiliar territory in a new and unaccustomed direction.

All the relevant data concerning individual turtles released from different homeward directions have been illustrated in Figures 4 and 5. Nos. 3, 23, 69 and 100 in Figure 4 are the best examples of Type III orientation. All but two headings of No. 3 were well-directed toward home. At Release Point G on 29 July an incorrect heading of 0 degrees closely resembled the previous heading, 348 degrees, at Release Point P. This appears to be Type II orientation. In the second example, M field, 3.95 miles from home, had almost the same homeward direction as Release Point P. In the third example shown, No. 3 returned to its home after ten days or less. Taking into account the rugged terrain the turtle had to traverse, the difficulty was considerable. Deep ditches, logs and dense mats of honeysuckle presented numerous obstacles.

Most of the headings of turtle No. 69 demonstrate good Type III orientation except for the 9 September record at Release Point G, which closely resembles previous releases at P, apparently another example of Type II orientation.

On 2 July, No. 23 was captured about three miles south of the pit where it was kept during the period of experimentation until the last week of August, when it escaped. On 24 September it was found only forty feet from the pit. On the assumption that it had made this place its new home, it was placed in a bag and taken to a large clearing 0.15 mile away. While walking to this release point I rotated and spun the turtle in various directions so as to remove any possibility of its remembering the movements. It was in my pocket for the remainder of the short journey. Upon release, No. 23 headed accurately toward the spot where it had just been recaptured. Following a similar procedure, it was again released from a point having a homeward direction 72 degrees different from the previous release

point. Again the heading was toward the spot where it had just been recaptured. It started in the direction it had proceeded from the previous release point, but after a short distance it headed correctly. Neither the previous direction nor the homeward direction corresponded to the direction the turtle was faced.

No. 100 was released from three different release points within a period of thirty-seven minutes; all headings are supportive evidence for Type III orientation.

Figure 5 is a compilation of records of turtles released from different homeward directions which showed evidence of Type II orientation or of an ambiguous type which may have been Type II or Type III. The others in this figure are less obvious but complete the collected data on this subject.

Another instance of Type II orientation was demonstrated by ten releases of eight turtles from New Jersey and Massachusetts, 180 and 400 miles, respectively, from the home grounds. In six out of ten cases the headings closely resembled the same directions taken when last released from points approximately one mile from home. The differences between directions headed from New Jersey and Massachusetts release points and those at close distances were 0, 3, 3, 10, 14, 17, 38, 43, 148, 155 degrees.

Discussion

While the turtle is moving on its course the head is held high and is directed forward so that if it is walking in a direction opposite to the sun's position it is necessary for the turtle to turn its head in order to see the sun directly. This very thing was frequently observed. The turtles would stop, look about them for thirty seconds to a minute or more, and then continue on their way. These stops were often made every two to four minutes, varying considerably with the individual turtle. Many turtles showed ability to maintain a straight course. One, for example, traveled 200 feet and kept within three degrees of a straight line for twelve minutes. Seven turtles released before sunrise moved but little until the sun was visible. Turtles released just before sunset either stopped after the sun had set or changed to a wrong direction after having followed the correct course while the sun was visible.

Twelve turtles were recovered after escape or release from a pit where they had been in captivity. Four turtles actually homed from distances of 405 to 563 yards. One returned to the pit from another release point after having been in captivity in the pit most of the summer. The remainder were picked up at points which were nearly in direct line with the homeward direction from their starting point. One of these had traveled 458 yards in less than six days in a presumed line which was only nine degrees from the homeward direction. The distance to home was 4.47 miles.

In some preliminary experiments turtle headings were recorded under natural sunlight and then observed in the shadow of a person or tree when the sun was reflected onto them from a mirror placed about 180 degrees from the sun's position. In all of twenty observations using ten turtles they changed their courses significantly when the sun's image fell upon them. In nearly all cases the turtles headed toward the mirror. Furthermore, in all cases the headings which were initially taken in the sun were resumed after the mirror was removed and the turtle was returned to natural conditions of direct sunlight. The exact meaning of these observations remains obscure, but they do support the hypothesis that it is the sun which enables these turtles to exhibit Type II and Type III orientation.

Summary

1. An investigation was made to test the hypothesis that box turtles [*Terrapene c. carolina* (Linnaeus)] employ a means of sun orientation similar to that found in birds. Box turtles from different localities were taken in closed containers to unfamiliar territory and released in large open fields 0.28 to 5.80 miles from their homes. (According to Stickel the home range is about 300 feet in diameter.) They were then observed over periods varying from ten minutes to two hours, and with the aid of a compass their headings during that period were plotted, and the distances traveled were paced off. After the observation they were again placed in closed containers and returned to a pit where they were kept until the next release.

2. Of forty-three turtles released and observed in this manner, twenty-two headed toward home; seventeen of the latter were released under sunny and overcast skies and in most cases this was done from at least two different release areas. Homeward headings were observed in sunny weather, but under overcast skies orientation broke down (Fig. 3). Twelve examples, two turtles each, of situations in which sixteen turtles with opposite homeward directions headed toward their respective homes at the same time from the same place, demonstrated that the heading was not dependent on some local environmental factor at the release point (Fig. 2).

3. Of sixteen turtles released from several completely different homeward directions, four showed definite ability to orient correctly (Fig. 4).

4. There were ten releases under sunny skies more than 150 miles from home. Nine turtles were used in these experiments. In seven of these headings the turtles went in a direction which seemed to correspond to the direction last chosen when close to home, regardless of the actual homeward direction. At short distances of a mile or less from home several turtles also headed in consistent directions regardless of the homeward direction.

5. Ten turtles in twenty experiments were first observed for directional heading in natural sunlight, and then observed in the shade while the sun's image was reflected upon them with a mirror. The direction of heading was altered in all cases and usually resulted in the turtles' heading for the mirror.

6. While walking, turtles stopped frequently and turned their heads as though looking at the sun.

7. These findings seem to support the hypothesis that turtle orientation resembles the type of orientation found in birds; however, more data are necessary to clarify numerous factors which may bring to light conflicting differences and close resemblances. Work is continuing and new developments will be reported as they are observed.

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AN EXAMPLE OF REVERSAL OF POLARITY DURING ASEXUAL REPRODUCTION OF A HYDROID

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A number of small and curious hydroids have been described over the years. These hydroids, in general, occur on soft bottoms as members of the fauna of the upper few millimeters and are of rather diverse asexual reproductive habits. This group, most of which are unrelated systematically, includes *Boreohydra, Psammohydra, Corymorpha, Heteractis, Euphysa* (the last three closely related), *Halermita* and *Microhydra*. In view of the apparently wide distribution of this type of hydroid in soft bottom communities it is probably not surprising that still another form has been discovered in San Francisco Bay, California.

On November 28, 1954, we recovered two live specimens of such a hydroid from a core sample 18 mm. in diameter taken at a depth of 30 feet off Pt. Richmond. The bottom at that location consists of grey mud overlain by about 5 mm. of loose debris, from which the hydroid to be described was taken. According to Sumner *et al.* (1914) the annual salinity in this area ranges from 18.0‰ to 32.3‰ with a mean of 27.0‰.

Morphology and Reproductive Behavior

The two specimens were a single polyp and a pair of polyps united at their base. Each had a small patch of debris adherent to its base. Figure 1 illustrates the pair. The polyps were quite extensible and were about 1–1.5 mm. long when maximally extended. The hydrocaulus was narrowest at the base and gradually enlarged toward the area of tentacular insertion, where the diameter was approximately twice that of the more proximal region. The tentacles were filiform and were capable of extending to about 1 mm. They were inserted in a single cycle at the base of the proboscis. The number of tentacles varied from as few as 4 to as many as 12, although, of the original specimens, the solitary individual had 6 and the pair had 9 and 10 at the time they were first observed. Due to the extreme contractility and extensibility of the hydroid, the relative length of the proboscis and the hydrocaulus was quite variable. In general, however, this relationship varied from 1 : 1 to 1 : 3 (*i.e.*, with the hydrocaulus about three times the length of the proboscis, at times). The color of the hydroids was a light, flesh-pink, and the whole animal was translucent.

The two original specimens were set aside in a stender dish to which was added a small amount of debris from the core sample. The specimens were observed at least once a week following this and were fed the nauplii of brine shrimp (*Artemia*) at each observation. Early in January it was noted that there were two pairs of polyps plus the single one in the culture dish. At the time it was assumed that the extra pair had been overlooked at the time of isolation. However, on January 24,

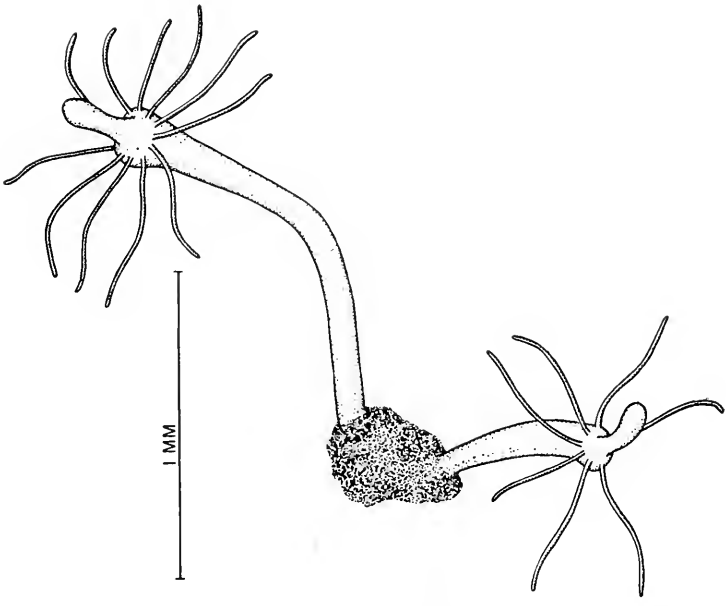

FIGURE 1. The original paired hydranths. The individual on the left has elongated preparatory to undergoing asexual division.

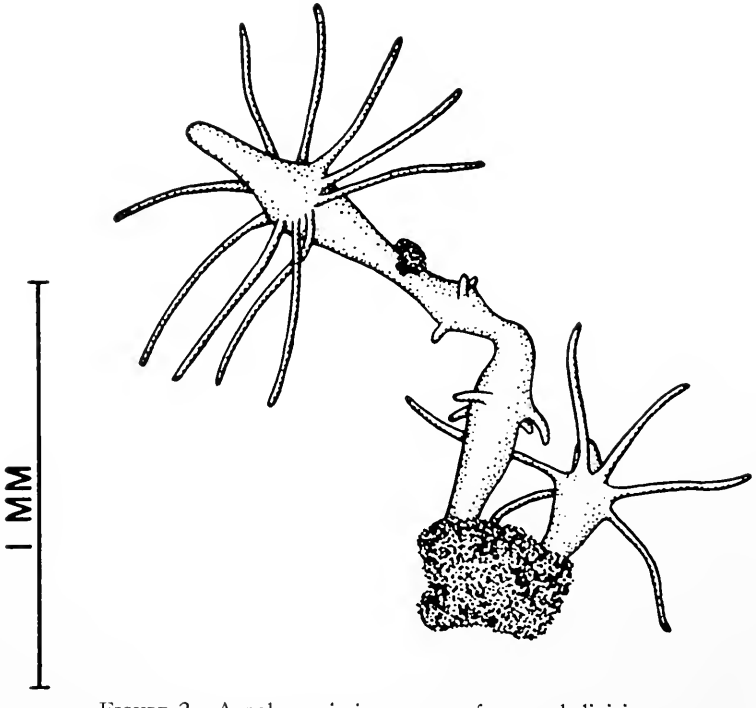

FIGURE 2. A polyp pair in process of asexual division.

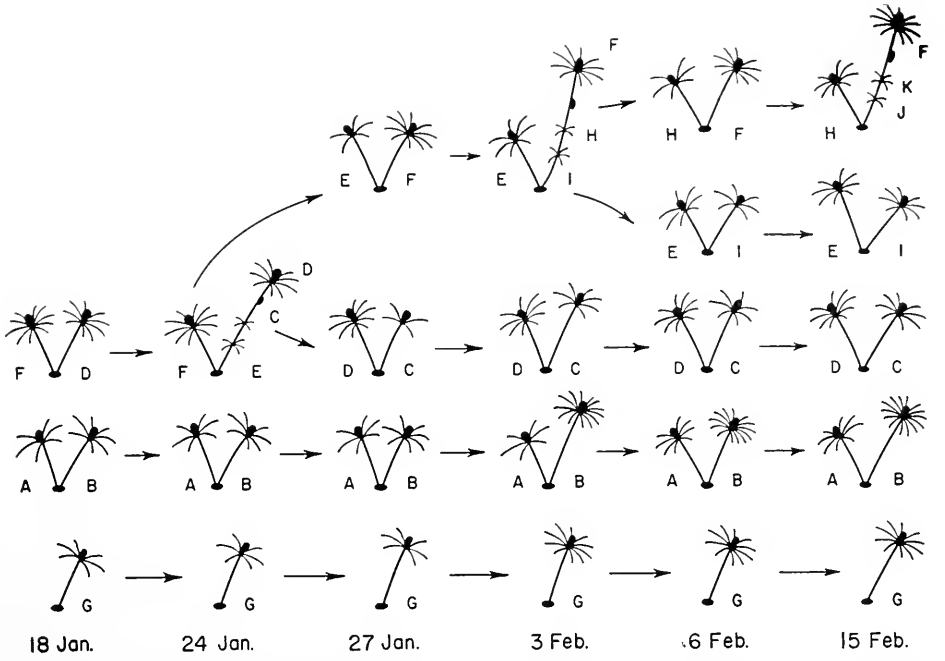

FIGURE 3. A schematic diagram showing results of observations on critical dates from January 18 to February 15, 1955.

1955, it was observed that a polyp of one of the pairs had become about twice the length of its partner and possessed two additional sets of tentacles, intercalated between the base and the original whorl of tentacles, and further, at a point between the new tentacles and old hydranth, debris had collected and was adherent to the hydrocaulus (Fig. 2). Daily observations following this made it clear that we were witnessing an unusual mode of asexual reproduction which resulted in the production of pairs of polyps. By January 27, fission had occurred between the two new sets of tentacles, and the area of adherent debris had become the base of the new polyp pair. Pursuant to this discovery we began a series of daily observations extending from January 24 to February 15.

For convenience in record-keeping, letters were assigned to the polyps (these letters will be referred to in the text, where necessary), and the accompanying figure (Fig. 3) illustrates, in a diagrammatic fashion, the results of our observations from January 18 to February 15. At this time there were four pairs and a single polyp. The polyps "F" and "D" were the only polyps to divide during this period, and one of these probably gave rise to polyp pair "A-B." In each instance the elapsed time between the first appearance of new tentacles and actual divisions was three days.

Following February 15 our observations became somewhat irregular, and a single observation on March 4 revealed that we now had seven pairs of polyps and that two of the pairs were preparing to divide once more (the single specimen had been sacrificed previously for nematocyst data). Immediately following this observation, the dish containing the hydroids became badly fouled (presumably as a re-

sult of over-feeding with *Artemia* larvae, and our subsequent failure to change the water), and on March 8, when this was discovered, the remaining hydroids were in what appeared to be very poor condition. Each polyp was tightly contracted to about one-third its normal height and was about 0.5 mm. in diameter. The tentacles were mere knobs and the animals had assumed a nearly hemispherical form. The water was changed, but this failed to save most of the specimens and by March 15 we were left with but a single pair of polyps.

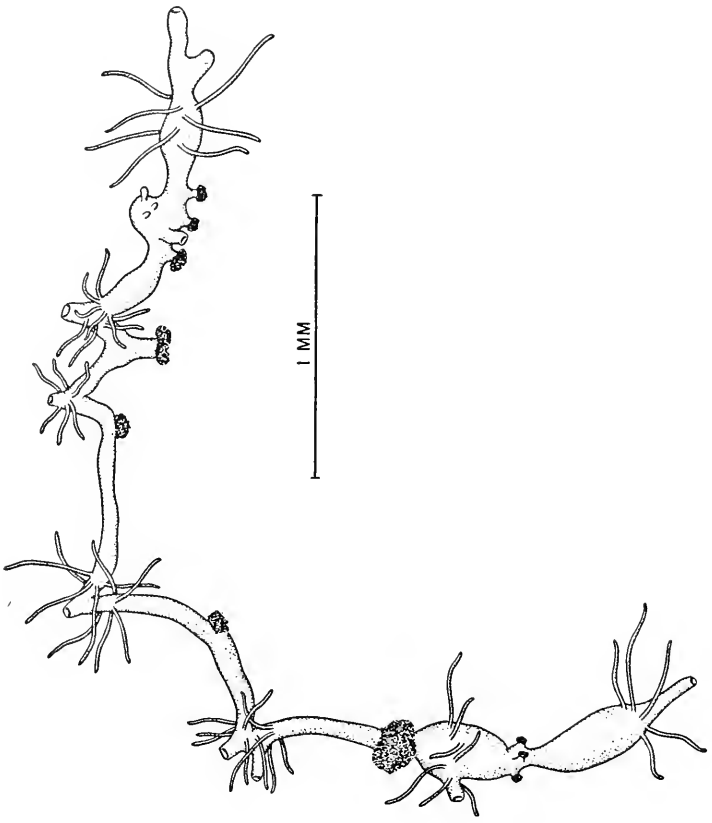

FIGURE 4. The monstrosity on March 25.

On March 21 we observed that our remaining pair had once more produced two new polyps, but although these were well-formed, they still had not separated. On the next day (March 22), although the two new polyps had not yet parted, there were signs that still another pair of polyps was being formed, giving us six polyps in series, and this specimen was apparently becoming a monstrosity. We also noted that near the middle of the central hydrocaulus there was a single protuberance and a group of four structures which looked like still another pair of incipient polyps with irregularly placed tentacles. By the next day (March 23) our specimen had opened another mouth between a pair of orally-directed polyps and we could now count 12 sets of tentacles and 4 open mouths. By March 25 the animal had grown

to about 4.5 mm. long (Fig. 4), was composed of 14 recognizable individuals and had 8 mouths which seemed to be functional (in that they were seen to open and close and brine shrimp larvae were ingested by several).

On March 28 the specimen had finally succeeded in dividing into pieces, and we were able to count two single polyps, two separate double polyps, one triple polyp (three mouths and sets of tentacles with a single base), one quadruple (four mouths, three sets of tentacles and a base), and two groups of polyps which had three and four mouths each. These last two groups consisted of rather curious assemblages of mouths, misplaced tentacles, and bits of adherent debris (suggesting bases) at irregular points over the masses.

This situation, that is, with eight variously assorted hydroids ranging from single individuals to groups of four, was maintained without further change until April 4. Following this date, the numbers were reduced by death, and by June 1, all the individuals had died.

As stated above, during the earlier part of our study, the original single polyp (polyp "G," Fig. 3) was sacrificed to study its nematocysts, and, unfortunately, none of the polyps or polyp pairs was preserved. Although we have taken more than 1200 core samples from the general area of the original collection, none have been found since.

Description of Asexual Reproduction

The sequence of events leading to the production of new pairs of polyps in this hydroid has been closely observed. The earliest sign that the process was underway

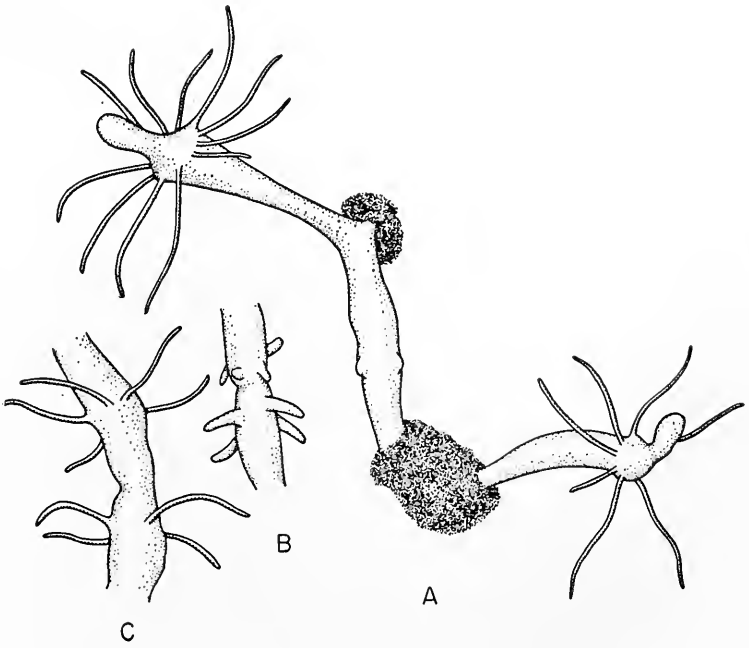

Figure 5. Development of tentacles prior to asexual division. A, the polyp pair with new tentacles barely recognizable (February 14); B, tentacle growth in 23 hours (February 15); C, tentacle growth in 40 hours (February 16).

was the adherence of a small bit of debris to the hydrocaulus at about its mid-point, although this was commonly nearer the tentacles than the base (Fig. 5, A). This phenomenon was associated with a slight increase in the opacity of the tissues at this point, and the protrusion of a small knob of tissue. This knob within 24 hours of its appearance became completely obscured by adherent debris. In addition, during this first phase of development, two sets of minute protuberances were seen in the mid-region of the hydrocaulus, between the adherent debris and the original base (Fig. 5, A). Within 24 hours, the protuberances grew into recognizable tentacles, usually three or four per whorl (Fig. 5, B). With the appearance of the tentacles, there is a marked increase in the opacity of the tissue in this area.

During the second 24-hour period, a translucent region developed in the area between the two new sets of tentacles, and subsequently, there was an indenting of the surface tissue of the hydrocaulus at this point. On the third day, there was a further continuation of the constriction of this area, leading to the actual separation of the two new polyps. This point of fission marked the distal end of the proboscis of each of the two new polyps, and each polyp possessed a functional mouth as soon as fission was completed; in addition, the point of adherent debris had now become the base of the newly released pair. The result of this process of fission was two pairs of polyps, each with one member (the older) about one-third larger than the new polyp.

Discussion

Many well-known studies have established the fact that hydroids possess gradients, particularly those of regenerative ability, which are more marked or rapid in more distal pieces of stems. Steinberg (1954), in studies on *Tubularia,* found that short pieces of stem (1–2 mm. long) regenerate bipolar or partial bipolar hydranths, while in longer pieces (2–12 mm.) a new hydranth is usually formed only at the original distal end of the stem, and, in still longer pieces (12–30 mm.), each end regenerates a hydranth. Steinberg also describes movements of cells toward the distal ends of cut stems and states that these cell movements precede the process of regeneration. In the hydroid described above, since the whole animal is less than 2 mm. long, one wonders if there can be effective or actual gradients such as those obviously present in larger hydroids. However, numerous studies on *Hydra,* an equally small organism, have shown that well-developed gradients also exist in small hydroids. In the animal described here, the appearance of two new orally-directed hydroids interposed in series between an extant base and hydranth must mean that a re-organization, involving a reversal of the original polarity of the hydrocaulus, has occurred, and it would seem that information obtained from experimental studies already completed on other hydroids should help to explain this observed event.

It should be pointed out that the reversal of polarity, which we assume to be present during the course of asexual reproduction, is a unique event. Hyman (1940, pp. 487–492) has reviewed much of the literature to that date relative to asexual reproduction and regulative processes in hydroids. She points out that in animals as small as hydras the polarity is well-developed and can only be altered by rather drastic treatments, *i.e.,* electrical currents, burial of the apical end of a cut stem, or by treatment with cyanides, anesthetics, and other depressing chemicals. Further, it is well known that pieces of hydroid stems and hydras retain their original polarity whether free or grafted to other pieces. We assume, therefore, that our

animal must also have a definite basal-oral polarity; that in the course of asexual reproduction by this animal, the polarity is reversed, at least for a short part of the hydrocaulus; and that this is a naturally-occurring and unusual event.

After considering all the evidence available to us, we have come to the conclusion that we can explain our observations on this hydroid if we are allowed to make one hypothesis. This hypothesis is concerned with the first event we observed during the course of asexual reproduction (*i.e.*, adherence of debris to the hydrocaulus) and is unique to the extent that it gives certain unusual features to the presumptive base of the new polyp pair. We suggest that the appearance or formation of the new base, as the first step in asexual reproduction here, must represent an effective block in the existing gradient, and that this new base must compete (in a certain sense) with the old base for the hydrocaulus between them. Since it is well-established that distal portions of hydroid stems form new hydranths, we can now consider that the portion of the stem between the bases is distal to each base and that it proceeds to develop two new orally-directed polyps simultaneously. In other words, we believe that the two bases with the common piece of hydrocaulus between them, are, individually, behaving in the same fashion as would pieces of decapitate stem, *i.e.*, that there will be regeneration of a new hydranth at the distal extremity. This, of course, must involve a reversal of the preëxisting gradient in the piece of hydrocaulus closest to the newly formed base, and we might postulate that this is the result of some disorganizational or re-organizational power of the base. To the extent that we observed a condensation or increasing opacity in the tissues at the points of formation of the two new polyps, our observations seem to fit the known facts, such as those presented by Steinberg (1954). If, therefore, our hypothesis is acceptable, we can interpret asexual reproduction in this hydroid as a phenomenon involving regeneration at distal extremities.

