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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

METABOLIC STUDIES ON THE GAS GLAND OF THE SWIM BLADDER [1]

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The main function of the swim bladder of teleosts is assumed to be that of a hydrostatic organ. It is filled with a mixture of oxygen, carbon dioxide and nitrogen, so that the bladder is normally inflated to a volume which in marine forms is approximately 5% of the total body volume (Jones and Marshall, 1953). This serves to bring the specific gravity of the fish close to that of the sea water in which it lives. The composition of the swim bladder gases varies with the depth of water at which the fish dwells. As the hydrostatic pressure due to depth increases, most species keep their bladder volume constant by increasing mainly its oxygen content. Thus one finds at depths where the hydrostatic pressure is equivalent to 100 atmospheres or so that the percentage of oxygen in the swim bladder gases reaches values well over 90% (Scholander and van Dam, 1953). In such cases this means, in round figures, that a volume of oxygen (as measured at one atmosphere of pressure) equal to 100 times the volume of the swim bladder has been introduced. This amazing feat of introducing oxygen into the swim bladder in relatively large amounts and against extremely high pressures is attributed to the functioning of the so-called gas gland of the swim bladder. The structure and function of this gland has been adequately described in two recent reviews (Fänge, 1953; Jones and Marshall, 1953) and needs no further description here. The mechanism as to how this is achieved by the gas gland remains unproven. At the time the work to be described here was begun in the summer of 1950, little attention had been paid to the metabolic activity of this gland. In view of the concept that acidification of the blood passing through this gas gland might furnish a mechanism for the release of oxygen and carbon dioxide from the blood, it seemed worthwhile to obtain some quantitative data on the gland's oxygen consumption and glucose utilization. It will be shown here that the gas gland possesses a very high capacity to convert glucose to lactic acid and the possible implications of this acid formation on the mechanism of gas production will be discussed. A preliminary report of a portion of this work has been published (Strittmatter, Ball and Cooper, 1952).

[1] This work was supported in part by funds received from the Eugene Higgins Trust through Harvard University.

[2] Lalor Foundation Fellow, Summer, 1952.

tures of equal parts of NaCl and sea water diluted so as to be isotonic gave still lower values.

The values given in Table I are all without added substrate. The addition to the flasks of 0.2 ml. of isotonic glucose (0.5 *M*) did not increase the rate of O_2 uptake (as shown in Curve 1, Fig. 1) and sometimes depressed it. This latter may be the result of the rapid lactic acid production from glucose which occurs, as data to be presented below will show.