Cases of reversal of polarity do not seem to have been reported in nature for any other hydroids, unless we can interpret the study of Rees (1937) on *Heterostephanus* (now *Heteractis aurata* according to Kramp, 1949) as such. In *H. aurata* the polyp bud develops from the side of a hydranth, usually a tentacle base, in such a fashion that the free end of the bud is the future hydrocaulus. This same phenomenon has been observed by us in a similar hydroid (*Euphysa* sp.) from San Francisco Bay. In all other cases where transverse fission occurs, a new polyp develops at the distal end of the remaining hydrocaulus, and the new free individual develops a base at the proximal end of its own hydrocaulus. In one genus, *Psammohydra*, Schulz (1950) has found that the new hydranth develops tentacles before fission actually takes place.

Recently Kinne (1956) has made observations on *Cordylophora caspia*, an athecate hydroid from fresh and brackish water. He noted that in approximately 20% of polyps adapted to a salinity of 24‰, there were various abnormalities developed. These consisted of: the formation of intercalary hydranths on a hydrocaulus ("uniaxial hydranth aggregates"); fusion of polyps ("multiaxial hydranth aggregates"); the formation of large coenenchymal bodies, which became detached from the colony and formed new hydranths; and "globular hydranth complexes," with no stolons and no stalks.

The formation of the intercalary polyps is remarkably similar to the phenomenon observed in the hydroids from San Francisco Bay. However, there are points of difference, for in *Cordylophora*, the two new polyps are joined by the sides of the

hypostome and the mouths are directed laterally, the original polyp is a member of a colony, and there is no interruption of the hydrocaulus between the old and new polyp head which might correspond to the new base of our local forms. Kinne mentions that when the hydranth groups are detached they frequently become established on the substrate and sprout new stalks, but no intimation is made that these persist as pairs, nor that further intercalary hydranths are formed.

In an early section of this report we described a monstrosity which developed from a pair of polyps following their recovery from a period of depression. Hyman (1940) has reviewed the general effects of depression on hydroids, and notes that after recovery from depression, hydras often regenerate doubled parts or other anomalies, and that this is followed by fission processes. Kinne explains his various anomalous forms as the results of disturbances of growth and differentiation processes and a consideration of our observations leads us to evaluate our monstrosities in the same light.

However, since we noted some six apparently normal divisions, culminating in seven polyp pairs, and since the original pair was taken in the field and had not, so far as we know, been exposed to abnormal environmental conditions, we reserve judgment as to whether or not this process is abnormal, for this organism. The possibility does exist that the unknown hydroid, upon which we based our observations, is actually a *Cordylophora,* for this genus has been collected at the Carquinez Straits, approximately 15 miles up the San Francisco Bay Estuary from Point Richmond.

Systematics

We have little concrete information to guide us in the identification of the hydroid we have described. Our study of the nematocysts revealed that it possesses a simple cnidom of desmonemes and euryteles, as follows:

Small desmonemes (common)	4–5 × 3–4 μ
Large desmonemes (rare)	9–10 × 5–4 μ
Microbasic euryteles (common)	9–11 × 3–4 μ

This cnidom suggests that the hydroid is a member of the gymnoblasts (anthomedusae) or limnomedusae (see Weill, 1934; Russell, 1938; Hand, 1954). The complete absence of perisarc also suggests that the hydroid may be a limnomedusan form. However, the perisarc apparently may be completely absent in some gymnoblasts and calyptoblasts. We also know that differences may occur between the cnidom of a hydroid and that of its medusa (Hand, 1954), but in this hydroid, we do not know what medusa, if any, is involved in the life history. Therefore, we can not place this hydroid in any order with certainty, although it seems clear that it can not be a calyptoblast.

Because of the proximity of a source of *Cordylophora,* the possibility of the unknown form's being an abberant type of *Cordylophora* was explored. The main points of difference are that *Cordylophora* grows in extensive colonies, its tentacles are scattered, and it possesses a well-developed perisarc. None of these characters applies to the form described here. In addition, Hand and Gwilliam (1951) reported only a single size-class of desmonemes for *Cordylophora,* while we have found two size-groups. Thus, it seems doubtful that this is actually a growth variant of *Cordylophora.*

Since so little can be done with the hydroid from the standpoint of systematics, we will refrain from assigning it the status of a new species, pending its further collection and a more exact taxonomic determination.

The authors wish to acknowledge the assistance of Mrs. Emily Reid, to whom we are indebted for the illustrations accompanying this report.

Summary

1. Asexual reproduction, involving reversal of the original oral-basal polarity, is described.

2. Asexual reproduction in this hydroid leads to the production of pairs of polyps sharing a common base.

3. The systematic position of the hydroid is not established, although the cnidom suggests possible affinities among the gymnoblasts.

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THE ELECTROCARDIOGRAM OF A STOMATOPOD

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It is generally accepted that the crustacean heart beat is of neurogenic origin. This has been concluded from pharmacological (Krijgsman, 1952; Prosser, 1942), electrophysiological (Prosser, 1950; Hagiwara and Bullock, 1956) and anatomical evidence (Alexandrowicz, 1932, 1934). Both *Limulus* and the lobster have been extensively studied, but relatively few studies of other species of crustaceans have appeared in the literature.

Alexandrowicz (1934) has described the innervation of the heart of stomatopods, and showed that there are nerve cell bodies in the elongated ganglionic trunk, which he considers as an automatic apparatus which rules the heart beat. Since the ganglia of the stomatopod are of simpler structure than those of *Limulus,* a physiological study of the heart of the mantis shrimp may be of considerable assistance in explaining the origin of the heart beat of the arthropod neurogenic heart. This paper describes the electrocardiogram of the mantis shrimp and the detection and localization of the pacemakers of this heart.

Materials and Methods

Marine mantis shrimps (*Squilla oratoria* de Haan, Crustacea, Malacostraca, Stomatopoda) were used throughout the present study. They were fixed on a cork board, the shell opened dorsally and the preparations placed in a plastic dish filled with sea water. The dorsal muscles were dissected and removed to expose the long segmented tubiform heart which extends from the posterior to the thoracic part of the body cavity. Since the heart is closely attached to the digestive tract the experiments were carried out without isolation of the heart from the body. The preparations were decapitated at the level of the fifth segment, to avoid central nervous effects and suppress muscle activity.

The indifferent electrode was placed remotely from the heart in the sea water, while the recording electrode was moved with the aid of a micromanipulator and microscope along the median line of the exposed heart. Low resistance microelectrodes (tip diameter 10–15 μ), originally described by Tomita and Funaishi (1952), were used. The surface action potentials were amplified with a condenser-coupled amplifier, displayed on an oscilloscope and photographically recorded. When longer recordings were needed a smoked paper electrometer (Hatakeyama, 1954) was employed.

Results

Structure of the heart. The heart consists of fourteen segments each of which has a single nerve cell, a pair of ostia and arteries. Except at the proximal and

distal ends of the heart tube, the single nerve cells of each segment lie individually in the median nerve trunk behind the ostial orifices. Alexandrowicz (1934) previously pointed out that the size of the nerve cell varies with the segment. In this study, on the basis of thirteen preparations, it was found that the thirteenth segment always contained the largest cell (average value: length 80 μ, width 63 μ). There is a progressive decrease in the size of the nerve cells anterior and posterior to the cell of the thirteenth segment.

The major components of the electrocardiograms. When the recording electrode was placed on the surface of the peripheral part of the heart, relatively slow triphasic action potentials were observed as shown in Figure 1 A. As can be seen

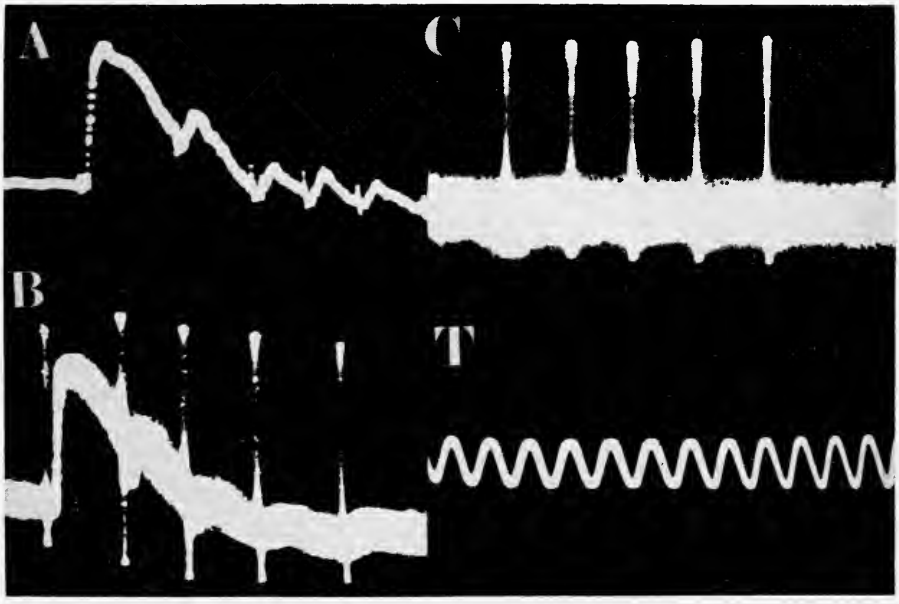

FIGURE 1. Surface action potentials of the heart of the mantis shrimp. A. Record obtained at some distance from the nervous trunk. Small rhythmic waves and small spikes can be seen. B. Record from the central part of the heart tube, characterized by a train of spike potentials and the slow muscle potentials. C. When the contraction of the heart is small or absent, only a train of spike potentials is recorded. T. Time: 60 cycles per second.

in this figure, the action potential usually reached its summit within 8 msec., and thereafter exhibited a slow exponential decay. However, when the recording electrode was placed on the mid-dorsal region of the heart, where the ganglionic trunks are located, a train of very rapid spike potentials was superimposed on the slow wave (Fig. 1 B). When the contraction of the heart tube was small or absent, only the spike train was recorded (Fig. 1 C). The spike components as observed in Figure 1, B and C had extremely brief durations not exceeding one millisecond. The height of these spikes remained relatively constant in the same preparation. However, attenuation of spikes was observed when the electrode was moved away from the median nervous trunks, as shown in Figure 1 A. The number of spikes in each heart beat varied widely from preparation to preparation, ranging from one

to about sixteen and averaging 7.2 spikes per heart beat in twenty-eight preparations. The number of spikes seemed to decrease with the duration of each heart beat.

The findings are compatible with the concept that the spike component arises from a stimulating discharge of the large single nerve cell of the heart segment and the slow potential from the heart muscle.

Location of pacemaker. Transection is a valuable method for the detection of pacemakers in this type of heart because of its segmentation. When a part of the heart muscle was dissected, no remarkable change in action potentials or heart rate was observed; however, when the nervous trunk was incised, definite changes occurred which varied according to the place of transection. It was not possible, however, to transect the nerve trunk without injuring part of the heart muscle.

TABLE I

The influence of successive isolating transverse sections upon the rate of contraction of the segments so isolated

Preparation number	Rate of heart before sectioning*	Rates of various isolated segments after sectioning anterior to segment thirteen			
		Segment number			
		13	12	11	10
1	84	84	52	30	18
2	96	96	42	16	†
3	48	48	42	18	†
4	60	60	24	†	†
5	60	60	30	†	†
6	84	84	48	†	†
7	54	54	30	14	†

* Figures in the table are rates in contractions per minute.

† Contractions stopped.

Sections were made serially between successive segments starting with a cut between numbers 12 and 13. Following the cut, a recording of the rate was made and the process repeated with the next segment.

Transection of the heart nerve trunk, starting with a cut anterior to segment thirteen, showed that the heart rate was reduced in sections anterior to the cut. The results of seven experiments in transection at successive levels are given in Table I. As can be seen from the table, the rate of beating of segment thirteen was unchanged by a section anterior to it but segments anterior to the cut beat at a slower rate. In terms of the rate of the thirteenth segment as 100 per cent, the first cut between segments twelve and thirteen reduced the rate of segment twelve to an average of 56 per cent. The next cut between segments eleven and twelve stopped the beating in three out of seven cases, but in the four that continued to beat the rate was reduced to 29 per cent of the original. The next cut stopped beating in all but one preparation.

These results clearly demonstrate that the thirteenth segment, which has the largest median nerve cell, has the fastest rate and governs the rate of the heart beat.

Discussion

The electrocardiogram of the Stomatopoda resembles that of *Limulus* (Prosser, 1950) and *Astacus* (Hoffman, 1912). However, the electrocardiogram of the mantis shrimp differs in that it consists of two clearly distinguishable component potentials: one of muscle and one of nerve. Since the ganglionic trunk in Stomatopoda lies on the surface of the heart tube, pure nervous spikes are obtained.

Prosser (1943) demonstrated bursts of impulses from the isolated *Limulus* heart ganglion. Welsh and Maynard (1951), and Maynard (1955) studied the cardiac ganglion of the lobster and showed that a burst of nerve impulses preceded and accompanied the early part of the mechanical response. Matsui (1955) found that the number of spikes within one heart beat varied extensively from preparation to preparation, ranging from several to eighty. Maynard (1955) distinguished two different spike potentials, namely, small and large components in his records. The type of spike-train which was obtained from a median ganglionic trunk of this stomatopod seems to be simpler than any other pattern of impulses obtained through the surface electrode from other crustaceans, and only comparable with those records from a single nerve cell of lobster (Hagiwara and Bullock, 1955; Watanabe, personal communication). Thus, the uniformity and the simplicity of this record seemed to be due to the simple structure of the ganglionic trunk: this was confirmed histologically (Irisawa and Irisawa, 1956).

Since the heart ganglion frequency is unchanged after the removal of the influence of the regulator nerve, the ordinary heart rate is probably regulated by the activity of the chain of ganglionic trunks. Possibly there is a dominating cell in the ganglionic trunk that synchronizes heart activity. Maynard (1955) stated that both large and small cells are the pacemaker cells; Hagiwara and Bullock (1955) suggested that the posterior small cells may be the pacemaker cells.

Our experiment demonstrated a remarkable gradient of the size of these heart ganglion cells and of the frequency of spontaneous firing by the respective segments when isolated from faster segments. Studies have also revealed variation in the sensitivity to a stimulus (Irisawa and Irisawa, 1956). In summation, the large cell of the thirteenth segment is the largest, most sensitive to a localized thermostimulus (Irisawa and Irisawa, unpublished data) and its segment has the highest automaticity. It is probable that this large cell of the thirteenth segment is the dominant cell and is the pacemaker in this heart.

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Summary

1. The electrocardiogram of the tubular heart of the mantis shrimp, *Squilla oratoria* de Haan, was studied. Structural findings are described which confirm Alexandrowicz's observation.

2. The electrogram consists of rapid spike components and slow action potentials. The spikes originate from the median nervous system of this heart, and the slow potential from the muscle.

3. The results of transection experiments on the nerve trunk support the view that the nerve cell of the thirteenth segment has a dominant role in the pacemaker activity of the heart contraction.

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THE AMINO ACID CONSTITUENTS OF THE PHYCOBILIN CHROMOPROTEINS OF THE RED ALGA PORPHYRA [1]

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The phycobilin chromoproteins, phycocyanin and phycoerythrin of red algae, because of their stability and water-solubility, have been studied physico-chemically by many investigators. They have been shown to have definite molecular weight, characteristic isoelectric points, mobility, diffusion and adsorption properties (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929; Svedberg and Eriksson, 1932; Tiselius, 1930, 1937; Swingle and Tiselius, 1951). Their behavior as accessory pigments in photosynthesis is also well established (Haxo and Blinks, 1950; French and Young, 1952; Blinks, 1954a, 1954b; Yocum and Blinks, 1954). A comparative study of the chromatographically separated phycobilins of red and blue-green algae has recently revealed the occurrence of individual phycobilin pigments other than the classical varieties (Haxo, O'hEocha and Norris, 1955; Tiselius *et al.*, 1956). The former authors also established the presence of allophycocyanin as a natural, (although minor) component of the chromoproteins of several of the red and blue-green algae. The water-soluble chromoproteins of *Porphyra naiadum,* after separation by column chromatography and electrophoresis, have been shown to contain an appreciable quantity of allophycocyanin as well as phycoerythrin and phycocyanin (Airth, 1955; Blinks and Airth, 1957). A highly ionized lavender fraction was also found to be present. This behaved as a homogeneous entity and moved as an anion in the electrophoretic cell even at a pH of 5.0 where phycocyanin and phycoerythrin are nearly isoelectric.

Quantitative analyses of crystallized chromoproteins by Kylin (1910), Kitasato (1925) and Fujiwara (1955) show very little difference in elementary composition. Wassink and Ragetli (1952) reported on the amino acid composition of phycocyanin isolated from a species of the blue-green alga *Oscillatoria.* These authors detected sixteen ninhydrin-reactive spots of which thirteen were identified.

The present paper is concerned with the amino acid composition of several chromatographically pure phycobilin chromoproteins isolated from *Porphyra naiadum, Porphyra perforata* and *Porphyra Nereocystis,* all primitive red algae.

Material and Method

The algae were freshly collected from the shores of the Monterey Peninsula, California. *P. Nereocystis* was found growing epiphytically upon the stripes of the large kelp *Nereocystis Luetkeana. P. naiadum* was collected from the leaves of the

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flowering plant, *Phyllospadix* growing at mean low tide. The species *P. perforata* was collected from the rocks high up in the intertidal zone. Each species occupied, therefore, a different ecological habitat.

Extraction of pigments

The algae were returned to the laboratory in sea water, then washed twice with distilled water before extraction. In the case of *P. naiadum* the washed tissue was covered with distilled water and allowed to stand in the refrigerator for 24 hours at 5° C. (see Blinks and Airth, 1957). With *P. Nereocystis* and *P. perforata* it was found necessary to macerate the algal tissue in a Waring Blendor for two minutes and to allow the macerated material to stand in the refrigerator for 48 hours at 5° C. to obtain maximum extraction. The material was then filtered through cheesecloth and the filtrate passed through Whatman No. 1 filter paper pulp to remove chloroplasts and other fine organic debris. In each case the clear filtrate, exhibiting intense fluorescence, was precipitated with ammonium sulphate. Although the extracted chromoproteins of *P. Nereocystis* and *P. perforata* were precipitated with ammonium sulphate at 50% saturation, with *P. naiadum* precipitation was only complete at 90% saturation. After the purple-red precipitate was allowed to settle out overnight in the refrigerator it was removed by centrifugation, redissolved in distilled water and dialyzed against running tap water for 24 hours at 10° C., followed by 0.1 *M* acetate buffer at pH 5.0 for a further 24 hours. The non-dialyzable pigment solution was finally concentrated by pervaporation.

Fractionation

For the separation of the individual phycobilins the concentrated solution was subjected to column chromatography as employed by Airth (1955). Columns were prepared using one part tricalcium phosphate (dry weight) to five parts of washed Celite filter air. Gentle suction was used in forming the column which was washed with NaCl (1%) followed by 0.1 *M* acetate buffer at pH 5.0. The pigment extract was introduced onto the column and the individual phycobilins eluted with the appropriate buffer solutions, details of which are shown in Table I. Each fraction was further chromatographed on individual columns to ensure complete separation and elution.

The phycobilins from *P. naiadum* were also separated using a Tiselius electrophoresis apparatus. At pH 5.0 with acetate buffer, the individual chromoproteins were found to be homogeneous.

Absorption spectra were determined for each pigment over the range 250–700 mμ using a Beckman Model DU spectrophotometer.

The fractions, phycocyanin, phycoerythrin, allophycocyanin and the "highly ionized fraction" from *P. naiadum* were concentrated by pervaporation and a quantitative analysis of the amino acids released on acid hydrolysis undertaken. Because of the small amount of *P. Nereocystis* available at the time of the experiments, only phycocyanin and phycoerythrin were investigated from this alga.

Acid hydrolysis

The protein fractions were hydrolyzed using a mixture of equal volumes of concentrated hydrochloric acid and glacial acetic acid containing 4 per cent of stannous

TABLE I

Buffer solutions used in the chromatographic separation of the various phycobilins

Species	Phycobilin	Buffer	Absorption data
P. naiadum	Phycocyanin	1 *M* acetate pH 5	λ max. 615 mμ
	Phycoerythrin	1 *M* acetate pH 5	λ max. 545 mμ "Shoulder" 560–565 mμ
	Highly ionized fraction	2 *M* acetate pH 5	λ max. 565 and 615 mμ
	Allophycocyanin	0.1 *M* phosphate pH 7	λ max. 650 mμ
P. perforata	Phycocyanin	1 *M* acetate pH 5	λ max. 557 and 615 mμ
	Phycoerythrin	1 *M* acetate pH 5	λ max. 495 and 565 mμ "Shoulder" 540–550 mμ
	Allophycocyanin	0.1 *M* phosphate pH 7	λ max. 650 mμ
P. Nereocystis	Phycocyanin	1 *M* acetate pH 5	λ max. 557 and 615 mμ
	Phycoerythrin	1 *M* acetate pH 5	λ max. 495 and 565 mμ "Shoulder" 540–550 mμ
	Allophycocyanin	0.1 *M* phosphate pH 7	λ max. 650 mμ

chloride dihydrate (Fowden, 1954). Protein concentrations were adjusted to about 10 mg. per ml. and the hydrolysis performed in sealed tubes heated at 105° C. for 24 hours. After completion of hydrolysis the mixtures were evaporated to dryness *in vacuo* to remove the volatile acids, and the residues redissolved in 5 ml. distilled water. The amino acids were absorbed on the cation exchange resin Zeo-Karb 225, eluted with 1 N NH_4OH and finally dried in a vacuum desiccator over $CaCl_2$ and NaOH. Before chromatographing, the amino acids were taken up in 0.2 ml. isopropanol (10%) to which was added a little HCl.

Chromatographic procedure

Whatman No. 1 chromatography paper was used throughout. Spots were applied to the paper with calibrated micropipettes, the size of the spot being kept as small as possible (about 5 to 8 mm. diameter).

For two-dimensional chromatography, n-butanol : acetic acid : water (4 : 1 : 5) was used for the first direction and phenol-water (80 : 20) containing 0.04% 8-hydroxyquinoline in an atmosphere of NH_3 (1%) for the second direction. The butanol solvent was run for 28 hours at 20° C. and the solvent allowed to drip on the paper. The phenol solvent was run for 24 hours at a temperature of 20° C. The butanol was removed from the paper by drying the sheets in a current of warm air for two hours. The phenol was removed by the ether-wash technique of Fowden (1951). The amino acids phenylalanine, leucine and isoleucine were resolved

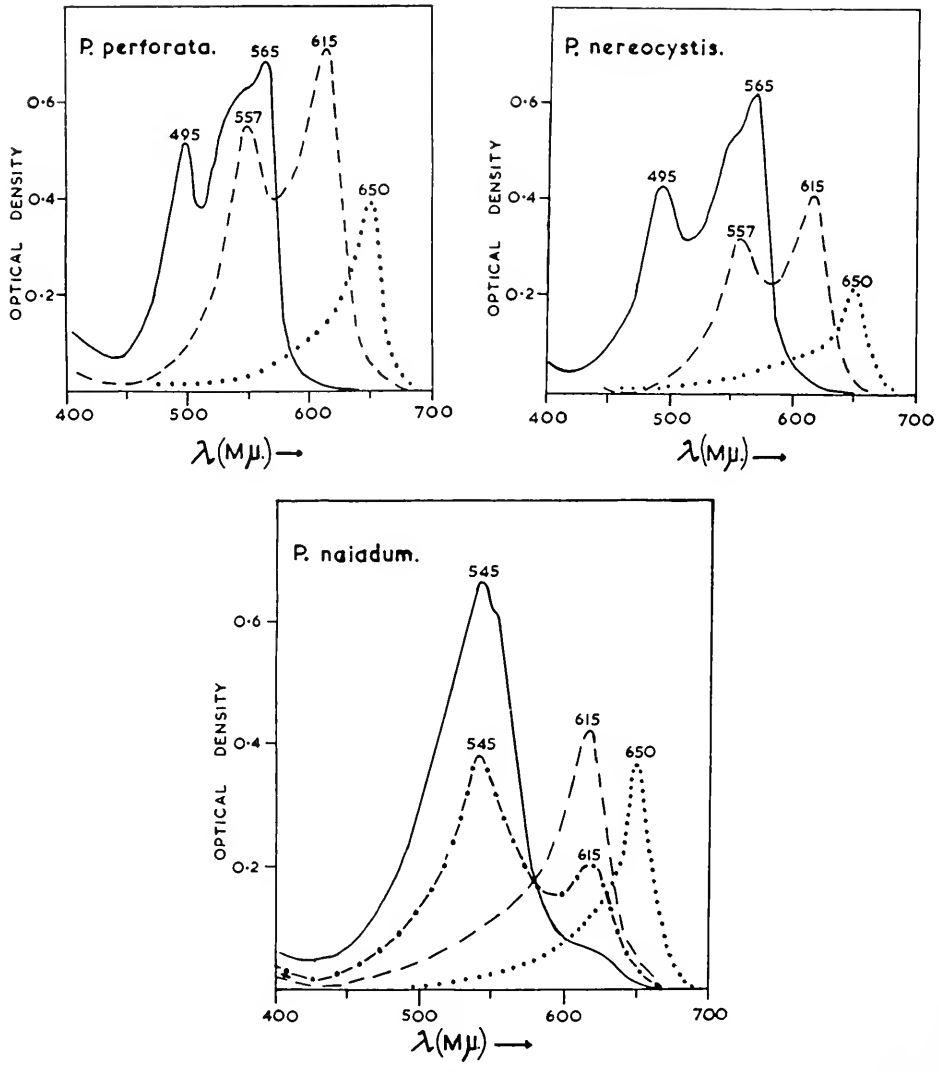

FIGURE 1. Absorption spectra of isolated phycobilins from three species of *Porphyra*. ———— phycoerythrin; – – – – – – phycocyanin; . . . . . allophycocyanin; –·–·–·–·– highly ionized fraction.

by one-dimensional chromatography using continued development for four days in water-saturated tertiary amyl alcohol in an atmosphere of 1% diethylamine. The solvent was removed from the paper by drying in a current of warm air for two hours.

The amino acids were located by dipping the chromatograms in 0.2% isatin in acetone and heating at 100° C. for three minutes to reveal proline and then dipping them into 0.2% ninhydrin in acetone and heating at 100° C. for 15 minutes to detect the other amino acids (Jepson and Smith, 1953; Smith, 1953).

TABLE II

Amino acid composition of phycobilin chromoproteins isolated from three species of Porphyra (Percentage by weight)

Amino acid	*P. naiadum*				*P. perforata*			*P. Nereocystis*	
	PC	PE	APC	"I"	PC	PE	APC	PC	PE
Aspartic	12.9	15.3	12.8	12.9	10.7	12.5	9.4	12.6	11.3
Glutamic	12.3	13.9	16.3	17.5	12.9	10.1	11.2	12.5	8.7
Serine	3.1	0.2	3.5	3.1	2.4	2.5	3.5	8.9	8.0
Glycine	5.4	5.7	7.2	5.2	6.8	9.1	9.2	11.9	13.5
Threonine	5.8	1.6	8.6	3.7	6.1	3.6	4.5	5.7	2.8
Alanine	12.3	18.3	10.9	9.5	12.6	20.3	12.5	12.3	11.7
Histidine	1.0	2.1	+	0.8	1.4	2.1	1.0	1.3	1.9
Lysine	2.6	1.4	5.1	2.3	3.1	4.9	3.6	3.4	6.5
Arginine	2.0	1.1	5.8	1.4	2.0	4.6	4.2	2.9	7.1
Proline	5.8	4.1	6.2	4.9	5.1	2.9	5.2	3.8	4.6
Valine+methionine	10.6	7.4	11.7	6.0	10.5	9.1	6.9	6.3	3.1
Phenylalanine	6.5	10.7	3.1	6.3	2.0	4.2	7.4	3.4	5.6
Leucine	11.2	10.1	5.1	10.5	12.2	8.9	11.1	7.8	6.8
Isoleucine	9.1	6.3	3.9	8.8	6.8	4.4	7.8	3.1	5.2
Tyrosine	−	2.0	+	5.0	4.4	1.9	+	4.1	3.7
Cystine	−	+	−	+	−	+	−	−	+

PC = phycocyanin; PE = phycoerythrin; APC = allophycocyanin; "I" = highly ionized fraction. + = present but too small to determine. − = not detected.

Quantitative estimation

The chromatograms, developed as described above, were utilized for quantitative estimation by a densitometric method. The maximum spot color density (*i.e.*, average blank reading minus the minimum reading for the given spot) multiplied by the spot area is a constant under the same conditions (Block, 1950). A densitometer suitable for the analysis of the chromatograms was constructed; this consisted of a photoelectric cell, a constant voltage light source and a galvanometer (Weston Model 440 No. 10623). Rapidity of operation was improved by fixing the photoelectric cell on a movable arm which held it over a circular light source of diameter 0.5 cm. Before use the light source was adjusted to produce a suitable standard transmission. For the paper blanks percentage transmission readings of 90–100 were obtained while the amino acid spots varied between 5 and 80 per cent transmission for the concentrations employed. The area of the amino acid spot was determined by tracing the spot on uniform paper and weighing the cut-out spot with a torsion balance. Standard chromatograms of known amino acid composition were developed at the same time as the hydrolysates. For the standard amino acids linear relationships were obtained over the range of 1–25 μgm. amino acid with an error of $\pm$ 12%.

All analyses were carried out in duplicate for each alga.

RESULTS

The three species of *Porphyra* investigated exhibit a graded increase in the ratio of phycocyanin to phycoerythrin. The deep water form, *P. Nereocystis*, has the

least phycocyanin. *P. naiadum* possesses comparatively large amounts of both phycocyanin and phycoerythrin. *P. perforata* has proportionately the most phycocyanin and the least phycoerythrin. Allophycocyanin is present in all three species of *Porphyra,* but is most abundant in *P. naiadum.* The absorption spectra for the various isolated and chromatographically purified phycobilins are shown in Figure 1.

The amino acid composition of these chromoproteins is given in Table II. The distribution of the amino acids is expressed as a percentage of the total weight of the amino acids in the protein. It is seen that, in the phycobilins isolated, the same

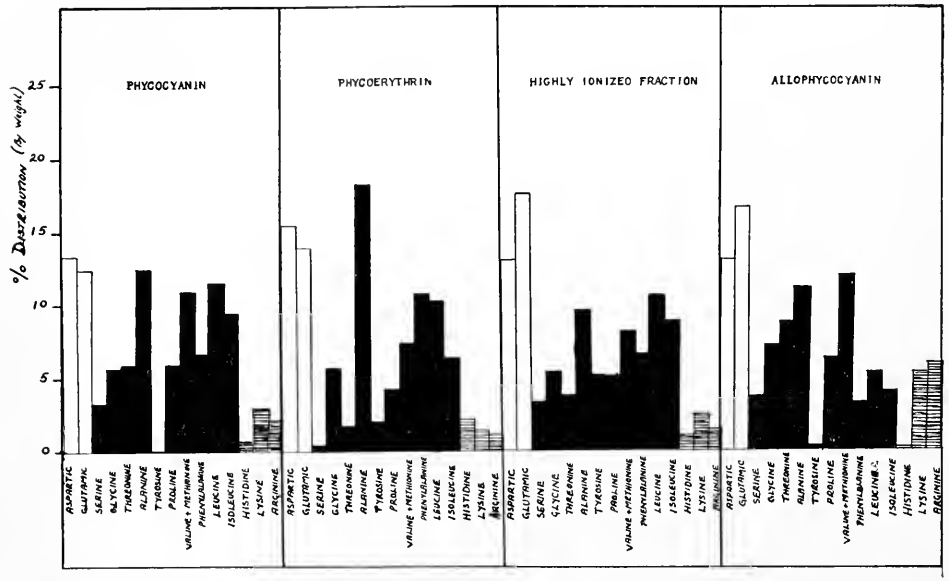

FIGURE 2. The major amino acid components of four chromoprotein fractions of *Porphyra naiadum.* Acidic amino acids are shown in white, basic, cross-hatched, and neutral, in black. The amounts are indicated as percentage of the total.

amino acids are present. Although tyrosine was not detected in the phycocyanin from *P. naiadum* it may well be due to the fact that this amino acid exists in concentrations considerably lower than that of any other amino acid present. The quantitative distribution of each amino acid varies for each protein analyzed. In all cases the dicarboxylic amino acids predominate and are comparable. Of the neutral amino acids, alanine and the two leucines are highest, although in the phycoerythrin isolated from *P. Nereocystis* glycine is present in relatively high concentration. It is interesting to note that alanine is present in greater amount than any other amino acid in the phycoerythrins isolated from *P. naiadum* and *P. perforata.* The basic amino acids of all the phycocyanins are low compared with the other phycobilin proteins. A histogram of the distribution in *P. naiadum* is shown in Figure 2.

DISCUSSION

The absorption characteristics of the isolated chromoproteins described in this work agree closely with those published by previous workers (Blinks, 1954a, 1954b; Airth, 1955; Haxo, O'hEocha and Norris, 1955). Typical R-phycoerythrins of the

higher red algae (Florideae) display absorption peaks at 495, 545, and 565 mμ. Of the lower red algae (Bangiales) here investigated, *P. perforata* and *P. Nereocystis* lack a pronounced second peak at 545 mμ. In *P. naiadum*, the phycoerythrin is characterized by a single peak at 545 mμ and a small shoulder at 560 mμ. This phycobilin has been designated B-phycoerythrin (Blinks, 1954b; Airth and Blinks, 1956), and is closest to the C-phycoerythrin of the Cyanophyta which has a single peak at 550 mμ. The phycocyanins of *P. perforata* and *P. Nereocystis* are similar in possessing the characteristic principal maximum at 615 mμ and a small one at 557 mμ. *P. naiadum*, however, contains a phycocyanin which has but a single absorption peak at 615 mμ. This is similar to the C-phycocyanin of blue-green algae. Allophycocyanin with an absorption maximum at 650 mμ is present in all three species of *Porphyra*, thereby substantiating the findings of Haxo, O'hEocha and Norris (1955) that it is a natural, although minor, component of the chromoproteins of marine algae. The highly ionized chromoprotein found only in *P. naiadum* has two absorption maxima, a major one at 545 mμ and a minor one at 615 mμ (probably due to the presence of phycocyanin).

The comparison of the above phycobilin proteins was undertaken to establish whether similar types of protein were present in the different species. The basis of comparison employed here, namely amino acid composition, although useful is subject to certain limitations. The physico-chemical properties of proteins depend not only upon their amino acid composition, but upon the arrangement of the amino acid residues within the protein and the nature of the helical configuration of the molecule. It is therefore realized that an amino acid analysis alone cannot account for all the biological or physico-chemical characters of the proteins.

The data presented show that the amino acid compositions of the various phycobilins differ significantly. Although *P. perforata* and *P. Nereocystis* possess phycobilins of similar absorption spectra, the amino acid composition of the proteins varies. The amino acid analysis of the phycocyanin from *P. naiadum* differs widely from the analysis published by Wassink and Regetli (1952) for the C-phycocyanin of *Oscillatoria* which has similar absorption characteristics. Of particular note is the presence of arginine which was absent from *Oscillatoria* phycocyanin. *P. naiadum* possesses phycobilin chromoproteins which differ from the other species of *Porphyra*, both in amino acid composition and absorption spectra. This is of particular interest since Professor G. J. Hollenberg (University of Redlands) has noted several morphological peculiarities which will probably remove *P. naiadum* from its present genus. The amino acid analysis of the highly ionized fraction present in this species offers little explanation for the high mobility of the molecule when subject to electrophoresis at pH 5.0. The degree of ionization is too great to be accounted for by the carboxyl groups of the amino acids. However, this fraction exhibited high absorption in the U. V. range of 265–280 mμ which suggests that the pigment may be attached to a nucleoprotein, in which case nucleic acid could be responsible for the high mobility.

Summary

1. The phycobilin chromoproteins of three species of *Porphyra* have been separated by column chromatography and their individual absorption spectra recorded. These "chromatographically purified" chromoproteins were subjected to acid hydrolysis and their constituent amino acids resolved by paper chromatography and determined quantitatively by a densitometric method.

2. The quantitative amino acid composition of each chromoprotein differed. The dicarboxylic amino acids alanine, glycine and the two leucines were most abundant. Alanine was found to be present in high concentration in the phycoerythrin of *P. naiadum* and *P. perforata*.

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HISTOLOGICAL CHANGES IN REGENERATING PIECES OF DUGESIA DOROTOCEPHALA TREATED WITH COLCHICINE

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It has been shown that colchicine inhibits or completely abolishes regenerative changes in pieces of *Dugesia dorotocephala* (McWhinnie, 1955). Because of its selective stathmokinetic action it can be assumed that cellular studies, of colchicine-treated planarian pieces, should give evidence as to the source of blastema and regenerate cells. As early as 1902 Stevens reported numerous parenchymal cells in stages of mitosis, when untreated short transverse pieces of *Planaria lugubris* were regenerating. He suggested the embryonic nature of these cells which through multiplication and differentiation replaced the elements lost in section. With the use of x-rays Bardeen and Baetjer (1904) showed marked inhibition of regeneration in *P. maculata* and *P. lugubris*. Histological study showed no change in the cells of muscle, nerve, endoderm or gonad and an absence of mitosis in parenchymal cells. Control pieces showed mitotic cells in the parenchyma. Wiegand (1930) also demonstrated this point with several planarian species. On an indirect basis Curtis and Schultze (1934) emphasized the role of parenchymal cells in regeneration by comparing their number in species known to have high regenerative capacities (*P. maculata; P. agilis*) with one limited in regenerative ability (*P. fluviatilis*). Subsequent studies (Curtis, 1936) with x-rays showed a reduction of free parenchymal cells in proportion to the reduction in regeneration.

Colchicine inhibition of development has been shown by Beams and Evans (1940). Fertilized eggs of *Arbacia punctulata* were unable to divide if exposed to colchicine during the pre-metaphase interval and also showed a considerable decrease in viscosity.

Despite the evidence for the role of parenchymal cells in planarian regeneration, several workers have reported the absence of mitosis in normal planarian regeneration (Steinmann, 1926; Bandier, 1936; Clement, 1944). In an effort to demonstrate a specific source of cells which contribute to planarian regeneration, histological studies were made at selected intervals after colchicine treatment.

Materials and Methods

A stock of *Dugesia dorotocephala* was collected and maintained in the manner previously described (McWhinnie, 1955). Animals were sectioned into two halves at the level of the mouth. After sectioning, the pieces were separated into two groups. In one group, anterior and posterior halves were placed into $M/5000$ colchicine at the time of section. These were prepared for study at the end of exposure periods 3, 6 and 10 days. Pieces in the second group were placed into aerated tap water and were allowed to reconstitute for 24, 48 and 72 hours. At the end of each time interval these pieces were transferred to $M/5000$ colchicine where they were

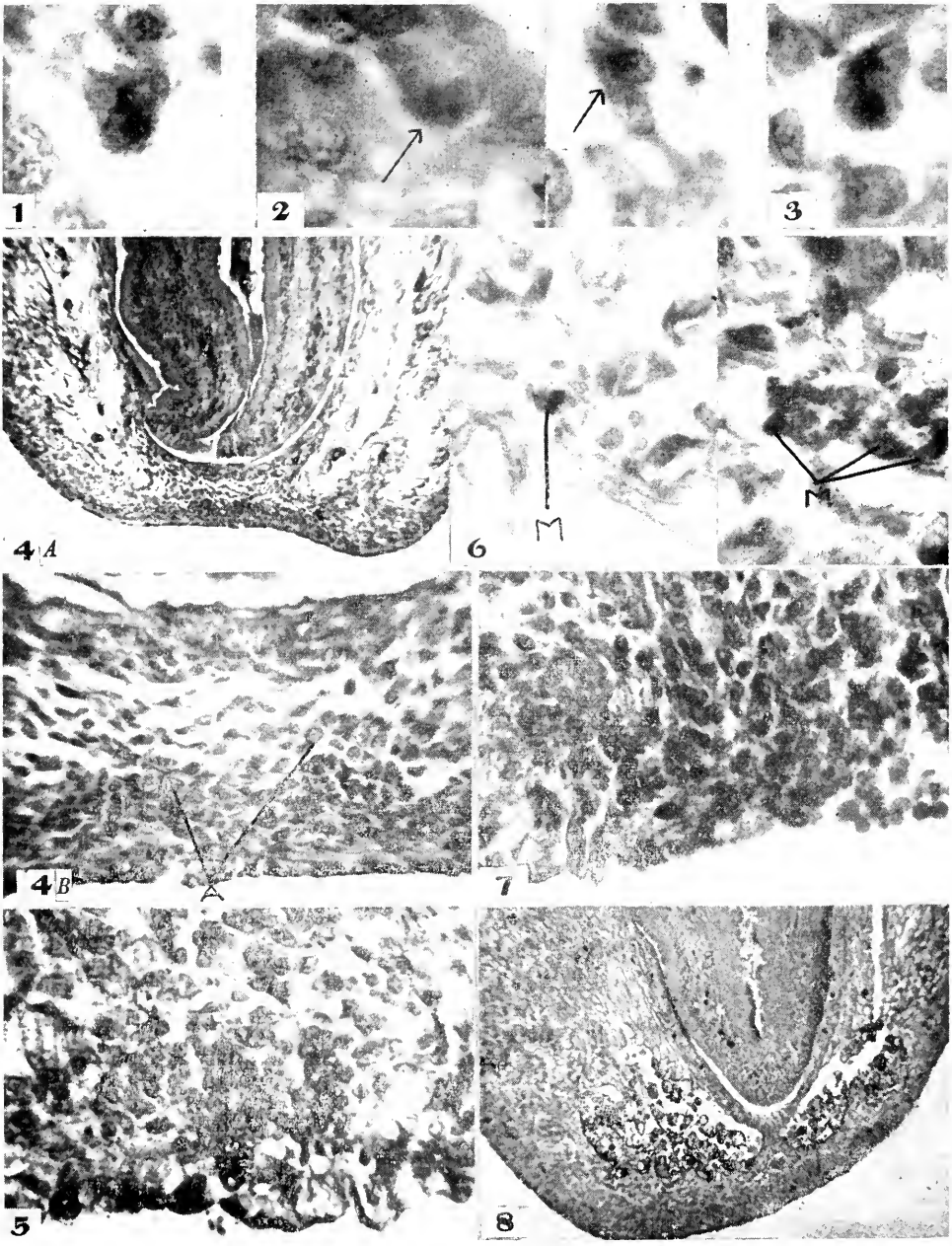

PLATE I

Explanation of Figures

FIGURE 1. Mitotic figure in a free amoebocyte in a three-day regenerating planarian piece, exposed to $M/5000$ colchicine from the time of section. $\times 1425$.

permitted to remain for 12 and 24 hours. All pieces to be prepared for cell study were narcotized in 0.05–0.1% chloretone, killed and fixed in Bouin's fluid and embedded using the standard paraffin technique. These were sectioned at 6 micra in such a manner that both frontal and sagittal sections were obtained. Delafield's hematoxylin was used without a counterstain. Study was with 1000 and 1500 diameters magnification.

Results

The history of parenchymal cells in regenerating planarian pieces treated with colchicine demonstrates the paramount role of these cells in this developmental process. Planarian pieces treated with $M/5000$ colchicine for three days following section show many free amoebocytes in mitosis. These are uniformly distributed with extremely few in the area of the cut surface. Many of these cells appear elongate and in strands oriented to the cut surface, indicating migration. Mitotic figures were normal (Fig. 1). However, after 6 days' exposure to colchicine from the time of section most of the mitotic amoebocytes had abnormal chromosomal configurations and considerable pycnosis (Fig. 2). At 6 days these cells were still generally distributed throughout the parenchyma with never more than one to two in the region of the cut surface. Also, the large free amoebocytes were oriented into longitudinal strands. At the end of 10 days' exposure to colchicine a larger number of cells were at metaphase or beyond. However, at this time there was extensive cellular degeneration as evidenced by granulation of the nucleus, mitotic abberations and cytoplasmic vacuolation. Of the free amoebocytes undergoing degeneration many were oriented into migration strands.

Despite the apparent increase in number of parenchymal cells in mitosis from three to ten days after section in treated regenerating planarian pieces, the untreated pieces do not show this same progressive increase in number of cells undergoing division in time. Three days after section, untreated planarian pieces show some free amoebocytes in mitosis (Fig. 3) but none were observed in the region of section. On a comparative basis the number of mitotic figures was less than in the three-day colchicinized pieces. A marked decrease in the proliferation of these cells is apparent by six days after section when pieces regenerate in aerated tap water. However, at this time orientation of migratory strands to the area of the cut is

Figure 2. Mitotic figures in free amoebocytes in a six-day regenerating planarian piece, exposed to $M/5000$ colchicine from the time of section. × 1425.

Figure 3. Mitotic figure in a free amoebocyte in an untreated three-day regenerating planarian piece. × 1425.

Figure 4. A. Long section through the cut surface of a planarian piece treated with $M/5000$ colchicine for 24 hours after 24 hours in water. × 150. B. Same as A. × 660. (A = amoebocyte.)

Figure 5. Long section of an untreated regenerating planarian piece 48 hours after section. × 660.

Figure 6. Parenchyma of a planarian piece treated with colchicine for 24 hours after 48 hours in water. Note free amoebocytes in metaphase and evidence of migration. × 1425.

Figure 7. Long section of an untreated regenerating planarian piece 72 hours after section. Note increased number of amoebocytes. × 660.

Figure 8. Long section of a planarian piece treated with $M/5000$ colchicine for 24 hours after 72 hours in water. An increase in the number of cells can be seen in the region of the cut surface. An increase in gut degeneration is apparent. × 150.

notable. By ten days after section, when the regeneration process is conventionally considered complete, there is no evidence of mitotic activity. Regenerating pieces of planaria had no mitotic activity in epidermis, muscle or endoderm tissue, whether the pieces had been treated with colchicine or permitted to regenerate in water.

When anterior and posterior halves of planarians were maintained in water for 24 hours and then transferred to *M*/5000 colchicine for 12 and 24 hours, large numbers of free parenchymal amoebocytes were in metaphase with considerably fewer in telophase. These cells had divided *in situ* as well as during migration to the area of the cut surface. Degenerative changes in the gut and parenchyma were not found after 12 hours and were minimal after 24 hours treatment. In this group some few mitotic cells showed degenerative changes as indicated by granulation of nuclear components and rupturing of the cytoplasm. Cells of the gut, fixed nuclei of the syncytium, muscle and epidermis were not in mitosis. Beneath the newly-formed epidermal covering there was a slightly larger number of free amoebocytes than in the rest of the parenchyma (Fig. 4 A and B). At this same time interval untreated pieces had extremely few mitotic cells but a considerably greater number of amoebocytes at the cut surface (Fig. 5).

A similar group of anterior and posterior halves was allowed to undergo regeneration for 48 hours before treatment with colchicine for 12 and 24 hours. These pieces showed a still greater accumulation of free amoebocytes and more metaphase figures beneath the epidermal covering than in the previous group, *i.e.*, colchicine treatment after 24 hours in water. Many amoebocytes *in situ* and in migration were at metaphase (Fig. 6).

In untreated 72-hour regenerates there were few mitotic figures, but numerous amoebocytes were densely packed at the region of the blastema (Fig. 7). Pieces treated for 24 hours showed fewer mitotic figures than those treated for 12 hours. Treatment with colchicine for 12 and 24 hours, after a reconstitution period of 72 hours in water, resulted in pieces with a greater number of amoebocytes in the region of the cut surface. At this time fewer mitotic cells were seen in all areas. Gut, as well as general parenchymal degeneration was more apparent than in the previous series (Fig. 8). On the other hand controls showed large numbers of amoebocytes in the blastema, little evidence of mitotic activity and no degenerative changes.

Discussion

Through the use of colchicine on regenerating planarian pieces it can be concluded that the cellular elements involved in restoration to wholeness are primarily the free amoebocytes of the parenchyma. In all cases observed, after a minimum of 24 hours of reconstitution, epidermis, co-extensive with the epidermis of the rest of the piece, covered the wound surface. In no case was mitosis observed in this tissue. Similar wound epithelial covering without cell proliferation has been demonstrated in amphibian limb regeneration (Lash, 1955). The cut surface is readily distinguishable from the rest of the piece by the lack of sub-epidermal pigment as well as by the localized density of the parenchymal cells. With the use of colchicine it would appear that the time course of mitotic activity during regeneration explains the short interval in which there is no apparent change after section, the time of onset of greatest cellular proliferation and the known difference in susceptibility to toxic influences through the regeneration period. Some studies made in this work show

that mitotic cells increase in number from the third to the tenth day after section when pieces are placed into colchicine at the time of section. However, observation of untreated pieces at the same time intervals strongly indicates a greater number of parenchymal cells in division at the third day after isolation than in pieces 6 or 10 days after isolation. The progressively larger number of metaphase cells found at longer time intervals after isolation and introduction into colchicine would simply emphasize the sustained inhibition in the presence of the alkaloid and consequently the accumulation of inhibited cells.

Evidence can be gained as to the time of greatest mitotic activity by permitting isolated pieces of planarians to initiate normal regeneration in water before placing them into colchicine. Halves of planarians placed into colchicine for 24 hours after initial regeneration in water for 24, 48, and 72 hours show a greater number of parenchymal cells in division at a final age of 72 hours (48 hours in water, 24 hours in colchicine). However, a substantial number of these cells were in division in pieces placed into colchicine for 24 hours after 72 hours' regeneration in water. While the number of mitotic figures found in this group was less than in the preceding it is also true that parenchymal degeneration was considerably greater than in that group. Under these conditions there is extensive gut degeneration, granular degeneration in patches of parenchyma as well as in the area of the cut surface. Mitotic cells at this time showed a range from normal metaphase to cells with chromosomes widely dispersed and granular in appearance. These modifications were not so apparent in pieces placed into colchicine after 48 hours in water.

The fixed 24-hour exposure to colchicine in these three groups with a greater susceptibility of pieces at the fourth day of reconstitution agrees with previous findings on the critical period in reconstitutional development. By studies of gross changes in planarian pieces and their susceptibility it was demonstrated that the fourth day in development is the most critical (McWhinnie, 1955).

While the highest mitotic activity appears at the third day after isolation and both gross and microscopic evidence show a high susceptibility at the fourth day, it would appear that the increased population of parenchymal cells and their migration to the cut surface constitute the most active and therefore the most susceptible period in planarian regeneration. This is visibly expressed by the toxic effects of colchicine on the fourth day.

It is suggested that the sequence of events in planarian regeneration includes an initial slow onset of division of parenchymal cells, rising to a peak at the third day after isolation. Associated with the rise in number of cells proliferating, oriented migrations of these cells to the cut surface follows. It would appear that the activity of migration is greatest through the fourth to the sixth day afer section. Some oriented strands found in 48-hour pieces indicate the onset of migration at this time. By the sixth day of regeneration, mitotic activity and cell migrations are subsiding and the remainder of the reconstitution period represents the time of morphogenetic changes to complete species organization, both internal and external.

It can be concluded also that the mechanism of colchicine inhibition of planarian regeneration is through its influence on parenchymal amoebocyte proliferation as well as reduced migration of those cells to the cut surface(s). It is entirely likely that colchicine-induced changes in viscosity (Beams and Evans, 1940) could account, in part, for the marked difference in cell density in the blastema of normal and treated regenerating pieces.

SUMMARY

1. Histological studies show that the mechanism of colchicine inhibition of regeneration in pieces of *Dugesia dorotocephala* is the stathmokinetic action it exerts on free parenchymal amoebocytes.

2. Parenchymal amoebocytes are the only cells exhibiting mitotic activity during the period of regeneration.

3. Mitotic activity reaches a peak at the third day of development while oriented migrations of amoebocytes appear to set in at the second day with marked movement through the fourth to the sixth day after section.

4. The free amoebocytes of the parenchyma constitute the exclusive source of cells participating in the replacement of parts lost when planarian worms are sectioned.

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GROWTH-PROMOTING EFFECTS OF HYDROLYZED NUCLEIC ACIDS, NUCLEOTIDES, AND NUCLEOSIDES ON ENDAMOEBA HISTOLYTICA

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Endamoeba histolytica has not yet been maintained indefinitely in pure culture, although bacteria-free cultures have been carried for periods of up to one month in growth factor-fortified media (Nakamura and Baker, 1956). Jacobs (1947) and Shaffer and Frye (1948) have also grown the amebas in media containing no or relatively few multiplying bacteria. Therefore, it becomes apparent that although bacteria contribute tremendously to the growth and multiplication of the amebas, they are not absolutely essential and that perhaps amebic growth can occur in a semi-synthetic medium if supplied the necessary growth-promoting factors. It has been shown that purines, pyrimidines, citrovorum factor, and ribose-5-phosphate can substitute partially for bacterial association and permit bacteria-free cultures of *E. histolytica* to multiply for a limited period. However, ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) tested singly or in combination failed to promote amebic growth; on the other hand, dialysates of media containing RNA and DNA preconditioned by bacterial growth contained ameba-stimulatory substances which permitted seven sub-cultures of the amebas in the absence of associated bacteria (Nakamura and Baker, 1956). It was postulated that bacterial action on the nucleic acids produced catabolic intermediate(s) which were essential to the nutrition of the amebas. In order to determine more exactly the specific components in the nucleic acid digest which were ameba-stimulatory, nucleic acids were hydrolyzed by enzymatic, acid, and alkaline hydrolysis; the dialysates of the hydrolysates were studied for their effects on *E. histolytica* under bacteria-free conditions. Furthermore, nucleosides and nucleotides, obtained from commercial sources, were also assayed for their activity on the growth of the amebas.

Materials and Methods

Organism and the assay medium

Strains of *E. histolytica* employed in these experiments consisted of: (1) NRS, obtained from Dr. Quentin M. Geiman, Stanford University School of Medicine, San Francisco, California, (2) HUS-100, isolated from the stool of a carrier during an outbreak of amebiasis in Indiana in 1953, obtained from Dr. Chia-Tung Pan, Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts, and (3) UC, also obtained from Dr. Pan. Stock cultures of the amebas, containing a mixed bacterial flora, were maintained in a modified Boeck-Drbohlav (1925) medium. The assay methods were essentially identical with those described earlier (Nakamura, 1955; Nakamura and Baker, 1956; Nakamura and

Jonsson, 1956). Coagulated egg slants were overlaid with a liquid phase consisting of glucose (0.5%), sodium thioglycollate (0.3%), penicillin G (10,000 units/ ml. final concentration), streptomycin (5000 units/ ml. final concentration), horse serum-Ringer solution (1/5), rice powder (approximately 10 mg.), and a vaspar (vaseline and paraffin, 1/1) seal. The volume of the overlay fluid was four ml. The dialysates and the nucleosides and nucleotides assayed were added to the liquid phase of the medium.

The inocula consisted of 2 drops of stock cultures adjusted to contain approximately 15–20 amebas per low power field. The bacteria introduced with the ameba inocula were sterilized within 4–6 hours by the combinations of antibiotics used. The culture tubes were incubated for 3–4 days at 37° C.; ameba counts were made by taking the sediment from each culture tube (in duplicate), placing a few drops on a clean slide, covering with a cover slide (22 × 22 mm.) and counting the number of amebas per low power field. Ten fields were counted and an average count recorded. At the same time the amebas were transferred to tubes containing identical nutritional components. Control tubes consisted of media lacking only the materials being assayed. Positive controls consisted of media fortified with the growth factors ribose-5-phosphate and adenosinetriphosphate.

Hydrolysis of nucleic acids

The method of Kerr *et al.* (1949) was used for the acid hydrolysis of RNA. Fifty mg. of RNA were placed in a test tube with 5 ml. of 2 N sulfuric acid. The tube was placed in boiling water for 30 minutes. After hydrolysis the contents of the tube were diluted to 25 ml. with water. Acid hydrolysis of DNA was accomplished by placing 50 mg. of DNA in 5 ml. N sulfuric acid and refluxing in boiling water for 2 hours. The hydrolysate was adjusted to pH 6.5 with alkali.

The method of Volkin and Carter (1951) was used for the alkaline hydrolysis of RNA. Fifty mg. of RNA, dissolved in 3 ml. of 0.5 N NaOH, were kept at 37° C. for 17 hours. The digest was diluted with water to 0.02 N NaOH and finally neutralized. DNA was hydrolyzed using a modified method of Marrian *et al.* (1951).

Enzymatic hydrolysis of RNA was accomplished by suspending 50 mg. of RNA in 5 ml. water and adjusting to pH 7.2 with dilute NaOH. Then 5 mg. of crystalline ribonuclease, dissolved in 1 ml. of 0.1 *M* phosphate buffer at pH 7.2, was added to the nucleic acid solution under stirring. As the reaction progressed the solution was maintained at pH 7.2 with the addition of 0.05 N NaOH. The temperature of the digest was maintained between 25–27° C. Hydrolysis was complete in two hours. The method of Smith and Markham (1952) was used for the enzymatic digestion of DNA; the DNA was digested with desoxyribonuclease (20 ugm./ml.) in 0.005 *M* magnesium sulfate at pH 7.0 for 18 hours. The digests were dialyzed in water and the dialysates tested for their growth-promoting activity.

Results

As is evident in Tables I, II, and III, enzyme-hydrolyzed nucleic acids (both RNA and DNA) yielded a product which was stimulatory to the growth of *E. histolytica*. In all of the experiments enzyme-hydrolyzed RNA consistently stimulated the amebas slightly more than the enzyme-hydrolyzed DNA preparation. Al-

TABLE I

Effect of hydrolyzed nucleic acids, nucleotides, and nucleosides on the growth of E. histolytica under bacteria-free conditions; strain NRS

Material assayed	Total no. of determinations	Aver. count per low power field
Basal (control)	15	1
Basal + enzyme-hydrolyzed RNA	4	79
Basal + enzyme-hydrolyzed DNA	4	55
Basal + alkaline-hydrolyzed RNA	4	47
Basal + alkaline-hydrolyzed DNA	4	50
Basal + acid-hydrolyzed RNA	4	0
Basal + acid-hydrolyzed DNA	4	0
Basal + unhydrolyzed RNA	8	10
Basal + unhydrolyzed DNA	8	7
Basal + adenosine (0.1 mg./ml.)	4	41
Basal + guanosine (0.1 mg./ml.)	4	49
Basal + thymidine (0.1 mg./ml.)	4	59
Basal + adenylic acid (0.1 mg./ml.)	4	70
Basal + guanylic acid (0.1 mg./ml.)	4	66
Basal + thymidylic acid (0.1 mg./ml.)	4	83
Basal + uridylic acid (0.1 mg./ml.)	4	47

TABLE II

Effect of hydrolyzed nucleic acids, nucleotides, and nucleosides on the growth of E. histolytica under bacteria-free conditions; strain HUS-100

Material assayed	Total no. of determinations	Aver. count per low power field
Basal (control)	15	0.4
Basal + enzyme-hydrolyzed RNA	4	64
Basal + enzyme-hydrolyzed DNA	4	40
Basal + alkaline-hydrolyzed RNA	4	51
Basal + alkaline-hydrolyzed DNA	4	54
Basal + acid-hydrolyzed DNA	4	1
Basal + acid-hydrolyzed RNA	4	0
Basal + unhydrolyzed RNA	4	0
Basal + unhydrolyzed DNA	4	1
Basal + adenosine (0.1 mg./ml.)	4	39
Basal + guanosine (0.1 mg./ml.)	4	34
Basal + thymidine (0.1 mg./ml.)	4	43
Basal + adenylic acid (0.1 mg./ml.)	4	57
Basal + guanylic acid (0.1 mg./ml.)	4	68
Basal + thymidylic acid (0.1 mg./ml.)	4	49
Basal + uridylic acid (0.1 mg./ml.)	4	33

kaline hydrolysates of nucleic acids were also stimulatory to the amebas. However, acid-hydrolyzed nucleic acids were without ameba-stimulatory properties in studies on all three strains of *E. histolytica*. Unhydrolyzed nucleic acids were inactive, except for a slight effect on the NRS strain, as was to be expected according to the earlier data of Nakamura and Baker (1956). The nucleosides adenosine, guanosine, and thymidine stimulated the HUS-100 and NRS strains but not the UC strain. The nucleotides adenylic acid, guanylic acid, thymidylic acid, and uridylic acid were active as growth factors for all three strains of amebas tested, although

TABLE III

Effect of hydrolyzed nucleic acids, nucleotides, and nucleosides on the growth of E. histolytica under bacteria-free conditions; UC strain

Material assayed	Total no. of determinations	Aver. count per low power field
Basal (control)	18	2
Basal + enzyme-hydrolyzed RNA	4	83
Basal + enzyme-hydrolyzed DNA	4	69
Basal + alkaline-hydrolyzed RNA	4	40
Basal + alkaline-hydrolyzed DNA	4	40
Basal + acid-hydrolyzed RNA	4	3
Basal + acid-hydrolyzed DNA	4	2
Basal + unhydrolyzed RNA	4	1
Basal + unhydrolyzed DNA	4	0
Basal + adenosine (0.1 mg./ml.)	4	0
Basal + guanosine (0.1 mg./ml.)	4	2
Basal + thymidine (0.1 mg./ml.)	4	5
Basal + adenylic acid (0.1 mg./ml.)	4	61
Basal + guanylic acid (0.1 mg./ml.)	4	68
Basal + thymidylic acid (0.1 mg./ml.)	4	90
Basal + uridylic acid (0.1 mg./ml.)	4	56

the degree of activity as growth-promoting factors varied slightly from compound to compound.

Attempts to maintain bacteria-free subcultures on media containing hydrolyzed nucleic acids, nucleotides, or nucleosides were generally unsuccessful. The longest culture maintained on enzyme-hydrolyzed RNA and enzyme-hydrolyzed DNA was 5 transfers for a total of approximately 15 days. Sterility tests indicated the absence of viable bacterial cells. In media containing alkaline-hydrolyzed nucleic acids, two to three subcultures were usually possible; however, the total amebic populations were considerably lower than in the enzyme-treated nucleic acid media. Only one subculture with a meager ameba count was possible in the experiments containing nucleosides and nucleotides as growth factors.

DISCUSSION

The data in this report are in agreement with earlier reports that nucleic acids preconditioned by bacterial growth produce some catabolic metabolite(s) which are essential for amebic growth in the absence of living bacteria. In these experiments, enzymatic and alkaline digestion, rather than bacterial preconditioning, yielded ameba-growth-promoting factors. It is indeed difficult to explain the absence of similar stimulatory activity in the acid-hydrolyzed nucleic acid solutions. In studies with *Trichomonas vaginalis,* Sprince *et al.* (1953) reported that acid hydrolysis of RNA destroyed the growth-promoting effect of RNA whereas alkaline hydrolysis of RNA left intact this growth-promoting factor. They also reported that acid, alkaline, and enzymatic hydrolysis of DNA destroyed the growth-promoting effects of DNA. These results, however, are not quite analogous to the data in this paper since Sprince *et al.* (1953) were dealing with DNA and RNA which were established as growth-promoting factors for *Trichomonas;* in the case of *Endamoeba histolytica,* DNA and RNA in themselves do not stimulate amebic growth.

It is highly probable that the ameba-growth-stimulatory action of nucleic acid hydrolysates was not due solely to the nucleosides and nucleotides formed during the digestion. Smith and Markham (1952) have found that enzyme digestion of DNA produces many dinucleoside monophosphates; Markham and Smith (1952) reported that products of enzyme hydrolysis of RNA were largely cyclic pyrimidine nucleotides and only traces of adenylic and guanylic acids were found. On the other hand, alkaline digestion of RNA produces guanylic, adenylic, and uridylic acids (Magasanik and Chargaff, 1951).

The growth factor effects of purified nucleosides and nucleotides indicate the importance of these substances in amebic nutrition; these substances play a role in the synthesis of nucleic acids and pyridine nucleotides. Diphosphopyridine nucleotide has been shown to be necessary for amebic growth in the absence of bacteria (Nakamura and Baker, 1956). Johnson (1953) similarly showed that cytidylic and guanylic acids were growth factors for *Paramecium multimicronucleatum*.

There is evidence that different strains of *E. histolytica* possess different growth factor requirements. The nucleosides, which were highly active for the NRS and HUS-100 strains, were without activity on the UC strain. It is possible that the UC strain can synthesize its own nucleoside but that it cannot phosphorylate the nucleoside into the nucleotide which it apparently requires. In the cases of the NRS and HUS-100 strains, it appears logical to assume that they can synthesize neither nucleosides nor nucleotides, yet when supplied these two growth factors exogenously, the amebas can synthesize their own nucleic acids. A strong point in favor of this assumption is the fact that pre-formed nucleic acids do not aid amebic growth appreciably whereas the nucleosides and nucleotides are highly stimulatory.

Summary

1. Enzyme and alkaline hydrolysates of ribonucleic and desoxyribonucleic acids contained growth-promoting factors for *Endamoeba histolytica*. Acid hydrolysates of nucleic acids, however, were without this stimulatory activity on the amebas.

2. Nucleosides, adenosine, guanosine, and thymidine, were stimulatory to the NRS and HUS-100 strains but not for the UC strain. Nucleotides, adenylic acid, guanylic acid, thymidylic acid, and uridylic acid, were highly stimulatory for the growth of all three strains of *E. histolytica* studied.

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ANAEROBIC RECOVERY OF ASCARIS EGGS FROM X-IRRADIATION [1]

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Lea (1947) in his classic work on radiation biology has pointed out in his analysis of the work of Henshaw (1940) that the recovery of unfertilized, x-irradiated eggs of *Arbacia* is probably not due to diffusion out of the egg of inhibitory substances; the correlation between recovery rate and oxygen uptake suggests that the effect of the radiation is to destroy some nuclear constituent, and recovery consists in the re-formation of this constituent as a result of the metabolic activity of the cell. The rate of oxygen uptake is presumably an indication of the general level of metabolic activity, and in *Arbacia* eggs appears to vary in different stages of the egg in much the same way as does whatever reaction is responsible for recovery. It does not follow necessarily that the rates of recovery in different organisms will be proportional to their respective rates of oxygen uptake. Some organisms have, in fact, been shown to consume oxygen at appreciable rates after irradiation although they do not show any recovery.

The eggs of *Ascaris lumbricoides suum* possess certain advantages for a test of the question whether oxygen is necessary for recovery from x-irradiation. Since they are facultative anaerobes they can be held for long periods of time in anaerobic conditions. Even at optimal temperatures for normal development, under anaerobic conditions the eggs do not develop. If recovery should occur during the enforced anaerobic metabolism, not only would the necessity of oxygen uptake for recovery be disproved for *Ascaris* eggs, but some other possible mechanism of recovery would be suggested. The present paper complements a preliminary report (Pahl and Bachofer, 1954).

Materials and Methods

A stock of eggs of *Ascaris lumbricoides suum* in the one-cell stage was prepared according to methods already described by Bachofer and Pahl (1955). The source of x-rays was a beryllium-window tube operated at 100 kvp. and 8 ma., without added filtration. Each irradiated sample consisted of 10^5 eggs suspended in one ml. of distilled water and placed in an open, flat-bottom vial 2.7 cm. in diameter. The egg suspension was approximately 1.8 mm. deep with the eggs resting on the bottom during the exposure. The dose was calculated to be 12,000 r/min. at the center of the irradiated layer of eggs. This value was determined by exposing ferrous ammonium sulfate as a dosimeter to a 325-curie cobalt-60 source of gamma rays, as described by Weiss (1952). *Ascaris* eggs were then exposed to the gamma rays under the same conditions, and the biological response was correlated with dose.

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Aliquots of the same sample of eggs were then exposed to the x-ray beam, and from their response the output of the x-ray tube was determined. All subsequent exposures to x-rays were carried out under identical conditions at the same dose rate.

Each irradiated sample was diluted 20-fold with *Ascaris* physiological saline solution (Baldwin and Moyle, 1947) immediately after irradiation. Aliquots of two ml. each were de-oxygenated by bubbling specially purified nitrogen through the saline solution containing the eggs. The eggs were then placed at the appropriate incubation temperatures. After certain designated incubation periods, the seals were broken and the supernatant de-oxygenated fluid was drawn off while the eggs remained settled on the bottom. *Ascaris* saline, in equilibrium with air, was then added and incubation completed at 30° C. The same procedure was followed for non-irradiated controls.

TABLE I

Effect of post-irradiation anaerobic treatment at 15° C., 20° C., and 30° C. over a period of two weeks on the survival of x-irradiated Ascaris eggs. Dose: 28,000 r

Post-irradiation treatment		Per cent survival			
		Days under anaerobic conditions			
		0	1	7	14
30° C.	Aerated	47.5	45.0	47.3	48.8
	De-oxygenated	47.3	58.0	57.3	60.3
20° C.	Aerated	49.8	46.5	43.0	35.0
	De-oxygenated	45.0	48.3	50.0	38.8
15° C.	Aerated	48.8	42.0	36.0	27.8
	De-oxygenated	47.5	51.8	45.5	34.3

The two criteria used to determine the effects of irradiation and anaerobic treatment were the rate of first cleavage and the percentage of eggs which developed to the motile embryo stage. The term "survival" is used to designate the development of irradiated eggs into motile embryos.

RESULTS

Table I shows the results of irradiation of *Ascaris* eggs which were de-oxygenated immediately after irradiation and stored at temperatures of 15° C., 20° C., and 30° C. After periods of 1, 7, and 14 days at these temperatures, the de-oxygenated samples were aerated and incubated at 30° C. The results show that at any given temperature there was a higher survival for irradiated eggs given anaerobic treatment than for those incubated only aerobically. Keeping the eggs under anaerobic conditions for more than one day did not increase survival. Irradiated eggs kept at 30° C. throughout the entire post-irradiation period showed the highest survivals

TABLE II

Per cent survival of x-irradiated Ascaris eggs as affected by anaerobic treatment immediately after exposure

Dose in roentgens	Per cent survival				
	Days of post-irradiation anaerobic treatment				
	0	½	1	2	7
0	97.5	96.8	97.0	97.3	96.6
28,000	47.3	56.7	58.0	57.1	57.3
40,000	26.9	36.3	36.4	36.6	36.4
48,000	12.5	20.8	21.1	21.7	22.0
60,000	2.5	6.7	6.4	6.8	6.6

for both anaerobic and aerobic treatment. This confirms a previous study of the authors (Bachofer and Pahl, 1955) on post-irradiation temperature treatment of *Ascaris* eggs. In view of this, all other experiments were performed at 30° C., which is approximately the optimal incubation temperature.

Table II shows that anaerobiosis is able to bring about recovery over a wide dose range, even when untreated samples give survivals as low as 2.5%. When there was no anaerobiosis there was no recovery, recovery being represented by the difference in survival between the first column (where there was no anaerobiosis) and the other columns (where there was anaerobiosis). The critical period of recovery is shown to occur during the first 24 hours of anaerobiosis, since survivals for 12 hours of treatment are not increased appreciably for longer periods of treatment.

The effect of delaying the anaerobic treatment after irradiation was next investigated, with both cleavage delay and survival as criteria of recovery. The results summarized in Table III for cleavage delay show that the eggs must be de-oxygenated before 15 hours have elapsed after irradiation in order to procure recovery. The low values in the column for *no delay* in anaerobic treatment indicate the greatest recovery. Conversely, in Table IV, the high values in the column for *no delay* in anaerobic treatment indicate the greatest recovery. The crucial period of approximately 15 hours, therefore, affects both cleavage delay and survival.

TABLE III

50% cleavage time in hours for x-irradiated Ascaris eggs as affected by 24-hour anaerobic treatment initiated at various intervals after exposure

Dose in roentgens	No anaerobic treatment	Delay before anaerobic treatment		
		0 hrs.	15 hrs.	24 hrs.
	50% cleavage time in hours			
0	40.0	39.5	40.3	40.0
24,000	53.1	46.7	52.7	53.1
40,000	58.4	51.4	57.7	58.2
48,000	60.0	53.4	59.4	59.5

The recovery phenomena summarized above have been verified over a dose range of 24 to 48 kr, both for delay of cleavage and for survival.

Since anaerobic conditions during irradiation give high protection to *Ascaris* eggs, an experiment was designed to test whether post-irradiation anaerobic recovery could be secured with eggs which had been protected by anaerobic conditions during irradiation. When a dose of 60,000 r was delivered to one sample of eggs

TABLE IV

Per cent survival of x-irradiated Ascaris eggs as affected by a 24-hour anaerobic treatment begun at various intervals after exposure

Dose in roentgens	No anaerobic treatment	Delay of anaerobic treatment			
		0 hrs.	15 hrs.	24 hrs.	60 hrs.
	Per cent survival				
0	97.0	97.0	97.3	97.5	97.0
24,000	63.5	73.9	64.8	62.3	62.0
40,000	26.9	36.4	27.2	27.6	25.8
48,000	12.5	21.1	11.8	12.5	12.0

that was in equilibrium with air and to another similar sample that was under anaerobic conditions, the survival was increased in both cases by post-irradiation anaerobiosis. These increases in survival were duplicated when treatment for 1½ hours in 0.1 *M* KCN was substituted for post-irradiation anaerobiosis. Immediately after the period of exposure to cyanide, the eggs were washed by centrifugation and incubated in *Ascaris* saline. The results in Table V clearly indicate that the same pattern of protection can be obtained with cyanide as with anaerobiosis.

TABLE V

Effect of 24-hour post-irradiation treatments immediately following the irradiation of Ascaris eggs under different conditions during irradiation. Dose: 60,000 r

Treatment after irradiation	Treatment during irradiation	
	In equilibrium with air	Anaerobic
	Per cent survival	
Aerobic	2.5	74.3
Anaerobic	6.4	82.1
KCN	6.5	82.2

In other studies (unpublished results) the authors have established that cyanide inhibits the oxygen consumption of *Ascaris* eggs. Cyanide, however, is a general inhibitor of respiratory cycles whether they include the cytochrome system or not. To demonstrate that *Ascaris* eggs do have a cytochrome system, they were subjected to the light-reversal inhibition test of carbon monoxide by use of a Warburg respirometer adapted to this purpose. The eggs showed the same rate of oxygen consumption in air and in a 5% oxygen–95% nitrogen mixture. When CO replaced the nitrogen, however, there was an immediate and persistent drop in oxygen

consumption. Within a few minutes this leveled off at approximately 40% of normal consumption. In the presence of light this value rose to 75% of normal consumption.

Discussion

Numerous studies on the effect of anaerobiosis and other factors *during* irradiation are not comparable to the present investigation, since the present study utilizes anaerobiosis *after* irradiation and is therefore concerned with recovery processes. Although a number of post-irradiation treatments have delayed the expression of injury or decreased its rate of development, most of them have had no effect on the final outcome. In work with mice, Bacq *et al.* (1950) found that NaCN given immediately after irradiation only delayed mortality, and they concluded that cyanide was ineffective when given after irradiation. Bachofer (1956) has shown that post-irradiation anaerobiosis of x-irradiated *Ascaris* eggs restores in part the normal rate of pronuclear fusion, which is slowed down considerably by x-irradiation; the restoration is a genuine recovery and is attributable to the period of anaerobiosis.

The problem proposed by Lea (1947), as to whether the recovery of irradiated invertebrate eggs demands oxygen uptake or whether this uptake is a mere concomitant action, has been solved for *Ascaris* eggs. The facultative anaerobic nature of *Ascaris* eggs makes possible a complete elimination of free oxygen during incubation at optimum temperature. When x-irradiated eggs were subjected to post-irradiation anaerobiosis, their power of recovery surpassed that of x-irradiated aerated eggs as shown by decreased cleavage time and by higher survival (Tables I–V). It has been established, therefore, that oxygen is not necessary for recovery in this case.

A possible mechanism to be considered is whether this recovery could be attributed to the concomitant delay in cleavage brought about by anaerobic conditions. In studies concerned with cell cleavage, Schjeide and Allen (1951) found that tadpole hematopoietic cells appear to be susceptible to x-rays in direct proportion to the amount of cell division allowed to proceed following the irradiation period. Recovery of irradiated *Arbacia* eggs (Henshaw, 1940) was obtained only if they were kept unfertilized; as the time between irradiation and fertilization was shortened, the recovery was likewise decreased. In unirradiated *Arbacia* eggs cleavage begins at optimal temperatures within an hour after fertilization. Cytological observations (Bachofer, 1956) show that all eggs of *Ascaris lumbricoides* removed from the terminal 25 mm. of the uteri, in which the sperm has entered the egg, are in the pronuclear stage. Upon incubation at optimal temperature, pronuclear fusion begins slowly and precedes first cleavage by approximately 1½ hours. The eggs begin first cleavage only after 25 to 30 hours of incubation at optimal temperature, and achieve 50% cleavage after 40 hours. Since anaerobiosis had to be initiated before 15 hours had elapsed after irradiation in order to secure recovery, and anaerobic recovery was reduced if the treatment was delayed even a few hours following irradiation, it appears that the recovery process in question is not directly associated with the delay of first cleavage. Furthermore, if delaying the time of cleavage were the important factor in recovery, it would be expected that the survival of the irradiated eggs which were de-oxygenated and placed at the various sub-optimal temperatures would have remained at the same peak as those placed at 30° C. The progressive decrease in survival of both the aerated and anaerobically incubated eggs kept for

increasing lengths of time at temperatures lower than optimal (Table I) indicates that some factor other than cleavage delay is responsible.

Further evidence that cleavage delay is not the contributing condition for recovery is shown by the fact that the anaerobic incubation facilitates recovery only during the first 15 hours of this treatment (Table II). Likewise, eggs which have been allowed to incubate aerobically for 15 hours before being de-oxygenated give no evidence of recovery as indicated by cleavage delay (Table III) and survival (Table IV). During this 15 hours of aerobic incubation the eggs have not yet begun their first division. It appears that one must look to other conditions than delay of cleavage to explain the recovery.

It should be borne in mind that cellular activity, including cell cleavage, is necessary in most cases to demonstrate the injury, since the injury is latent. Postponement of cellular activity after irradiation may not involve recovery; once cellular activity is allowed to proceed the damage may be manifested. If the damage is as great as that which would have been manifested by permitting cellular activity to proceed immediately after irradiation, then there was no genuine recovery. True recovery was reported by Cook (1939) for survival of irradiated eggs of *Ascaris megalocephala* held at low temperatures after irradiation, but the opposite was found to be true for *Ascaris lumbricoides* (Bachofer and Pahl, 1955). Both studies agreed, however, in that post-irradiation treatment did not affect the time required for first cleavage. Pertinent to the present case, therefore, is the fact that forestalling cell cleavage and cellular activity after irradiation does not in itself produce genuine recovery.

The seat of the recovery from irradiation may involve various reactions of the respiratory cycle. The increased survival of irradiated eggs which have been subjected to cyanide or to anaerobiosis after irradiation suggests that the effects of irradiation operate to some extent through the cytochrome system, since both cyanide and anaerobiosis inhibit the cytochrome system. The mechanism of protection afforded by respiratory inhibitors may be either the prevention of the products of irradiation from reacting with the cytochromes or the prevention of the radiation-affected cytochromes from participating in the chain of reactions that normally bring about the observed effects of irradiation. Insofar as the cytochromes may be involved, the second possibility appears more pertinent in the present study, since the anaerobic condition would be too late, in time, to prevent a highly activated radiation product from reacting with the cytochromes, but it could prevent the affected cytochromes from reacting further.

There is, however, a function more important than holding the cytochromes in abeyance (Bachofer, 1956). It appears that checking aerobic metabolism permits anaerobic metabolism to restore essential molecules needed for normal development. The fact that recovery was greater for eggs held anaerobically at 30° C. than at sub-optimal temperatures indicates that anaerobic metabolism is associated with the recovery under consideration.

Summary

1. X-irradiation of *Ascaris lumbricoides suum* eggs produced delay of cell cleavage and reduced the percentage of eggs that completed embryogenesis. The time required for cleavage of irradiated eggs was reduced by an anaerobic treatment after

irradiation. The percentage of eggs that completed embryogenesis was increased by the same post-irradiation anaerobiosis. After the anaerobic treatment, eggs must be incubated aerobically since there is no perceptible development under anaerobiosis, although recovery takes place during this period. This recovery is greater at 30° C. than at sub-optimal temperatures.

2. Maximum recovery was obtained for eggs placed immediately after irradiation under anaerobiosis for periods of approximately 15 hours or more at 30° C. If the anaerobic treatment is delayed for 15 hours, the recovery is negligible.

3. Post-irradiation treatment with cyanide also fostered recovery from x-irradiation comparable to that secured with anaerobiosis.

4. Recovery was not due to delay of cleavage: the critical period for recovery took place long before cell division occurred even in air-saturated non-irradiated controls.

5. A cytochrome system in the eggs was demonstrated. The effects of cyanide treatment and anaerobiosis suggest that the mechanism of recovery may involve inhibition of the cytochrome system, which is prevented from participation in the reactions producing the expected deleterious effects of irradiation. There is, however, a positive contribution attributable to anaerobic metabolism, since recovery is greatest at optimal temperatures under anaerobiosis.

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RADIOCOBALT ACCUMULATION IN TETRAHYMENA

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Previous studies have indicated that cobalt is essential for growth in *Tetrahymena* (Slater, 1952; Roth, 1956), but there is no information available about cation uptake in this animal. Although the major function of cobalt is believed to be that of serving as part of the vitamin B_{12} molecule (Marston, 1952), cobalt is also implicated as the element *per se* in important hydrolytic reactions (Johnson and Berger, 1942).

The present experiments were designed to study the accumulation of cobalt in protozoans during growth. Elucidation of some of the factors regulating cobalt transport between the medium and the organism was also attempted. This study included the growth phase, the influence of deficient medium on exchange, the influence of population density on uptake per animal, and the effect of ion concentration on uptake.

Materials and Methods

Strain E of *Tetrahymena pyriformis* was used in this investigation, and all the experiments were performed in synthetic medium (Slater, 1952). Calcium, uracil, and adenylic acid were omitted from the media in all instances and cytidylic and guanylic acids were reduced to 10 μgm./ml. levels. In some experiments, growth effects were eliminated by use of media deficient in essential growth factors.

Cobalt-60 was used as a tracer and adjusted to 0.1 μc./ml. (final concentration) except where indicated. Cultures were grown in 10 ml. of synthetic medium in 18-mm. Pyrex tubes, and growth was measured turbidimetrically with a Lumetron (Model 400) colorimeter equipped with a red (650 mμ) filter. Radiations were detected with a deep-well scintillation detector and a Nuclear Instrument and Chemical Corporation Scaler (No. 162). Constriction chamber centrifuge tubes enabled clear separations of organisms from supernatant upon mild (100 G, one minute) centrifugation. The culture was washed with non-radioactive synthetic medium to remove excess fluid. The histidine in this medium is known to form a strong complex with cobalt (Burk *et al.*, 1946).

Prior to the introduction of Co^{60}, no cobalt was detected in the synthetic medium by ultraviolet emission spectroscopy, the porous cup technique being used. One

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liter of synthetic medium was concentrated 500-fold by evaporation for this analysis, and it was estimated that less than 0.01 μgm./ml. of cobalt ion was present.

Radiation effects from the tracer used have, in many instances, been known to influence physiological processes. The extreme resistance of *Tetrahymena* to radiation (Elliott and Slater, 1951), however, makes it unlikely that any influence from the tracer's radiation was significant during these experiments.

After the protozoans were separated from the supernatant by centrifugation, they were placed in two-ml. volumetric tubes and adjusted by micropipettes to 2.0-ml. volumes with distilled water. They were then transferred quantitatively to

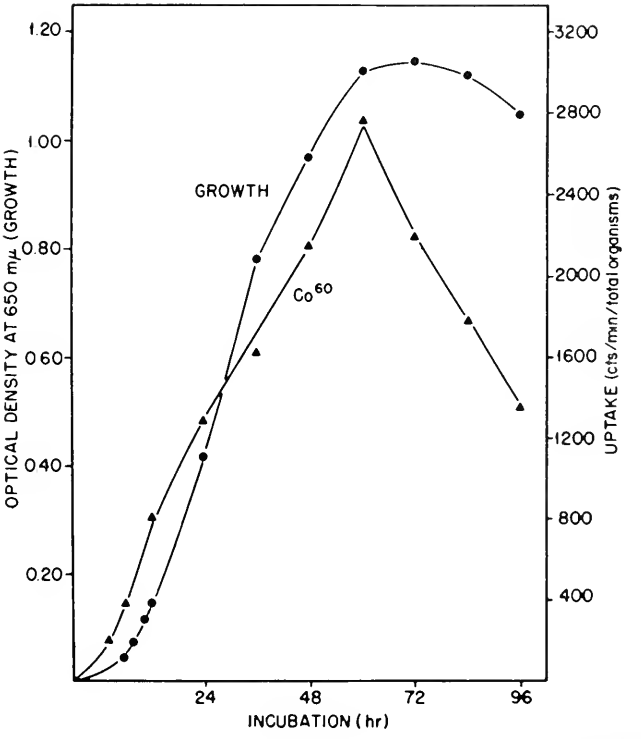

FIGURE 1. Cobalt-60 uptake and release during growth in *Tetrahymena*.

plastic tubes. Since the volume of the column of radioactive substance materially affected the number of counts registered by the scintillation detector, exactly 2.0-ml. volume adjustments were used throughout.

RESULTS

1. Uptake during growth

In the first series of experiments, Co^{60} at 0.01 μc./ml. (final concentration) was introduced into each culture at the beginning of the experiment as a tracer for the movement of this element during growth. Spectrographic analysis revealed that the

added cobalt amounted to 3.7 μgm./ml. (final concentration). No growth effects were noticed from cobalt at this concentration in preliminary experiments.

There was a steady uptake of cobalt during growth and an abrupt release of this ion shortly after the stationary phase was reached (Fig. 1). The temperature in the typical experiment reported was 27.5° ± 0.5° C., and the initial inoculum from mid-log phase cultures amounted to 185,000 ± 5% animals per tube. The total uptake during 60 hours of growth amounted to 0.97 μgm. of Co per total mass of cells, or about 26% of the cobalt present. This amounted to 4.6×10^{-8}% of the total cobalt present per hour per organism. Further calculations revealed that each *Tetrahymena* at 60 hours possessed about 10^9 atoms of cobalt. The number of animals remained constant from the sixth to the twelfth hour after inoculation (Table I) although the optical density measurements increased steadily. Uptake of cobalt during this period was probably associated with the increase in volume of the individual

TABLE I

Cobalt-60 uptake during growth for the first 12 hours

Time (hr.)	Optical density at 650 mμ	Number of animals	Uptake for total population (cts./min.)
0		185,000	
¼			77
2			208
4			252
6	0.04	479,000	381
8	0.07	459,000	489
10	0.11	457,000	743
12	0.14	466,000	806

organism. The volume of *Tetrahymena* reaches a maximum near the upper third of the log phase and falls off to about one-half this value upon reaching the stationary phase (Slater and Elliott, 1951).

The release of Co^{60} during the stationary phase was studied for only two days to avoid the possibility of measuring cobalt release from disintegrating cells. Microscopic observation of the protozoans during this period failed to reveal any obvious morphological breakdown, although an imperceptible physiological breakdown is certainly not an impossibility. Fifty per cent of the accumulated cobalt was released into the medium in 36 hours with a rate amounting to 1.4×10^{-6}%/hour/organism.

2. Effect of number of animals on uptake

The influence of number of animals on uptake per animal was studied. Eight-hour periods of time were selected to minimize growth effects and any influence from the accumulation of metabolic wastes. The number of animals present had a definite effect on uptake (Fig. 2) per animal. Populations of the order of 10^4 animals became nearly ten times as radioactive as populations of 2×10^6 organisms. At these high population densities it is not improbable that there was a great deal of competition for oxygen and also that harmful metabolic wastes resulted in inhibitory effects. Population densities of 2×10^5 cells, however, were far from being crowded under the experimental conditions and yet contained only one-third the ac-

tivity of the lowest concentration. In the experiment shown, the temperature was maintained at 26.5° ± 0.5° C., and cobalt was introduced at the 0.01 μc./ml. level.

3. Cobalt release in deficient medium

Release of cobalt was studied in media deficient in essential growth factors, salts, and glucose. Log-phase cultures were allowed to incubate initially in complete synthetic medium containing 0.01 μc./ml. of Co^{60} for 24 hours. The animals were

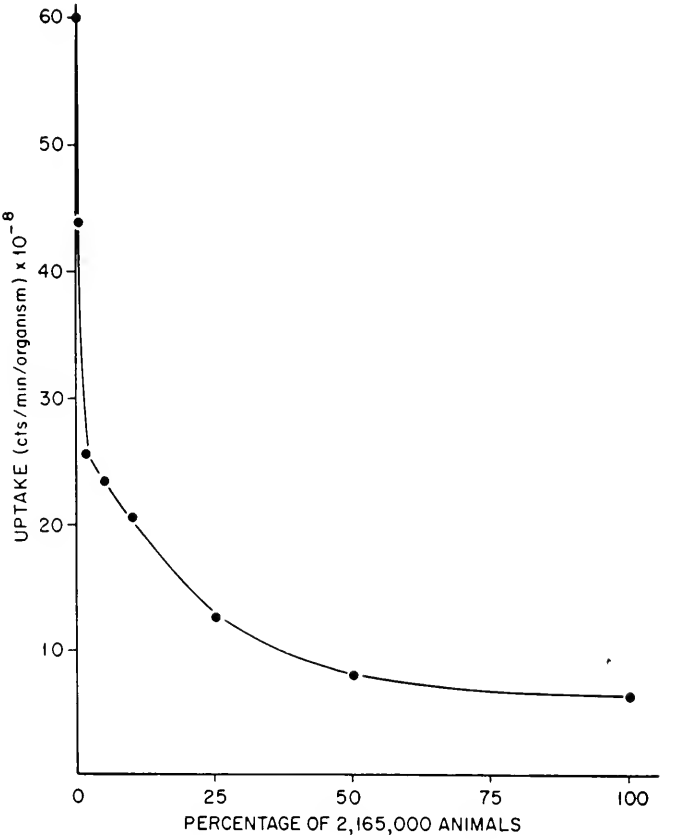

FIGURE 2. Influence of number of animals on uptake per animal.

then removed by centrifugation, washed once with deficient medium, and resuspended in deficient medium. This medium was used to prevent growth effects. In the typical experiment illustrated (Fig. 3) the initial population amounted to 415,000 cells/tube. These had grown to about twice this number at the time of introduction to the "cold" medium.

Two mechanisms are evident during cobalt release under these conditions. The first is very rapid and takes place within two hours. The rate of release during this time was 13%/hour/organism. The second mechanism is much slower and

amounts to 1.7%/hour/organism. Under these conditions, 50% of the cobalt was released in about 20 hours.

4. Cobalt concentration ability

The ability of protoplasm to concentrate certain elements has been known for a long time. *Tetrahymena* is no exception in this capacity. In the first series of experiments, uptake in relation to cobalt concentration was studied during 12 hours.

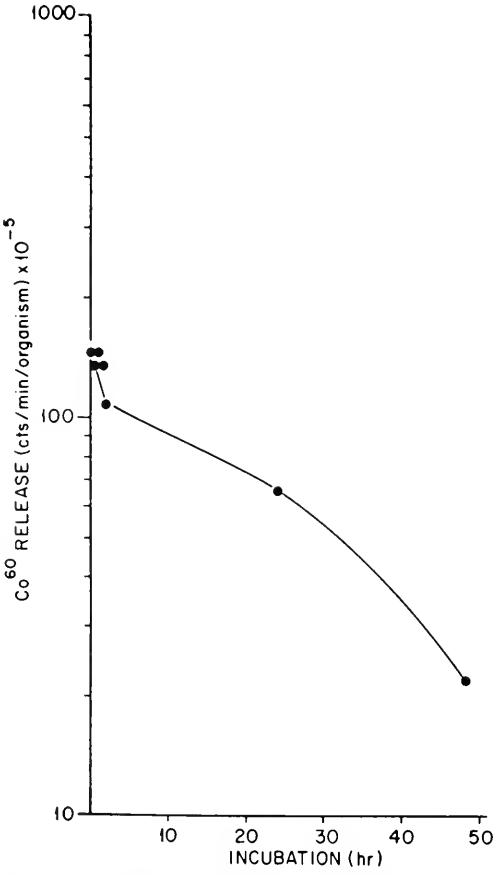

FIGURE 3. Release of cobalt-60 in deficient medium.

Complete synthetic medium was used in all instances, and the relatively short time interval was used to minimize growth effects. The concentrations used varied from 0.0005 μc./ml. of Co^{60} (3×10^{-6} *M*) to 0.0100 μc./ml. of Co^{60} (6×10^{-5} *M*). Uptake during 12 hours' incubation was found to be directly proportional to concentration (Fig. 4). The animals concentrated cobalt to the extent of 4.4–5.7 times that present in similar volumes of the environment regardless of the amount of isotope present (Table II). The volumes of individual animals used in these calculations are adapted from data presented earlier (Slater, 1951).

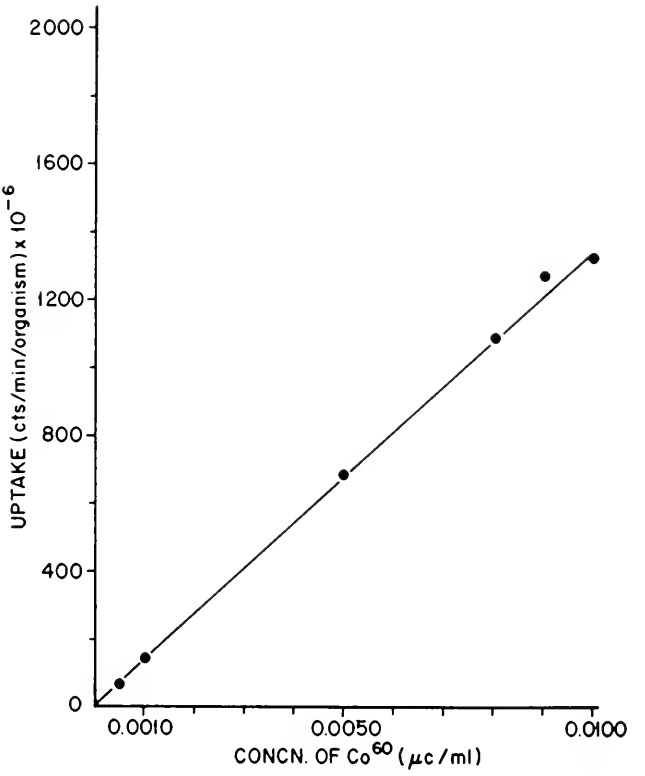

FIGURE 4. Effect of cobalt-60 concentration on 12-hour uptake.

TABLE II

Influence of cobalt concentration on uptake in 12 hours

Conc. of Co^{60} (μc./ml.)	Co^{++} (μgm./ml.)	Cts./min./total population	Cts./min./vol. occupied by population*	Degree of conc.** cts./min./total pop. / cts./min./vol. occup.
0.0100	3.70	1104	221	4.9
0.0098	3.63	942	216	4.4
0.0096	3.55	1211	212	5.7
0.0094	3.48	1126	207	5.4
0.0092	3.40	1169	203	5.7
0.0090	3.33	1062	198	5.4
0.0080	2.96	910	176	5.2
0.0050	1.85	573	110	5.1
0.0010	0.37	117	22	5.3
0.0005	0.185	58	11	5.3

* Calculated from μc./ml. times cts./min./μc. times volume occupied by protozoans after 12 hours growth. One ml. containing 0.01 μc. of Co^{60} gives 10,500 cts./min. The volume occupied by these populations equaled 0.0212 ml. after 12 hours growth.

** Representing the concentration of cobalt by the entire population of animals over that contained in comparable volumes.

TABLE III

Influence of cobalt concentration on maximum uptake

Conc. of Co^{60} (μc./ml.)	Co^{++} (μgm./ml.)	Max. uptake cts./min./total population	Cts./min./vol. occupied by population*	Degree of conc. cts./min./total pop. / cts./min./vol. occup.
0.01	0.764	2064	265	7.8
0.005	0.508	1373	132	10.4
0.001	0.148	399	27	14.8
0.0005	0.061	165	13	12.7
0.0002	0.025	68	5	13.6

pH, 7.4; temperature, 25.7° ± 0.5° C.

* Calculated from μc./ml. times cts./min./μc. times volume occupied by protozoans at maximum uptake. One ml. containing 0.01 μc. of Co^{60} gives 10,500 cts./min. The volume occupied by these populations equaled 0.0253 ml. of maximum uptake (Slater, 1951).

The influence of cobalt concentration on maximum uptake during growth was also studied. The greatest absolute amount of this ion was taken up by cultures in the presence of 0.764 μgm./ml. of Co^{++} (Table III). The ability to concentrate this ion over that contained in the medium, though, reached maximum at 0.1–0.5

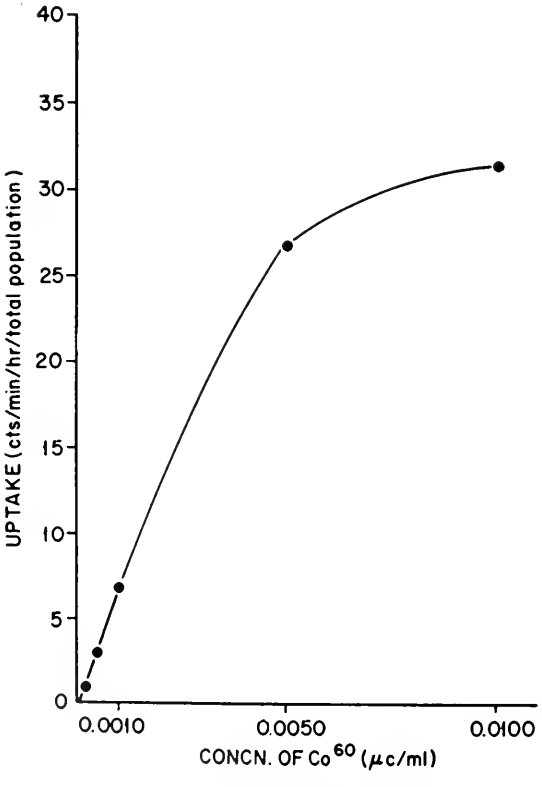

FIGURE 5. Uptake of cobalt-60 with concentration at 36 hours.

μgm./ml. of Co^{++}. The maximum uptake was about one and three quarters times that with 0.764 μgm./ml. of Co^{++}. Correspondingly, the rate of uptake seemed to be linear with concentrations up to 0.508 μgm./ml. of Co^{++} (Fig. 5) but was less at higher concentrations.

Discussion

The uptake of cations by animal cells involves a complex spectrum of interrelated processes, any one of which may alter the total cellular absorptive capacity. Among these controlling factors are the rate of utilization and probably intracellular translocation. Probably the uptake of inorganic substances first involves a combination with organic cell constituents (Sutcliff, 1954) ; specific metabolic pumps may also be involved. These cations then may become distributed to different cellular sites in response to varying chelation forces, which appear as the environmental mixture of cations changes. Thus during periods of major synthesis and growth, forces primarily concerned with cell divisions and external membrane changes might be most active, while during periods of relative inactivity, forces concerned with exchange, turnover, and simple release might well become more active. Chelation forces may also be involved in mitochondrial physiology and are probably involved in ion transport in these particulates. Studies of the relative binding abilities of various sites within the cell must certainly be done if the intricacies of cation transport and translocation are to be elucidated.

In *Tetrahymena*, it was shown that the population density may greatly influence the uptake of cobalt per cell. At great densities, cellular chelate forces may compete for environmental cations. It is also considered likely that competition for dissolved nutrients in general may inhibit the transport mechanism for any given cation.

It was demonstrated earlier that cobalt was essential for growth in purified synthetic medium without glucose. The physiological role of this cation in protozoans where an extraneous source of the B_{12} molecule is not required is not clear. It has been suggested that cobalt ion in these experiments may act non-specifically to increase the availability of other cations by releasing them or displacing them from complexes (Ford and Hutner, 1955, pp. 101–136) at the cell surface (Hutner *et al.*, 1950). Since Roth (1956) has shown that nickel does not have the same effect as cobalt on growth in *Tetrahymena*, and since the present experiments have demonstrated that cobalt is differentially bound to the animal, depending on the concentration of the cation, any non-specific effect may be minimal.

The uptake of cobalt during growth has been studied in *Bacillus subtilis* (Tanaka *et al.*, 1952), *Neurospora crassa* (Ballentine and Stephens, 1951), and *Saccharomyces cerevisiae* (Nickerson and Zerahn, 1949). No studies involving protozoan uptake of this ion seem to have been published.

Sizable losses of cobalt were observed with advancing age of the population in all the above organisms. In both *Tetrahymena* and *B. subtilis*, the release of the ion began abruptly after the stationary phase began. A comparison of the concentrating abilities of *Saccharomyces* and *Tetrahymena* showed that *Saccharomyces* had nearly 70 times the ability to concentrate cobalt possessed by the protozoan. Two washes of *Tetrahymena* did not appreciably remove the ion, and over 20 hours' washing of *Saccharomyces* also showed that cobalt was firmly held. Nickerson and Zerahn (1949) suggested that the presence of peripherally located metaphosphate might be significant in the accumulation of "metals" from dilute solution by yeasts.

In *Neurospora* (Ballentine and Stephens, 1951), at least 40% of the cobalt accumulated was present in stable cobalto-proteins. Fractionation of these compounds revealed the presence of a soluble fraction comprising 57% of the stably bound cobalt and a nearly submicroscopic particulate fraction. Similar cobalto-proteins were also found in *Chlorella vulgaris* and in the leaves of the musk melon and tomato. As with *Neurospora, Saccharomyces,* and *B. subtilis, Tetrahymena* concentrated cobalt against a concentration gradient.

Scott and Ericson (1955) reported that cobalt was absorbed by the marine alga, *Rhodymenia palmata,* and became bound within the plant as a complex quite different from B_{12}. Analysis of this complex revealed the presence of several components. Thus cobalt may play a multiphysiological role in protoplasm. Ericson (1952) reported that sea weed possessed an unusual ability to absorb and concentrate Co^{60} but the absorption of vitamin B_{12} was very limited and could not account for the concentration of the cation. In earlier work on the essentiality of cobalt for growth in *Tetrahymena* (Slater, 1952), it was shown that a definite response could be obtained in purified synthetic medium when as little as 0.5 μgm./ml. of cobalt ion was present. Under the conditions of those experiments, this amount of cobalt was equivalent to about 5×10^{10} atoms of cobalt per animal. In the present experiments, nearly 10^9 atoms of cobalt were accumulated per animal, presumably for non-growth purposes since growth progressed in controls without added cobalt. Thus, in purified synthetic medium, when extraneous cations are removed to a large degree, nearly 50 times as much cobalt is used for growth as is accumulated when growth proceeds under the influence of other cations.

In a study on cobalt localization in pooled white mouse cells, Rosenfeld and Tobias (1951) reported that most of the element was present in the cytoplasm and about 1% of it was firmly bound to cellular protein. Very little was discovered in the nuclei. Most of the cytoplasmic association was with the globulin in the bound fraction. The slowly released fraction of cobalt in *Tetrahymena* (Fig. 3) may be associated with a firmly bound fraction of this type, but this fraction appears to be about 75% of the total. The intracellular particulate localization of cobalt remains to be elucidated.

Summary

1. A steady uptake of radioactive cobalt was observed during the growth of *Tetrahymena* in synthetic medium. When the initially-added amount of cobalt was 3.7 μgm./ml., nearly 26% of the total was accumulated during 60 hours of growth. This amounted to approximately one billion atoms of cobalt per animal.

2. Upon reaching the stationary phase, sizable amounts of cobalt were released from the population. Fifty per cent release was observed in 36 hours with a rate amounting to 1.4×10^{-6}%/hour/organism.

3. The number of animals present had a definite effect on the accumulation of cobalt per animal. Populations of 10^4 animals became nearly ten times as radioactive as populations containing two million organisms.

4. In nutritionally-deficient media, the release of cobalt was biphasic. The first was very rapid and took place within two hours. The initial rate of release of cobalt amounted to 13%/hour/organism. The second mechanism was much slower and amounted to 1.7%/hour/organism. Fifty per cent release was noticed in 20 hours under these conditions.

5. As the concentration of cobalt was varied, the rates of uptake increased rapidly. Concentrations higher than 1.85 μgm./ml. had little effect. The greatest absolute amount of cobalt was accumulated when 3.7 μgm./ml. was initially present in the medium, but the ability to concentrate this ion over that contained in the environment reached a peak at 0.37 μgm./ml. of cobalt ion.

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CONTRIBUTIONS TO SURVIVAL MADE BY BODY CELLS OF GENETICALLY DIFFERENTIATED STRAINS OF MICE FOLLOWING X-IRRADIATIONS[1]

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Our strains of inbred mice have inherited relatively stable differences in their sensitivities to absorbed radiant energy (Gowen, 1950; Gowen and Zelle, 1945; Gowen and Stadler, 1956; Grahn, 1954) from whole-body irradiation. These differences are related to fixed variations in the normal cell structures of the strains (Gowen, 1945; Gowen and Calhoun, 1943; Gowen, 1952; Weir, 1949). Progress in understanding the mechanisms of irradiation damage may be advanced if genetic differences in resistance are traced to the cells as a whole or to particular organ systems. Regional body irradiation affords a technique for localizing organs significant to radiation resistance. This technique along with surgical removal and/or shielding before organ exposure has been tried with some indications that given organs are significant to irradiation resistance. Examination of irradiation effects on genetic strains of mice having known ranges in resistance combined with partial body exposure without confounding by surgical interference offers a promising means of attacking this problem.

Materials and Methods

The host constitutions in these experiments were differentiated into 5 distinct lines through 30 or more generations of brother-by-sister matings accompanied by selection for specific inherited types. The strains are homozygous albino but differ in coat color at the agouti locus. They are differentiated for resistance to *Salmonella typhimurium*, the typhoid-causing bacteria of mice. Under comparable conditions over a period of more than 20 years the mice of these strains maintain their relative resistances to 200,000 organisms to that observed in these experiments, S 100%, Z 45%, K 39%, Q 0% and Balb/Gw, hereafter called Ba, 0%. The strains differ genetically in body weight, growth rate, heart, kidney, liver, spleen and testis weights, serum globulins, leucocyte number, fixed phagocytic cells, macrophages, and cells metabolizing fat and storing glycogen as well as other significant physiological characteristics. The environment of the animals throughout the experimental period, as well as through many breeding generations, was that of a controlled laboratory where feed, water and management were uniform.

All mice were 46 ± 3 days of age at the time of irradiation. Strains and sexes were randomly distributed across the different treatments. Thirty-seven weeks

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were required to completely fill the experiment as designed. A General Electric Maxitron operated at 250 pkv, 30 ma with 0.25 mm. Cu + 1 mm. Al filtration at a distance of 47.5 cm. from anode to mid-mouse was used for the x-irradiation of all mice. The dose rate varied from 160 r/minute to 180 r/minute. Part of this range was accounted for by a change of x-ray tubes. Mice were held for x-irradiation in perforated plastic tubes with cork stoppers. These tubes were arranged in a wooden rack in two rows of eight tubes placed side by side with the closed ends of each row facing each other. The field of radiation covered by this rack of tubes permitted a range of only 12 r per minute over the entire area.

The experiment performed and analyzed was designed as a factorial having four elements. Five inbred strains of mice having known differences in x-ray and typhoid sensitivities were utilized in comparable numbers. The numbers for the two sexes were balanced for each strain. There were four x-ray exposure doses: 0, 320, 480 and 640 roentgens. The levels of x-ray dosage were chosen to span the range from no effect to nearly complete lethality when the mice were exposed to whole-body irradiation.

There were eight combinations of body coverage or exposure. The body of the mouse was divided into three regions—head, mid and rear. Shielding was with $\frac{1}{8}$ inch-thick strips of lead laid across the tubes covering the region or regions of the mice for a given treatment. The eight combinations of regions exposed are shown below.

Region Exposed

None
Head
Mid
Rear
Head-Mid
Head-Rear
Mid-Rear
Whole-Body

The head region or anterior third of the body extended into the thorax. The middle third of the body, mid region, included that part of the body containing lower thorax, and abdominal cavity containing stomach and upper intestinal tract, liver, spleen, adrenals, ovaries and kidneys. The posterior third of the body, rear region, included the lower intestinal tract, bladder, and urinary system and testes of the males. These regions are illustrated in Plate 1.

The eight groups of different regional exposures were treated with 320 r, 480 r and 640 r making a total of 24 different x-ray treatment groups. In addition the mice of one group were put in tubes and completely lead-covered for a time comparable to that of the longest dose, 640 r, but were not exposed. This group acted as a control on handling as well as for un-irradiated, 0 r group.

There were 25 treatment groups with 5 strains and 2 sexes making up 250 cells in the experiment. Each cell represented a different strain, sex, and treatment. A minimum of 25 mice were treated in each cell. Some cells contained a few extra animals. The completed experiment involved a total of 6904 mice.

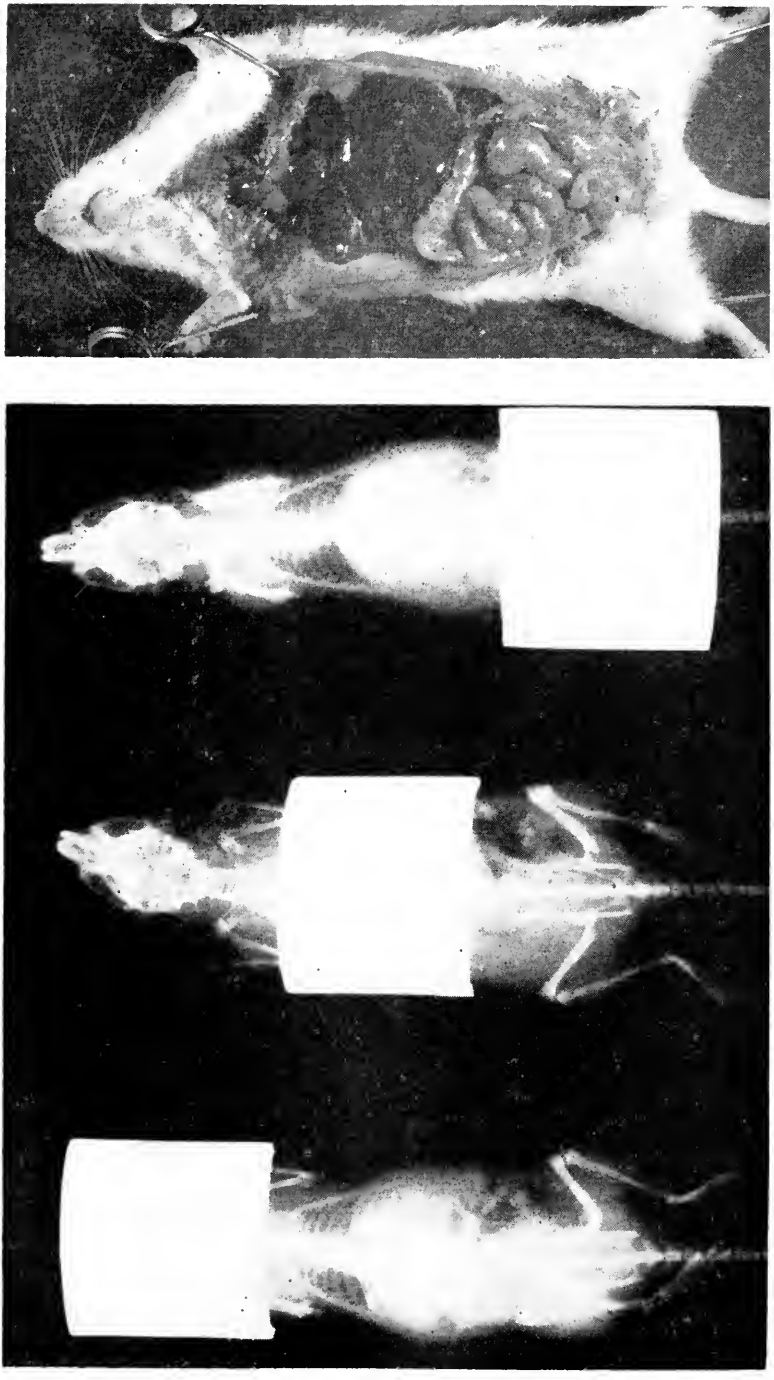

PLATE 1. Radiographs showing shielding marking off the three regions and their relation to the anatomy of the mouse.

The immediate effects of x-ray were largely completed 12 days following irradiation. Although there were marked differences in survival times between the different strains, few deaths occurred after the twelfth day. A 15-day interval following irradiation was allowed to cover the direct effects of exposure. Deaths were recorded daily and survivors of the fifteenth day were considered as surviving mice.

The $\frac{1}{8}$-inch lead sheets used to delimit the body regions exposed to irradiation protected the shielded regions from the greater portion of irradiation. But this protection was not complete. The $\frac{1}{8}$-inch lead was sufficient to absorb all but 0.75 r per minute. The back scattering of the radiation into the shielded regions accounted for larger doses of radiant energy in the protected regions. Measurements made when the tubes were placed on a nylon mesh screen under the same conditions showed that the back scattering largely came from the wooden rack used to hold the plastic tubes containing the mice. Only 7.4 r or 1.2 per cent of the 640 r dose was absorbed in the lead-covered mid-region when head and rear regions were exposed, whereas 78 r or 12.2 per cent of the x-rays was absorbed when the wooden rack was used. The amount of radiation absorbed in the different shielded regions varied from 3 to 12.2 per cent of the dose given. The region receiving most scattered radiation was the mid-region when the head and rear regions were exposed. This could be expected as the scattering could enter from two directions. The amount of radiation absorbed in any shielded region should tend to magnify somewhat the effects of the different treatments and reduce the differences between the treatments. The effects of scattered radiation were distributed through all treatment groups and tended to balance from one treatment to another. That this amount of radiation absorbed into the protected regions could lessen in some degree the recovery potentials of the mice treated in the region is recognized. The total roentgens scattered and absorbed should make this factor a minor contributor to the total variation.

The results of localizing radiation to particular regions of the body have been determined by comparison of sexes, strains and dosages across all regional exposure treatments in the factorially designed experiment. Two separate criteria have been used to measure the effects of irradiation, the percentage survival of the treated mice and the length of survival in days within the observational period.

Because of the unequal numbers of mice in the different treatment groups, all data have been analyzed throughout this paper by using disproportionate frequency analyses. Binomial analyses were used for the survival data and the customary methods for mean length of survival within the observational periods.

The data based on percentage survival did not allow for the full expression of the differences between strains, etc. Survival range had fixed limits at 0 and 100 per cent. These limits confined the quantitative estimates of the effects, *i.e.*, strain or radiation, to values within this range. Consequently all conclusions drawn from the percentage survival data led to minimal estimates by the nature of these limitations of the scale.

The data on length of survival were confined by one limiting measurement, the total length of the observation period. This fixed measurement again limited the full expression of the effects of irradiation on the mice. Any differences observed in both sets of data for each response were therefore minimal estimates of these consequences following the different treatments.

Experimental Results

The main purpose of this experiment was to examine the effects of irradiation to particular organs (body regions) and to their summation of effects on the cells of the body as a whole. Two variables are intrinsic in the data. Known genetic differences may assist in these analyses by introducing reliable differences in the resistance levels of the mouse strains. Sex of the mice utilized in the tests could affect the results although previous experience has shown that this is a minor factor in the expression of the mouse typhoid syndrome. The effects of sex and strain will be analyzed separately. The sex effects are considered first.

Effects of Sex

Irradiation may cause acute effects leading to death. In the range of x-ray exposures of this experiment these radiation deaths were largely over by 12 days post-irradiation. Consequences of the x-ray treatments were measured by percentages

Table I

Effect of sex on radiation sensitivity. Per cent survival in 15 days

Dose (r)	Region exposed	Mean per cent survived		Variance analyses			
				Within sex		Between sex 1 d.f.	
		Male	Female	d.f.	M.S.	M.S.	F
0	None	100	100	289	—	—	—
320	None	100	99	266	.004	.004	1.0
	Head	100	100	270	—	—	—
	Mid	100	99	267	.004	.004	1.0
	Rear	100	100	268	—	—	—
	Head-mid	99	100	270	.004	.004	1.0
	Head-rear	100	100	267	—	—	—
	Mid-rear	99	100	268	.007	.015	2.0
	Whole-body	97	95	271	.035	.019	0.5
480	None	98	100	257	.011	.034	3.0
	Head	100	100	258	—	—	—
	Mid	99	98	269	.018	.003	0.2
	Rear	100	100	265	—	—	—
	Head-mid	99	100	263	.004	.004	1.0
	Head-rear	100	100	269	—	—	—
	Mid-rear	99	100	276	.007	.015	2.1
	Whole-body	59	62	284	.241	.062	0.3
640	None	100	100	279	—	—	—
	Head	99	99	295	.010	.004	0.4
	Mid	97	97	286	.027	.000	—
	Rear	99	100	289	.003	.004	1.0
	Head-mid	92	95	284	.062	.092	1.5
	Head-rear	99	99	286	.010	.003	0.3
	Mid-rear	80	81	287	.159	.005	0.0
	Whole-body	7	3	271	.045	.090	2.0

TABLE II

Effect of sex on radiation sensitivity. Mean days survived in 15-day period

Dose (r)	Region exposed	Mean days survived		Variance analyses			
				Within sex		Between sex 1 d.f.	
		Male	Female	d.f.	M.S.	M.S.	F
0	None	15.0	15.0	289	—	—	—
320	None	15.0	15.0	266	0.06	0.06	1.0
	Head	15.0	15.0	270	—	—	—
	Mid	15.0	15.0	267	0.00	0.00	1.0
	Rear	15.0	15.0	268	—	—	—
	Head-mid	15.0	15.0	270	0.06	0.06	1.0
	Head-rear	15.0	15.0	267	—	—	—
	Mid-rear	14.9	15.0	268	0.15	0.30	2.0
	Whole-body	14.9	14.8	271	1.43	0.39	0.3
480	None	14.9	15.0	257	2.90	0.75	0.3
	Head	15.0	15.0	258	—	—	—
	Mid	14.9	14.8	269	1.20	1.02	0.9
	Rear	15.0	15.0	265	—	—	—
	Head-mid	15.0	15.0	263	0.14	0.13	1.0
	Head-rear	15.0	15.0	269	—	—	—
	Mid-rear	14.9	15.0	276	0.52	0.93	1.8
	Whole-body	13.6	13.8	284	5.02	1.91	0.4
640	None	15.0	15.0	279	—	—	—
	Head	14.9	14.9	295	0.38	0.06	0.2
	Mid	14.8	14.7	286	2.34	0.09	0.0
	Rear	14.9	15.0	289	0.28	0.28	1.0
	Head-mid	14.2	14.6	284	5.00	8.79	1.8
	Head-rear	15.0	15.0	286	0.06	0.03	0.5
	Mid-rear	13.2	13.3	287	14.18	0.42	0.0
	Whole-body	9.4	8.9	271	9.52	12.70	1.3

of the animals which survived for 15 days following exposure and by the average lengths of survival of the irradiated groups. The data are subdivided for each sex into the x-ray dose and the region of the body in which the mouse received the irradiation. The strains were nearly balanced in numbers and were combined in these tests.

Table I gives the data on the mice which survived the different x-ray treatments. The first column presents the x-ray dosage in air at the mid point of the mouse's body. Column 2 lists the body regions exposed to x-rays. Columns 3 and 4 give the mean survival as per cent of the mice surviving 15 days after the x-ray exposure according to the sex. The variance analyses give the degrees of freedom (d.f.) for the variances of the mice within sexes, the mean square (M.S.) between sexes with 1 d.f. and the F values for the mean squares. Throughout this paper * shows significance at the 0.05 level and ** at the 0.01 level.

Table I shows that only animals which received whole-body irradiation had appreciable mortality. In the 15-day period no mice died in the untreated group; the

320 r whole-body exposed mice showed a few scattered deaths, 3 and 5 per cent; the 480 r dose was more lethal, about 40 per cent of the mice died; and the 640 r dose was almost completely lethal, only 3 and 7 per cent survived of the 273 mice treated. Consideration of sex effects on radiation survival was consequently almost entirely limited to the 480 r and 640 r whole-body irradiation groups. The differences in responses of the sexes to irradiation were small throughout the 25 comparisons. In no case was a significant difference observed. The 480 r and 640 r whole-body exposures were severe enough to test any real biological changes in the physiologies of the sexes suffered through irradiation exposure. No sex differences appeared. In view of these facts the data for sexes have been combined in further consideration of these experiments on survival to x-radiation.

The severity of the x-ray effects may be measured by the number of days a mouse survives following x-ray exposure. Table II gives these data in the same form as Table I, with columns 3 and 4 showing the average number of days the males and females survived of the 15 days subsequent to x-irradiation. All treatment groups except the whole-body exposures at 320 r, 480 r and 640 r showed practically complete survival. The mean day survivals for whole-body exposures were 0 r—15 days, 320 r—14.9 days, 480 r—13.7 days and 640 r—9.1 days. The mean length of survival of the mice to whole-body irradiation was but slightly reduced by 320 r, was lowered by 480 r and was severely reduced by 640 r. The tests for sex effects on length of survival were only critical for the 480 r and 640 r whole-body irradiation groups. The sexes showed comparable mean days of survival. In no case was there a significant difference between sexes.

These results further support the conclusion that the sexes in mice are equally affected by x-irradiation. The conclusions on x-ray effects which are derived from these data will not be altered by combining the observations of the two sexes.

These tests also furnish a measure of the degree of protection afforded the animals by the coverage with lead plates. Animals of 0 r group were not exposed to x-rays but the bodies of the mice were completely shielded by ⅛-inch lead plates. The unexposed and 640 r groups had 100 per cent survival. In the 320 r treatment group exposed and lead-shielded, one female out of 268 animals died; the 480 r group had three males out of 259 mice die. Mortality at the three dose levels appeared fortuitous and unrelated to the irradiation. It was concluded that the protection with the lead shields was adequate for all groups.

Genetic Effects of Strains

The mice utilized in this experiment were known to exhibit differences in their abilities to withstand radiation. These differences have become isolated into different strains. The data on the genetic effects on resistance to radiation as evidenced by different strains within a treatment are presented in Table III.

Five treatments displayed strain effects on x-ray sensitivity. These exposures were: whole-body at 320 r and 480 r and mid, head-mid and mid-rear regions at 640 r. Examination of the mean strain survivals indicates that these differences came largely from the high susceptibility of the Ba strain. All strains were affected by the whole-body exposures, and the severity increased with dose. At 640 r the effects were so severe as to have overreached strain differences as judged by the F test, even though the strains still retained their relative order of resistance.

TABLE III

Genetic effects of strain differences on radiation sensitivity. Per cent survived 15 days post-irradiation

Dose (r)	Region exposed	Strains Per cent survival					Variance analyses			
							Within strains		Between strains 4 d.f.	
		S	Z	K	Q	Ba	d.f.	M.S.	M.S.	F
0	None	100	100	100	100	100	286	—	—	—
320	None	100	98	100	100	100	263	.004	0.004	1.1
	Head	100	100	100	100	100	267	—	—	—
	Mid	100	100	100	100	98	264	.004	0.004	1.0
	Rear	100	100	100	100	100	265	—	—	—
	Head-mid	100	100	98	100	100	267	.004	0.004	1.0
	Head-rear	100	100	100	100	100	264	—	—	—
	Mid-rear	100	100	100	100	97	265	.007	0.014	1.9
	Whole-body	98	100	94	100	90	268	.034	0.107	3.2*
480	None	100	100	96	100	98	254	.011	0.015	1.3
	Head	100	100	100	100	100	255	—	—	—
	Mid	98	100	98	98	97	266	.018	0.008	0.5
	Rear	100	100	100	100	100	262	—	—	—
	Head-mid	100	98	100	100	100	260	.004	0.004	1.0
	Head-rear	100	100	100	100	100	266	—	—	—
	Mid-rear	100	100	100	100	97	273	.007	0.013	1.9
	Whole-body	79	75	54	66	31	281	.213	2.116	9.9**
640	None	100	100	100	100	100	276	—	—	—
	Head	98	100	100	98	98	292	.010	0.005	0.5
	Mid	100	100	95	100	92	283	.026	0.082	3.1*
	Rear	100	100	100	100	98	286	.003	0.003	1.0
	Head-mid	98	98	96	100	75	281	.054	0.633	11.7**
	Head-rear	100	100	98	98	98	283	.010	0.006	0.5
	Mid-rear	92	96	71	98	49	284	.124	2.624	21.1**
	Whole-body	11	4	4	6	0	268	.045	0.085	1.9

Strain differences were separated most clearly when the animals were exposed to the 640 r. These strain differences appeared in all treatments. The 480 r treated mice showed the strain effects only in the whole-body irradiated class. The 320 r treated groups were at the border line where a reduction in survival was only beginning to appear in the whole-body treated mice of the most susceptible strain. The whole-body 480 r irradiations ordered the strains for resistance; the S was most resistant followed in order by the Z, Q, K and the most susceptible Ba.

In the 640 r dose range S, Z and Q strains were not very different in their responses. The Ba strain again showed the greatest radiation sensitivity with the K strain somewhat less sensitive than the Ba and noticeably less resistant than the other three strains.

The mean days of survival for the 15-day interval following irradiation and the analyses of the strain differences are presented in Table IV.

Table IV confirms the major features of Table III. Strain differences in resistance to irradiation were brought out in the 480 r whole-body treatment and with 640 r exposure when the mid, head-mid, mid-rear and whole body were treated. The significance tests identify a difference in radiation effects which made the two measures, per cent survival and length of survival, desirable. The 320 r whole-body exposure was sufficient to make the strain differences in per cent survival significant, whereas the mean length of survival did not show such differences. The mice that died, died late in the 15-day observation period. Following 640 r whole-body exposure, the mice showed only an insignificant difference in strain survivals even though all survival values were markedly reduced, whereas in length of survival the strain differences were accentuated to give a highly significant F value. The other three treatments, the mid, head-mid and mid-rear regions, suggest the mid region as the most sensitive to radiation damage.

TABLE IV

Genetic effects of strain differences on radiation sensitivity. Mean days survived in 15 days

Dose (r)	Region exposed	Strains Mean days survived					Variance analyses			
							Within strains		Between strains	
		S	Z	K	Q	Ba	d.f.	M.S.	M.S.	F
0	None	15.0	15.0	15.0	15.0	15.0	286	—	—	—
320	None	15.0	14.9	15.0	15.0	15.0	263	0.06	0.07	1.1
	Head	15.0	15.0	15.0	15.0	15.0	267	—	—	—
	Mid	15.0	15.0	15.0	15.0	15.0	264	0.00	0.00	1.0
	Rear	15.0	15.0	15.0	15.0	15.0	265	—	—	—
	Head-mid	15.0	15.0	14.9	15.0	15.0	267	0.06	0.06	1.0
	Head-rear	15.0	15.0	15.0	15.0	15.0	264	—	—	—
	Mid-rear	15.0	15.0	15.0	15.0	14.8	265	0.15	0.28	1.9
	Whole-body	14.8	15.0	14.9	15.0	14.5	268	1.41	2.30	1.6
480	None	15.0	15.0	14.8	15.0	15.0	254	0.29	0.52	1.8
	Head	15.0	15.0	15.0	15.0	15.0	255	—	—	—
	Mid	14.9	15.0	14.8	15.0	14.7	266	1.20	0.91	0.8
	Rear	15.0	15.0	15.0	15.0	15.0	262	—	—	—
	Head-mid	15.0	14.9	15.0	15.0	15.0	260	0.14	0.15	1.1
	Head-rear	15.0	15.0	15.0	15.0	15.0	266	—	—	—
	Mid-rear	15.0	15.0	15.0	15.0	14.7	273	0.52	0.84	1.6
	Whole-body	14.4	14.2	13.4	14.2	12.2	281	4.44	45.52	10.3**
640	None	15.0	15.0	15.0	15.0	15.0	276	—	—	—
	Head	14.9	15.0	15.0	14.9	14.9	292	0.38	0.20	0.5
	Mid	15.0	15.0	14.6	15.0	14.3	283	2.26	6.81	3.0*
	Rear	15.0	15.0	15.0	15.0	14.9	286	0.28	0.26	1.0
	Head-mid	14.9	14.8	14.8	15.0	12.8	281	4.32	53.74	12.4**
	Head-rear	15.0	15.0	15.0	15.0	15.0	283	0.06	0.03	0.5
	Mid-rear	14.4	14.7	12.4	14.8	10.2	284	11.01	235.54	21.4**
	Whole-body	10.6	10.4	8.8	10.4	5.4	268	5.72	265.42	46.4**

TABLE V

Effects of x-ray dosage to different body regions on survival 15 days after irradiation

Region exposed	Variance analyses							
	Doses 3 d.f.		Strains 4 d.f.		Dose×Strain 12 d.f.		Within dose and strain	
	M.S.	F	M.S.	F	M.S.	F	d.f.	M.S.
Percentage survival for 15 days post-irradiation								
None	No analysis—Practically all survived							
Head	No analysis—Practically all survived							
Mid	.048	3.9**	.044	3.6**	.017	1.4	1099	.012
Rear	No analysis—Practically all survived							
Head-mid	.291	18.6**	.156	10.0**	.162	10.3**	1094	.016
Head-rear	No analysis—Practically all survived							
Mid-rear	2.66	75.3**	.873	24.7**	.593	16.8**	1108	.035
Whole-body	54.00	734.9**	.869	11.8**	.480	6.5**	1104	.074
Mean days survived in 15 day post irradiation period								
None	No analysis—Practically all survived							
Head	No analysis—Practically all survived							
Mid	4.08	4.7**	3.27	3.7**	1.49	1.7	1099	.88
Rear	No analysis—Practically all survived							
Head-mid	22.12	19.1**	13.56	11.7**	13.46	11.6**	1094	1.16
Head-rear	No analysis—Practically all survived							
Mid-rear	220.15	73.7**	72.83	28.4**	54.61	18.3**	1108	2.99
Whole-body	2078.36	726.7**	131.31	45.9**	60.64	21.2**	1104	2.86

The mean days of survival of the five strains for the 25 different treatments showed somewhat less differentiation in the reactions of the strains to x-irradiation. The head-mid-rear exposure to 640 r showed the widest separation between Ba and K and these from the other three, S, Z, and Q strains. The order of the strains in resistance to radiation effects did not correspond to the order of these same strains in their natural resistance to mouse typhoid as noted earlier.

REGIONAL EFFECTS OF IRRADIATIONS AND GENOTYPES

Three elements were operating on the survival of the mice in this experiment, dosage of the x-rays, the region of the body exposed to x-rays and genotype as represented by strain of mice under treatment. Table V measures these effects first in terms of the mice surviving for 15 days following irradiation and second, the lower half of the table, in terms of mean length of survival within the 15-day period.

The data of Table V are presented for the body regions exposed, eight different categories in all. Of the eight groups four have not been analyzed as the deaths within these groups were few and scattered.

When the mid region only was exposed to x-rays, differences in dosage and in strains were evident and of equal significance. The dosage × strain interaction

was minor. Interaction between dosage × strain was a factor in survival when radiations were absorbed in the head-mid portion of the body. Both dosage and strain effects were large, with the dosage effects nearly double those of the strains. The interaction, however, was as large as the strain effects, indicating that the strains reacted differently to the different exposures.

X-rays to the rear two-thirds of the body gave even more noticeable effects. The effects of the dosages were markedly greater than the strain differences and the strain effects showed more than random deaths. The whole-body irradiations, those involving the head, mid and rear regions, were more severe than those of other treatments. The x-ray effects were so pronounced at the 640 r level (Table III) that they overshadowed the strain differences. The strain effects were clearer in the 480 r and 320 r whole-body treatment groups.

The data on length of survival within the 15-day interval following irradiation show essentially the same features as those for survival. Irradiation to the head-mid portion of the body was not as effective as that to the mid-rear portion, but both showed more change than when the x-rays were directed to the mid region alone. The mid region was sensitive but added irradiation to the rear or head regions increased the sensitivity. Irradiation to all regions leaves the body with no unexposed tissue. Under these conditions the survival values were materially reduced over those of all other types of treatment.

Quantitative Analyses of Regional Effects of X-ray Exposure

Quantitative estimates of the relative sensitivities of the different body regions were obtained by relating the survival values within the x-ray dosages for the different treatments. The basic theory was as follows.

A factor common to mice of each strain was assumed to represent the natural resistance of the strain. This factor was considered as alone responsible for the survival values attained by the unexposed control groups. It was common to mice of all groups before treatment and was in a sense the potential resistance of the strain against which the x-ray or other treatments operated to reduce viability. The factor was designated a. Irradiation to the head region contributed a factor, h, to extend or reduce life. Its value depended upon the dosage of radiation to the head but within any one dosage its effect was regarded as a constant. In the same manner irradiation effects to the mid region were regarded as due to a factor, m, and those to the rear region were considered due to a factor, r. When two regions were irradiated the effects of radiation were assumed to be additive, *i.e.*, $h + m$ for total effects of irradiation to the head-mid regions. Any unexposed cells within the body would contribute possibilities of continuing normal functions. Even a small fraction of the cells having normal functions might be of vital importance to the mouse. In consequence it was assumed that when the whole body was irradiated there were effects above and beyond those of $h + m + r$. These effects were represented by d. The full effects of whole-body irradiations were viewed as due to $h + m + r + d$. The d factor measured the importance of even a fraction of the body cells being normal in function. A system of eight equations was available for the analysis of these effects as expressed in the eight regional body treatments.

$$a = \text{value of control, unexposed mice}$$
$$a + h = \text{value of control} + \text{head exposed}$$
$$a + m = \text{value of control} + \text{mid exposed}$$
$$a + r = \text{value of control} + \text{rear exposed}$$
$$a + h + m = \text{value of control} + \text{head and mid exposed}$$
$$a + h + r = \text{value of control} + \text{head and rear exposed}$$
$$a + m + r = \text{value of control} + \text{mid and rear exposed}$$
$$a + h + m + r + d = \text{value of control} + \text{whole body exposed}$$

The values for a, h, m, r and d were obtained from the system of five simultaneous equations derived from the above by the method of least squares. These simultaneous equations are presented below. The P values in the equations were obtained as the sums of the observed values. Wherever a given factor entered into the basic equations it contributed to the corresponding P, *i.e.*, P_1 = the sum of the constants for all eight, P_2 = the sum of four equations where h entered, etc.

$$P_1 = 8\,a + 4\,h + 4\,m + 4\,r + 1\,d$$
$$P_2 = 4\,a + 4\,h + 2\,m + 2\,r + 1\,d$$
$$P_3 = 4\,a + 2\,h + 4\,m + 2\,r + 1\,d$$
$$P_4 = 4\,a + 2\,h + 2\,m + 4\,r + 1\,d$$
$$P_5 = 1\,a + 1\,h + 1\,m + 1\,r + 1\,d$$

The general solution for each of the constants was as follows:

$$a = 1/8\ (5\,P_1 - 3\,P_2 - 3\,P_3 - 3\,P_4 + 4\,P_5)$$
$$h = 1/8\ (-3\,P_1 + 5\,P_2 + 1\,P_3 + 1\,P_4 - 4\,P_5)$$
$$m = 1/8\ (-3\,P_1 + 1\,P_2 + 5\,P_3 + 1\,P_4 - 4\,P_5)$$
$$r = 1/8\ (-3\,P_1 + 1\,P_2 + 1\,P_3 + 5\,P_4 - 4\,P_5)$$
$$d = 1/2\ (1\,P_1 - 1\,P_2 - 1\,P_3 - 1\,P_4 + 4\,P_5)$$

The constants for the effects of irradiation, as measured by percentage survival, for different intensities to the different regions are shown in Table VI. The constants fitted the observations well as shown by the variations accounted for by the five factors as against the residual variation left after the fits were made.

As expected the severity of the effects of the x-rays to the different regions increased as dosage increased. The 320 r dose was not severe enough to separate the effects of exposure to particular body regions. The whole-body treatment even at this level of exposure showed the reduction in survival, d, to be greater than can be accounted for by the additive effects of $h + m + r$; d may be thought of as representing the recovery potential when any unexposed cells were present within the animal's body.

In the 480 r dose range the separation of body regions by irradiation effects was clearer. The values of m confirmed the greater sensitivity of the mid region in lowering the survival rates of the mice following x-irradiation. The head and

TABLE VI

Effects of x-irradiation to particular body regions on percentage survival of 5 different strains of mice

Constant	Dose (r)	Strains					Average
		S	Z	K	Q	Ba	
a	320	100	100	100.23	100	99.99	100.04
	480	99.55	100.25	99.54	99.53	99.54	99.68
	640	100.83	100.69	103.04	100.03	106.84	102.29
h	320	0	0	−0.68	0	0.88	0.04
	480	0.45	−0.75	0.46	0.47	1.29	0.38
	640	.01	−0.25	2.96	−0.92	−1.51	0.06
m	320	0	0	−0.68	0	−1.76	−0.49
	480	−0.45	−0.75	−0.46	−0.47	−2.13	−0.85
	640	−3.29	−2.07	−13.40	−0.04	−29.23	−9.61
r	320	0	0	0.23	0	−0.87	−0.13
	480	0.45	0.25	0.46	0.47	−0.37	0.25
	640	−2.47	−1.13	−9.91	−0.98	−14.63	−5.82
d	320	−1.75	0	−4.65	0	−8.41	−2.96
	480	−21.05	−24.00	−45.90	−34.48	−67.30	−38.55
	640	−84.37	−93.59	−78.91	−92.53	−61.46	−82.17

Variations in survival accounted for by the constants

Dose	d.f.	Mean squares				
		S	Z	K	Q	Ba
320 r	acc. 5†	15,935	16,000	15,711	16,000	15,404
	res. 3††	0	0	1.7	0	1.0
480 r	acc. 5	15,175	15,045	14,512	14,783	13,923
	res. 3	0.5	0.5	0.6	0.6	1.5
640 r	acc. 5	13,574	13,784	12,547	13,783	11,004
	res. 3	9.8	1.5	73.4	1.1	196.0

† acc. = Variation accounted for by the different regions.
†† res. = Residual or unaccounted for variation.

rear regions appeared of almost equal resistance in the strains. With the two exceptions of the *h* in the Z strain and *r* in the Ba strain, the *h* and *r* values for the other four strains showed a slightly stimulatory effect on viability. When the whole body was exposed to the 480 r x-rays the effects attributable to *d* were very severe. Since the effects as measured by the *h*, *m* and *r* values were not extreme and were quite consistent in the five strains, the order in magnitude of the *d* values for the five strains represented the levels of resistance of the strains to x-irradiation. The S mice were most resistant followed by the Z, Q, K and Ba in that order.

The effects of irradiation were more marked in all regions when the exposure

dose was 640 r. Except in the Q strain irradiation to the mid region, *m*, resulted in the severest reaction. The *r* values showed the rear region to be intermediate in resistance to irradiation. The head region as measured by *h* was least sensitive. The *d* values again portrayed the severity of exposure to x-rays in the absence of any unexposed cells or organs. The order of the strains in resistance to irradiation as observed from the *d* values with 480 r exposure and to a lesser degree with 320 r was not followed by the 640 r dose. As the *h*, *m* or *r* effects became greater the values of the *d* effects were decreased due to the limited range in which these constants operate. In the Ba strain survival from radiation had full expression from 0 per cent survival from whole-body exposure to 100 per cent survival for the unirradiated controls. The sensitivity of the mid region accounted for 29 per cent of the mortality. The sensitivity of the rear region accounted for 15 per cent more and that of the head region only 1.5 per cent. The *d* effect contributed 61 per cent additional mortality and was restricted by the 0 limit for survival.

The variation left unaccounted for after fitting the five constants was practically negligible when compared with that accounted for. The increased, but still minor, variation observed for the Ba strain at the 640 r exposure can best be attributed to the limitations of the scale for death and survival.

A like analysis of the data on length of survival (Table VII) added a little information to that already gained (Table VI). The values for *d* were more consistent with the innate resistance levels of the strains than were those based on percentage survival because the scale of measurement was not so restricted. However, at the lower x-ray doses the length of survival did not give more information than the percentage data because most mice lived the full 15 days. The mid region was again most susceptible to the x-rays with the rear next. Irradiations to the head showed little effect.

Discussion

The analyses of these data showed that sex had only inconsequential acute effects on the response of mice to irradiation. This observation agrees with that of Abrams (1951) for whole-body irradiation. Kaplan and Brown (1952) also found like reactions of the sexes in an experiment involving 1700 mice when sexes were equally distributed across several x-ray doses and fractions of the doses. Sex differences may be greater when life span effects are considered.

Comparisons of the mice without regard to strain show that the lethal effects of the whole-body x-rays increase with increase in kilovoltage, the 600 r at 250 pkv and 0.25 Cu + 1 Al filter being about as lethal as 960 r at 100 pkv Coolidge tube without filters (Gowen and Stadler, 1956).

Differences between the responses of the five strains of mice to x-irradiation were evident. Strain differentiation depended upon the x-ray dose. The more susceptible strains, Ba and K, reacted to the lower dose, 320 r, which had no effect on the survival of the S, Z and Q strains. As the exposure was increased to 480 r and 640 r the five strains were more clearly separated in their resistance levels. After exposure of the whole body to 480 r, 79 per cent of the S mice survived with a mean of 14.4 days. They were followed in order by Z, 75 per cent, 14.2 days; Q, 66 per cent, 14.2 days; K, 54 per cent, 13.4 days and Ba, 31 per cent with 12.2 days survival. The higher x-ray exposure of 640 r (250 kvp) delivered to the

TABLE VII

Effects of x-irradiation on body regions as measured by length of survival

Constant	Dose (r)	Strains					Average
		S	Z	K	Q	Ba	
a	320	15.00	15.00	15.01	15.00	15.00	15.00
	480	14.96	15.02	14.95	14.99	14.95	14.97
	640	15.06	15.06	15.24	15.01	15.64	15.20
h	320	0	0	−0.03	0.0	0.02	0.00
	480	0.03	−0.04	0.05	0.01	0.11	0.03
	640	0.01	−0.03	0.34	−0.01	−0.06	0.05
m	320	0	0	−0.03	0	−0.06	−0.02
	480	−0.03	−0.04	−0.05	−0.01	−0.18	−0.06
	640	−0.24	−0.18	−1.15	−0.06	−2.74	−0.87
r	320	0	0	0.01	0	−0.05	−0.01
	480	0.03	0.01	0.05	0.01	−0.02	0.02
	640	−0.19	−0.08	−0.84	−0.08	−1.36	−0.49
d	320	−0.21	0	−0.11	0	−0.41	−0.15
	480	−0.60	−0.73	−1.56	−0.83	−2.62	−1.27
	640	−4.03	−4.32	−4.76	−4.50	−6.04	−5.53

Variations in survival accounted for by the constants

Dose	d.f.	Mean squares				
		S	Z	K	Q	Ba
320 r	acc. 5†	360	360	359	360.0	356.
	res. 3††	0	0	0	0	.00
480 r	acc. 5	356	355	350	355	341.
	res. 3	0	0	.01	0	.01
640 r	acc. 5	333	334	312	335	277.
	res. 3	.06	.01	.61	.00	1.80

† acc. = Variation accounted for by the different regions.
†† res. = Residual or unaccounted for variation.

whole body was severe enough to appreciably narrow the range between the two extreme strains, S and Ba. The Z and Q strains interchanged positions in rank of resistance as measured by percentage survival, but were equal when the degree of severity was measured by length of survival. The strain differences were expressed only in the treatment groups where the radiation was of sufficient intensity to reduce survival of the more sensitive strains (Tables III and IV). The strains responded differently to the graded x-ray doses as shown in Table V. The interactions between x-ray doses and strains in the exposure treatment groups were about equal to the effects of the strains alone. Each strain appeared to have a reaction curve of its own.

This genetic differentiation of the strains in their response to radiation has been expanded by more recent observations on the LD50 values for 15 days under the same conditions of x-irradiation. The actual x-ray doses required to reduce survival of each strain 50 per cent were determined by exposing mice of different strains to a range of x-ray doses. The LD50 values obtained experimentally for whole body irradiations were S, 537 r; Q, 528 r; Z, 522 r; K, 481 r and Ba 438 r. From this information the Q and Z strains were more nearly alike in radiation sensitivity than was indicated in the 480 r whole-body treatment group of the experiment under discussion, but comparable to the levels determined by the 640 r exposure.

The genetic differentiation of these five strains of mice in their resistance to x-irradiation confirms the observations of Gowen and Zelle (1945) on some of these same strains and by Henshaw (1944b) on other strains. Henshaw found that C_3H mice were more sensitive than LAF_1 mice to whole-body irradiation as measured by survival and the effects on the blood picture. Kaplan and Paull (1952) showed differences between strains A and C57 black to radiation response. Differences between four strains were shown by Reinhard *et al.* (1954) with minimal lethal doses of x-irradiation. The four strains ranged in MLD values from 570 r for the Marsh strain to 492 r for C_3H. Grahn (1954), in this laboratory, observed genetic differences between six strains of mice (including S, Z and Ba) in radiation response as related to body weight changes. With respect to dosage relationships these observations are in agreement with the findings of many other investigators. Heineke (1905) reported that increased x-ray exposure reduced the efficiency of the blood-forming organs in mice. Lawrence and Tennant (1937) concluded that length of life of Swiss mice following x-irradiation was directly related to the dose given. As dose was increased they noted the increased frequency of diarrhea in the mice. Like conclusions were reported by Osborne *et al.* (1952) and by Kaplan and Brown (1952). This latter work involved adequate samples of mice for each of nine x-ray doses, 283 r to 1131 r, given in single exposures and in fractions of these total doses. From the single exposures mortality increased from 4 per cent at 283 r to 83 per cent at the 566 r dose level.

In this work the S, Z, K and Ba strains maintained the same order in both radiation response and in subsequent response to mouse typhoid. This experiment introduces a new strain, Q. The Q strain showed a difference in its reactions. It was quite resistant to x-rays but extremely susceptible to mouse typhoid. Q mice did not fit in the observed pattern of the other mice with respect to the two responses. The leucocyte level for the Q strain has not been determined but is of real interest. The level of the white blood count of this strain would be indicative of the causal relationship of leucocytes to disease resistance (Weir *et al.*, 1953; Gowen and Calhoun, 1943) or to radiation sensitivity (Gowen, 1952). The K strain, not included in the six strains previously tested, followed in line with the other strains in both disease resistance and numbers of leucocytes (Thompson, 1952).

Differences in regional sensitivities to x-rays became evident when the body regions and combinations thereof were irradiated. The order of increasing sensitivity was head, rear, head-rear, mid, head-mid, mid-rear and head-mid-rear or whole-body. The dosages were not adequate to appreciably affect survival when the head, rear or head-rear were x-rayed. Only those four groups in which the

mid third was involved gave noticeable differences for the strains and dosages. Comparison of the mid-rear to the head-mid indicated a greater radiation sensitivity of the rear third as contrasted with the anterior third of the body. The reduction in survival and length of survival was between four and five times as severe following exposure of the head-mid as it was following that of the mid alone. The mid-rear reaction was around 16 times as severe as that of the mid alone. The increased reduction was affected by the particular regions rather than by the proportion of the body exposed.

Although no attempt was made in this investigation to associate specific organs to radiation sensitivity, specific organs or tissues were implicated by their inclusion within a given region as shown in Plate 1. The posterior third of the body implied the intestines, testes, bladder, etc., whereas the mid portion included the spleen, liver, etc. Our observations on the sensitivity of the mid-rear region in part confirmed those of Warren and Whipple (1922). They found in dogs that the abdomens, comparable to the rear plus a good portion of the mid region in these data, were more sensitive to x-irradiation than the head and thoracic region. They attributed this increase in mortality from x-rays to severe toxemia and septicemia enhanced by exposure of the intestines. Bond *et al.* (1954) arrived at similar conclusions. Chrom (1935) separated the effects of exposure to the rear region from those to the rest of the abdomen. He concluded that the rear region, including the intestines, was not as sensitive as the upper part of the abdomen. These data also supported the conclusion of Osborne *et al.* (1952) that bacteremia and intestinal damage were not closely correlated. The observations of these investigators were supported by those which have been derived from our studies.

The greater sensitivity of the mid region as shown in our data may be related to the response of the lymphoid tissues, spleen and nodes. Heineke's (1905) observations, supported by those of Läwen (1909), showed the blood and blood-forming tissues to react strongly to x-radiation in rats, rabbits, mice and guinea pigs. Further emphasis on the hematopoietic tissues in radiation response as well as in recovery comes from the investigations of Lawrence and Tennant (1937), Ellinger (1945), Henshaw (1944a, 1944b, 1944c), Bloom and Jacobson (1948) and Barrow and Tullis (1952) to name but a few. Again the data of this paper may be interpreted as indicating the significance of the proper functioning of these organs as they affect survival.

The important role of the spleen, a center of hematopoiesis, has been demonstrated by the increased survival obtained by shielding this organ from x-radiation (Jacobson, 1954; Wissler *et al.*, 1953; and Bond *et al.*, 1950) and by the partial protection afforded irradiated animals by injections of splenic or bone marrow tissue homogenates (Jacobson, 1954; Cole and Ellis, 1953; Lorenz *et al.*, 1952; and Barnes and Loutit, 1955). Again the behavior of these organs under irradiation has parallel significance to the data on x-ray survival presented in this paper.

The quantitative interpretation of these data is, however, somewhat different. The authors cited above have tended to consider each organ studied as all-important to irradiation survival. Our data show that while the different regions were significant, their effects on survival were less impressive than these other investigators suggested.

The fitting of constants to the regional body effects offers a new approach to the evaluation of radiation effects to different regions of the body. This quantitative

estimation of regional effects confirms our previous observations that exposure of the head region or anterior third of the body is less effective in reducing survival than either of the other regions. X-ray doses in the range used did not appear to influence this minor effect. Exposure of the rear region was shown to be detrimental to survival at the higher exposure level of 640 r. This effect was consistent in the five strains although of minor importance to the Q strain. Irradiation to the mid region showed the greatest consistent reduction in survival for all strains. Increase in dosage increased the severity of the reaction in the strains. Greater mortality was observed in the more susceptible strains, Ba and K. Only the Q mice showed a different reaction; exposures of the rear or head were nearly as detrimental as those to the mid region, although the effects of the three regions were small.

The greatest reduction in survival resulted from whole-body irradiation. The amount of this reduction as measured by the constant, *d,* was above that due to the combined effects of head, mid, and rear exposures. The *d* values represent that percentage of the total mortality that resulted when all cells of the body were exposed and is in addition to the mortality resulting from the combined exposures to the three portions of the body. The magnitudes of these *d* values particularly after 640 r, but also after 480 r, indicate the importance of at least some unexposed cells in facilitating the recovery of the irradiated animal. The large differences between the combined effects of the three regions as compared to the body irradiation, *d,* suggest that any unexposed cells contribute materially to the protection of the mouse from irradiation. This was also indicated by the work of Gershon-Cohen *et al.* (1951), who showed that shielding areas comparable to 15 per cent of the total body area of mice resulted in reduced mortality from radiation. Almost equal protection was afforded the mice by shielding the liver, or lung or abdomen. They concluded that viability of the animal was increased by shielding any part of the hematopoietic system and was not confined to special organs. Jacobson *et al.* (1951) compared the hematopoietic recovery in mice irradiated with regions or organs shielded from exposure to 1025 r. Shielding of the spleen gave the greatest increase in survival as well as in hematopoietic recovery. Shielding of the liver lobe or portion of the intestines increased survival, but to a lesser degree. These results were associated with only somewhat less recovery of hematopoiesis. However, shielding of the head gave nearly the same increase in survival as that of the intestine, but recovery in blood formation was only partial. The protection of the right hind limb, but not of the kidney, was also beneficial to survival. The results from the head and intestine shielding point to the influence of cells, tissues or systems, other than those involved in hematopoiesis, as contributing to the radiation response. Although injections of suspensions of splenic tissues contributed most, bone marrow and liver tissues as well as body tissues of embryos contributed to recovery of the irradiated host (Jacobson *et al.,* 1955). Our own observations do not rule out a major role for the hematopoietic system, but the increased severity of radiation to the whole body over that to the regions suggests that any cells, regardless of their apparent morphological specificity, could stimulate recovery. The scattered radiation absorbed in the shielded regions would tend to increase the effects to the exposed regions. Consequently the presence of scattered radiation would tend to decrease the *d* value as obtained here (Table VI).

In all cases the Ba mice show the largest effects of the regional exposures; the

d values have been minimized, however, by the limitation of 0 per cent survival. The K strain as previously shown is next in susceptibility to radiation followed by the Q, Z and S strains. The resistance of the S strain appeared related to the resistance of its cells to better withstand radiation even though exposures to the mid and rear regions were more detrimental to survival of the S mice than to the Z or Q mice. The resistances of the Z and Q strains appeared to be determined by the interactions of all cells and regions. The increased susceptibility of the K and Ba strains was contributed to by the proportionately greater sensitivity of the mid and rear regions. The five strains, however, showed quite similar reactions but to different degrees in their responses to x-irradiation. These results were in contrast to those of Reinhard *et al.* (1954). They found marked differences in four strains of mice in radiation sensitivity of the head as compared to that of the remainder of the body. Kaplan and Paull (1952) also showed strain differences between A and C57 black mice in the results of spleen shielding. Protection of the spleen was more important to A mice than to C_3H mice. This observation of the A strain was in accord with that of Lorenz *et al.* (1952). They observed differences between four strains of mice in their responses to the protection afforded by bone marrow cell suspensions. Intravenous and intraperitoneal injections of the homogenate increased survival for two strains, L and LAF_1. For strains A and C_3H intraperitoneal injections were of little value in decreasing mortality.

The data indicate the importance of maintaining at least some cells free of irradiation if the organism is to survive. The body cells retain a significant totipotency which contributes to maintaining the organism as a whole even though the cells may have differentiated to extreme types anatomically or physiologically.

Summary and Conclusions

1. The influence of x-irradiation absorbed in three body regions and in the combinations of these regions has been measured by three subsequent responses: survival to radiation, natural resistance to disease and ability to acquire resistance following contact with the disease agent, *S. typhimurium*. The effects of irradiation are presented in this paper. Papers on natural and acquired resistance will follow. The experiment was designed as a factorial with five genetically differentiated strains of mice, S, Z, K, Q and Ba; four levels of radiation: 0 r, 320 r, 480 r and 640 r; eight treatment groups and two sexes. All mice were 46 ± 3 days of age when irradiated from a 250 pkv x-ray source operated at 30 ma with 0.25 mm. Cu + 1 mm. Al filter at a dose rate averaging 170 r/minute. For the initial treatment the strains and sexes were well balanced, at least 50 mice in each of the 25 different treatment groups. The bodies of the mice were marked off in three regions, head *h,* mid *m,* and rear *r,* each comprising one-third of the body length. These regions, their combinations and their controls with each irradiation account for the 25 treatment groups. Shielding was done with ⅛-inch lead. As most deaths occur between 7 and 12 days, an interval of 15 days was allowed for expression of any direct effects due to radiation. Deaths were recorded daily.

2. Percentage survival and length of survival were the two measurements used for determining the reactions in each response.

3. The sexes responded in like manner to x-irradiation. A penetration or wavelength effect was indicated in these data. The reactions of the mice to the whole

body irradiation at 250 pkv, 0.25 Cu + 1 Al filter 600 r were similar to those for 100 pkv, Coolidge tube, no filtration, 960 r.

4. Within the x-ray dose range used the responses of the strains to x-irradiation were shown to be partially genetically determined.

5. The levels of radiation resistance were in the order from resistant to sensitive: S, Z, Q, K and Ba. After 480 r total-body exposure the survival percentages of the five strains were: S, 79; Z, 75; Q, 66; K, 54 and Ba, 31. This order does not coincide with the order known to be followed in natural resistance to mouse typhoid: S, Z, K, Q and Ba.

6. Shielding of one-third of the body protected the mice of the five strains from 320 r and 480 r x-radiation, and to much lesser degree, depending upon regional exposures, from 640 r. The dose of 640 r was not of sufficient intensity to allow full expression of strain differences for the different regional exposures.

7. Whole-body exposure to 320 r reduced the 15-day survival for the more sensitive strains Ba and K, 480 r decreased survival in the five strains; 640 r was severe enough to largely overcome the genetic differences between the strains.

8. The mid region of the mouse was most sensitive of the three single regions, and more sensitive than the combined head and rear regions. The radiation effects were determined by the region rather than by the area of the body exposed.

9. The mid region in combination with the rear region showed greater sensitivity than the head-mid region. All strains were reduced in survival by exposure of the mid-rear to 640 r, whereas only the less resistant strains Ba and K showed the effects from the exposures of the less sensitive regions.

10. The decrease in survival showed the mid region as most sensitive for S, Z, K and Ba, followed by the rear portion with the anterior third of the body resistant. These four strains responded in the same manner but to different degrees.

11. The reactions of the Q strain separated it from the four other strains. Its level of radiation resistance with respect to the other strains was in contrast to its low level of natural resistance to mouse typhoid. Radiation resistance and natural resistance to this disease have been found highly correlated in seven of our strains of mice. The Q mice show comparable though slight sensitivity to x-radiation in the three body regions. Mortality in the Q strain was largely confined to whole-body exposures of 480 r and 640 r. This suggests that the Q mice have no particular center of radiation sensitivity, but that mortality is the result of the interactions of the cells throughout the body.

12. The data on length of survival confirmed the results from percentage survival and contributed additional information for those reactions that resulted in 0 or near 0 per cent survival.

13. The lead shielding, ⅛-inch in thickness, was adequate to protect the given regions from radiation. The three groups completely shielded when exposed to the three dosages of x-rays did not quite duplicate the 0 r group in their reactions. Mortality appeared unrelated to the x-ray dose, as 100 per cent survived 640 r, 98.4 per cent the 480 r and 99.6 per cent the 320 r.

14. The strains exhibited their own characteristic responses to different x-ray doses as was evidenced by the large values for dosage × strain interactions. These interactions were real, representing the expressions of genetic resistance and as such would contribute to the strain effects.

15. The effects of the relative sensitivities of the body regions were estimated quantitatively as well as qualitatively. The quantitative estimates compared favorably with the qualitative observations.

16. The additivity of the regional effects is supported by the little unaccounted-for variation remaining after fitting the constants derived on the assumption that *h, m, r* and *d* were additive in effect.

17. Mortality from whole-body irradiation was only partially accounted for by the combined mortalities resulting from the exposures to the different regions of the body. The effect of total-body exposure over and beyond that of the combined regional effects, *d,* was interpreted as a measure of the reaction when all cells of the body of the mouse had been exposed, or when all recovery potential had been affected.

18. The whole-body effect, *d,* was large and suggested that all cells may contribute to recovery regardless of the organ or system involved. As a consequence, protection of any cells of the body during exposure to radiant energy may stimulate recovery.

19. In terms of host resistance the unexposed cells over-compensate. The extreme over-compensation initiated by cells in different unexposed regions when cells of other regions are inactivated points to the significance of all body cells in resistance whatever their degree of tissue or organ differentiation.

20. These results indicate that the body cells retained a totipotency to assist in maintaining the organism as a whole despite the differentiation which these cells may have undergone since their stem cells left the embryologically differentiating primitive tract. They further show the importance of maintaining at least a small portion of the body free from irradiation if irradiation exposure should occur through accident or calculated risk.

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SEXUAL DIFFERENTIATION IN THE TELEOST FISH XIPHOPHORUS HELLERII, AS MODIFIED BY EXPERIMENTAL TREATMENT

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Attempts to alter the pattern of sexual differentiation in the Mexican swordtail fish, *Xiphoporus hellerii,* show a stability in sexual development usually not credited to this species. The swordtail has long been used as a classic example of lability of sexual differentiation and determination. The widespread reference to sex-reversal occurring in the species suggested a need for further investigation.

This work was aided by the guidance and kind supervision given by the late Dr. Carl R. Moore of the University of Chicago. I am indebted to Dr. Myron Gordon for his generosity in supplying the fishes used in this study. It is a pleasure to acknowledge the cooperation of the Schering Corporation which supplied the hormone preparations used.

Animals and Their Treatment

The fishes used were descendants of ten pairs from the Genetics Laboratory of the New York Zoological Society. They were reared under conditions similar to those described in earlier papers (Vallowe, 1952, 1953). The original ten pairs produced 618 males and 490 females in a period of approximately three years. The presence of a modified anal fin was the criterion used to establish maleness. Fish not possessing the gonopodium were anesthetized in Chlorobutanol and examined before a strong light. The characteristic amber color of the ova apparent in the ventral region of the posterior end of the body cavity was the criterion used to classify fish as females.

The sex ratios which have been established for this species show wide variations. Witchi (1939) considers *X. hellerii* to be a species without any hereditary sex determining mechanism although the presence of such mechanisms has been shown for other species in the genus. Geiser (1924) presents a comprehensive table showing the sex ratios reported by various authors. Harms (1926) gives a ratio of 24 males to 35 females. Bellamy (1922) reports 100 males to 66.7 females. Friess (1933) also reports a high proportion of males and considers temperature an important influence on the sex ratio. Breider (1935) made a study of the effects of light, nutrition, space and water and found that these had no certain influence on the sex ratio. In light of many conflicting reports, one cannot assume that a preponderance of one sex is due to sex-reversal unless all factors which may influence sexual differentiation are duly considered.

As a basis for interpreting the experimental results reported, a large series of fish were killed at various stages of development, beginning two weeks before birth

and continuing up to more than two years of age. The gonads of these fish were examined to determine the normal pattern of sexual development and differentiation. As early as three days after birth, the gonads could be recognized as potential testes or ovaries by the relative size, number and arrangement of the primordial germ cells. In the young male the germ cells were slightly smaller than in the female. In addition, there were fewer germ cells in each gonad primordium and they were concentrated at the periphery of the gland. In the young female the slightly larger cells were in greater numbers and were well distributed. The embryonic development of this species closely parallels the description given by Goodrich *et al.* (1934) and Dildine (1933, 1936) for *L. reticulatus,* that by Wolf (1931) for *X. maculatus,* and those by Geiser (1924) and Medlen (1950) for two species of *Gambusia.* Although sexual differentiation is conspicuous in these species shortly before birth, Essenberg (1923), Bailey (1933) and Regnier (1938) have shown it to be somewhat delayed in *X. hellerii.*

The differences which distinguish the early testes and ovaries are very subtle characteristics. The appearance of the early ovary and the indifferent gonad are so similar that Regnier (1938) considers that all the fish are born female and that the males later undergo a sex change. This introduces an unnecessary complication which is easily resolved if one assumes a longer period of indifferent development. Once differentiation begins, it progresses in an orderly sequence of events. Chavin and Gordon (1951) describe the differentiation of the testes in *X. maculatus* by using a series of six stages. With only slight modifications, these stages were used in the present study to classify the testes of *X. hellerii.* Gordon and Aronowitz (1951) make the observation that the histological structures of the testes of adult *X. maculatus* and *X. hellerii* are practically identical. The present study has shown that the development of the testis and the pattern of the differentiation of its structures are strikingly similar in the two species. Except for the time spent in the late stages of immaturity, the sequence of events is identical.

The process of sexual differentiation in *X. hellerii* as described by Essenberg (1923) and Van Oordt (1925) traces the origin of the definitive sperms to the epithelial cells of the testis tubules. Wolf (1931), Goodrich *et al.* (1934), and Chavin and Gordon (1951) were unable to find comparable stages of transformation in the species which they studied. The testis in *X. hellerii* is the acinus-type characteristic of the vivaparous Cyprinodonts. The arrangement and development of the structures within the testis indicate that the acini form from pre-existing acini at the periphery of the testis. As the acini are formed, they are pushed along the tubules toward the central ducts by the growth of new acini at the periphery. The peritoneal covering of the testis, the stroma cells, and the epithelium of the ducts and tubules gave no evidence of transforming into germ cells.

It is slightly more difficult to distinguish the newly differentiated ovary from the indifferent gonad because the arrangement of the cells remains about the same. However, the relative size and number of the cells characterize the gonad as an immature ovary. Early in the differentiation process the primordial germ cells are found scattered throughout the stroma of the ovary. When the fusion of the paired ovaries is completed, the germ cells come to lie within the wall of the cavity formed between them or in the stroma layer which supports this wall. In the mature female oogonia are found developing within the wall of the cavity, hence the name germinal epithelium. The larger and more advanced cells are pushed

outward toward the peritoneal covering. Essenberg (1923) feels that all the primordial germ cells disintegrate and do not take part in the formation of the germinal epithelium. Although there is great difficulty in determining the origin of the cells within the germinal epithelium, no disintegrating primordial cells were encountered in the early stages of gonad fusion and cavity formation. The primordial cells appeared to remain in normal active condition and were arranged in small groups surrounded by stroma cells. In the germinal epithelium it is possible to see all stages of transition in the shape of the nuclei from elongate oval, typical of the epithelial cells, to the spherical nuclei of oogonia. A comparable situation exists in *L. reticulatus* according to Goodrich *et al.* (1934) and leads them to the conclusion that epithelial cells as well as primordial germ cells may give rise to the definitive ova. Wolf (1931) arrived at a similar conclusion. The origin of the sex cells appears to be different in the two sexes of these species. While the primordial germ cells are the only source of definitive sperms, the peritoneal cells forming the wall of the ovarian cavity may contribute to the formation of definitive ova.

The following series of experiments reports the effects of estrogenic and androgenic hormones on gonad development. The results of the treatments described are interpreted in light of their effect on the normal sexual differentiation.

Experiments and Results

Immature fish

Young sexually immature fish (49 to 55 days old) were given nine weekly injections of 0.01 cc. of sesame oil containing 0.25 mg. of testosterone propionate into the body cavity. During this period of time there was a conspicuous thickening and elongation of the anal fin rays, a growth and pigmentation of the sword-like extension of the caudal fin and an intensification of the lateral line and dorsal fin coloration. In short, the young fish appeared to be miniatures of the sexually mature adult males. The histological picture presented by the gonads of these fish was one of radical change in the immature ovaries and one of general stimulus in the young testes.

The ovaries of the testosterone-injected immature females lacked any sign of ova. There were follicles present but these were filled with a loose collection of cells. This indicates that ova had been present previously, but that resorption had taken place. In other follicles primary spermatocytes, spermatids and spermatophores were observed. The ovarian cavity was obscured and much stroma filled the gonad. The brood-mate control females showed ovaries in which fats and oils were being deposited in the maturing ova.

The testes of immature males treated with this androgen showed an acceleration in spermatogenesis. The tubules and sperm ducts were filled with all stages of spermatogenesis including well formed spermatophores. The epithelium lining the sperm ducts was greatly hypertrophied but otherwise the testes appeared very similar to those of mature males. Brood-mate control males showed testes in less advanced stages of development.

Another group of immature fish at comparable ages was given nine weekly injections of 0.01 cc. sesame oil containing 0.00083 mg. of estradiol benzoate. At the end of the treatment period these fish were deep-bodied, showed only faint

indications of coloration and, in general, appeared to be miniatures of the adult females. The histological picture presented by these fish was the reverse of the situation found in those injected with testosterone. The ovaries of the estradiol-treated females showed a general stimulus while the testes of the treated males showed radical changes.

The testes of the estrogen-treated males showed a modification from the bipartite gonad to a fused structure in which the two lobes were no longer distinct. The peritoneal covering of the gonad had thickened, the germinal elements were no longer concentrated at the periphery of the gonad, the sperm ducts were obscured, and the blood vessels were enlarged. Some acini contained what appeared to be oogonia. Other acini contained disintegrating cells which resembled primary spermatocytes. The testes were larger than those of the brood-mate controls. In some of the gonads the sperm ducts appeared only as spaces with no organized epithelium. Two new dorso-lateral cavities had formed and were lined with a well organized epithelium. Oocytes were common in the testes showing this degree of modification.

Ovaries of the estradiol-injected females showed ova in the stage of oil deposition. There was a slight increase in the amount of stroma present but the blood vessels and follicular epithelium appeared normal. The germinal epithelium appeared very active and abundant oogenesis was observed.

Mature fish

The histological picture presented by the testes of sexually mature males after ten weekly injections of 0.02 cc. sesame oil containing 0.00166 mg. estradiol benzoate was one of general suppression and destruction. The most conspicuous effects obtained were those of an enlargement of the sperm ducts and the destruction of the spermatogenic elements. In normal mature males the sperm ducts were paired, centrally located tubes which contained spermatophores suspended in a lightly staining, non-granular fluid. In estrogen-treated males the ducts were greatly enlarged and in some cases occupied most of the testes when viewed in cross-section. They seemed to be distended with fluid and contained spermatophores in various stages of disintegration. Quantities of free spermatozoa were observed in the lumen of the ducts; this condition is not found in normal mature males. However, free spermatozoa are found in the ducts of senile males which have passed the reproductive age and are no longer capable of fertilization.

The effect on the germinal elements was most drastic in the intermediate stages of spermatogenesis. While spermatozoa and spermatogonia were still abundant, primary and secondary spermatocytes and spermatids were usually reduced in number or entirely absent. The acini which, by their position, should have contained the intermediate stages of spermatogenesis, had hypertrophied walls in which the cell outlines were conspicuous. These acini were smaller than normal and were either filled with disintegrating germ cells or were completely empty. The reduction of the acini was accompanied by a slight increase in the amount of stroma tissue and the appearance of a fibrous tissue network. The blood vessels were numerous and enlarged. The testes appeared less bipartite than the normal condition, but none were found in which fusion was as complete as that found in the ovary.

The histological picture presented by the ovaries of females given ten weekly injections of 0.02 cc. sesame oil containing 0.5 mg. testosterone propionate was also one of general suppression and destruction. None of the ovaries showed any signs of spermatogenesis even when injections were continued for as long as eighteen weeks. A conspicuous activity was noted in the germinal epithelium but there was no indication that the primary germ cells being proliferated could be spermatogonia. The cells were in groups of as many as ten, but their arrangement, size, and staining properties were strikingly similar to the oogonia found in normal ovarian development. The larger ova that were present in the gonad were in various stages of disintegration. Only a few of the ovaries examined showed the presence of mature ova; in most cases, there remained only large, empty follicles which were in various stages of collapse. There seemed to be little effect of the hormone treatment upon the smaller ova; they were still firm, were surrounded by a well organized follicle, and were in their normal arrangement in the ovary. There was very little increase in the amount of stroma in the ovary and only isolated areas in which a fibrous network was formed. On the other hand, the blood vessels had increased in size and were found throughout the ovarian tissue. In gross appearance, the ovaries of the androgen-treated females resembled those of immature normal females.

In the empty follicles and ovarian cavity of many of the virgin ovaries the presence of a secretion was observed. This secretion appeared as a mass of crumpled membranes which is normally found only in the non-virgin ovary. The frequency of its appearance indicated that this is a typical response to androgenic injections and it may be indicative of an expulsion of mature ova from their follicles. However, no mature eggs were found in the ovarian cavities or oviducts. If the eggs were completely evacuated from the body, they were never found in the aquaria.

The ovaries of the gravid females did not differ from the ovaries of virgin females given the same treatment. In two cases where the females were killed three weeks after the initial injection of testosterone propionate, embryos in early stages of development still remained in the follicles. The characteristic membrane-like secretions were observed in the follicles of some of the gravid females.

Discussion

The results obtained from the study of the normal sexual differentiation of *X. hellerii,* and from the attempts to alter the pattern of this differentiation by experimental treatment, point to a stability in sexual development and differentiation which has usually not been credited to this species. Much of the experimental and descriptive work dealing with Xiphophorin fishes has emphasized the ease with which the secondary sexual characteristics of these species can be made to respond to hormonal treatment. The effects of many hormone substances on the primary sex organs have also been described but the results are not always in agreement. However, in all cases of sex-reversal reported for adult *X. hellerii* the change has always been from female to male, whether such reversals were naturally occurring phenomena or whether they were induced by hormone treatments. During the course of this investigation no case of natural sex-reversal was observed in the laboratory population; further, the sex ratio obtained for this

population indicates that unobserved sex-reversals could not have occurred in large numbers. The adult sex ratio and the sex distribution of the fishes used in the study of the normal development of the gonads did not indicate that a shift in the ratio occurred between the juvenile and mature populations. Essenberg (1923) postulated that a sex-reversal of 50 per cent of the immature females would explain the shift he observed in the sex ratio (a change from a ratio among immature fish of three females to one male to the adult condition of one female to three males).

A possible explanation of naturally occurring sex-reversal in adult fish, in which virgin females become males, lies in improper initial classification. In this study only those fish which possessed amber ova that could be observed through the body wall when viewed against a strong light source were designated as females. This procedure reduced to a minimum the possibility of erroneous sex classification. The classification of immature males as females may account for a few, but certainly not all, of the cases of hormone-induced sex-reversal reported by other investigators.

Another factor which may play a significant role in the discrepancies of the results obtained from experiments with *X. hellerii* lies with the variations in the fish used. The very important reviews by Gordon (1931, 1937) concerning the history of Xiphophorin species as aquarium fishes give substantial evidence to indicate that the *X. hellerii* available from commercial hatcheries have possibly been produced by hybridization with *X. maculatus*. For the aquarist the hybrid fish has many traits that are desirable. The hybrid is usually more highly colored, more robust and larger than either parent species, and more prolific in the production of large broods of young. These factors would account for its selection and propagation by commercial hatcheries. Gordon and Rosen (1951) suggest that the hybrid possesses an imbalanced chromosomal arrangement that may have endocrinological significance in that this imbalance may initiate abnormal or nonfunctional gonads in the hybrid. Although hybrids may usually be identified by color or color patterns, some are practically identical with the wild-type swordtail. The few cases of natural sex-reversal that have come to my attention have always been in hybrid fishes or in fish with unknown origins. A strong possibility exists that the sex-reversals reported by Essenberg (1926) occurred in hybrid stocks. (He gives the origin of the stock as the Crescent Fish Farm, a commercial fish hatchery.) Further application of this possibility suggests that the work of other investigators may also be based on commercially available hybrid strains rather than on pure species stocks.

The results of the experimental treatments given to the pure species used in this study were found to be in close accord with the results obtained by many investigators using the closely related species which are not known for their tendencies to produce sex-inversions. The response to the techniques employed indicated that these fish were in no way aberrant but were in close agreement with the other members of the viviparous Cyprinodont group.

Although the immature fish developed the germ cells of the opposite sex under hormone treatment, the sexually mature fish did not when subjected to the same hormone preparations. Since the gonads are homologous structures, it is possible that they retain a few common properties until differentiation becomes so advanced that these must be sacrificed. Evidence from the treatment of maturing males

with estradiol benzoate shows that the ability of the testis to produce ova is retained from a short time after gonopodium elongation. However, this response is lost after the later stage of male development, *i.e.*, gonopodium differentiation, is initiated. In the mature male the effects of estrogenic treatment were a destruction and suppression of existing elements. Unlike the results obtained in immature and maturing males, there was no stimulus to develop female characteristics.

Immature females produced spermatozoa within their ovaries under the influence of testosterone propionate. This response, however, was not found in the ovaries of mature females given twice the amount of the same hormone preparation.

Summary

1. The normal sexual development and differentiation of *Xiphophorus hellerii* have been observed in the light of the adult sex ratio and the differentiation of the gonads from birth to maturity. The sexual differentiation has been shown to follow a definite pattern in both sexes. The gonads of both sexes are homologous and indifferent at birth, but within a few days testes and ovaries can be distinguished by the relative size, number and arrangement of the primordial germ cells. No atypical gonads were discovered in the course of this study which would indicate the possibility of sex-reversal.

2. Injections of testosterone propionate induced spermatogenesis in the ovaries, of immature females but had no comparable effect in mature specimens.

3. Injections of estradiol benzoate induced oogenesis in the testes of immature males but had no comparable effect in mature specimens.

4. Improper initial sex classification and hybrid origin of the fish used are suggested as possible explanations for some of the discrepancies between the results obtained in this study and those reported in earlier investigations.

5. The results of the study of the normal sexual development and the attempts to alter the differentiation pattern by experimental means indicate a stable sexual development and differentiation for this species.

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