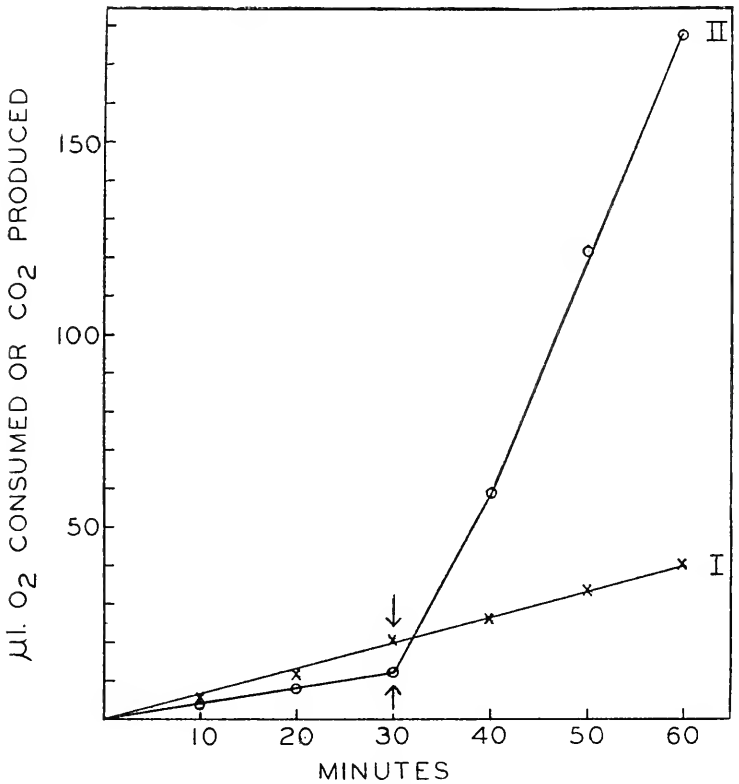

FIGURE 1. Oxygen consumption and anaerobic glycolysis of the gas gland. Curve 1: Oxygen consumption, tissue weight 189 mg. Curve 2; Glycolysis as measured by CO_2 evolution, tissue weight 122 mg. In both cases glucose was added at the point indicated by the arrow so as to yield a final concentration of 300 mg. per 100 ml. Temp. 29.45° C. Media as described under Methods.

Homogenates of the gas gland were prepared with the aid of a glass homogenizer of the Potter-Elvehjem type (Potter and Elvehjem, 1936) in cold NaCl-phosphate medium. The oxygen consumption of such homogenates without added substrate ranged from 2–6 μl. of O_2 per 100 mg. of wet tissue. The addition of succinate or para-phenylene diamine brought the oxygen consumption values of these homogenates up to the range found for the intact gland without added substrate. If cytochrome *c* was added as well, a further small increase in O_2 uptake

was observed. Addition of glucose to gland homogenates caused no significant alteration in oxygen consumption. The addition of sodium hexose diphosphate did stimulate respiration but to only half the extent that succinate did. It was observed that flasks to which hexose diphosphate had been added were strongly acid to phenol red at the end of the run. This suggested that glycolysis was occurring and turned our attention to this aspect of the problem.

Glycolysis

The conversion of glucose to lactic acid by the gland is most conveniently followed manometrically by following the release of CO_2 from bicarbonate. In

TABLE III

Anaerobic glycolysis

	Experiment I				Experiment II			
	No glucose added		Glucose added to give 75 mg./100 ml.		No glucose added		Glucose added to give 150 mg./100 ml.	
Vessel	1	2	3	4	1	2	3	4
Gland wt., mg.	70	74	111	106	85	141	69	82
Initial glucose, μmols	0.20	—	13.21	—	0.67	—	25.06	—
Initial lactate, μmols	0.40	—	0.70	—	1.03	—	1.07	—
Final glucose, μmols	—	0.13	—	8.17	—	0.67	—	21.56
Final lactate, μmols	—	0.66	—	7.64	—	1.75	—	7.41
CO_2 evolved, μmols	—	0.57	—	7.57	—	0.61	—	6.59
Total glucose consumed, μmols	0.07		5.04		0.00		3.50	
Total lactic acid produced, μmols	0.26		6.94		0.72		6.34	
CO_2 release accounted for by lactic acid production	46%		92%		118%		96%	
Glucose consumption accounted for by lactic acid production	186%		69%		—		91%	

Gas phase 5% CO_2–95% N_2. Temp. 30.1° C. Total fluid volume 3.0 ml.

Figure 1, Curve 2, data are plotted for a typical experiment. There is a fourteen-fold increase in the rate of CO_2 production after tipping glucose into the medium. The data given in Table III clearly show that CO_2 production in such an experiment is due to the conversion of glucose to lactic acid. In each of these experiments two pairs of vessels were set up, each containing an intact gas gland. Usually an effort was made to have approximately equal amounts of tissue in each vessel of a pair. One pair of vessels contained glucose in the side arm. All vessels were gassed with 5% CO_2—95% N_2 and equilibrated in the constant temperature bath for five minutes. The vessels containing glucose were tipped and immediately aliquots of the contents of one flask of each pair were removed for

glucose and lactic acid analysis. The other two vessels remained in the bath for one hour during which CO_2 production was measured. At the end of the hour the contents of each of these flasks were immediately prepared for glucose and lactic acid measurements. Total glucose consumption and lactic acid production were then obtained by the difference in values of the initial and final measurements for each set of vessels. It can be seen from the calculations presented in Table III that in each of the two experiments where glucose was added lactic acid production accounted for 92 and 96% of the CO_2 released and 70 and 90% of the glucose that disappeared was accounted for by lactic acid. (It is assumed that each glucose molecule yields two of lactic acid.)

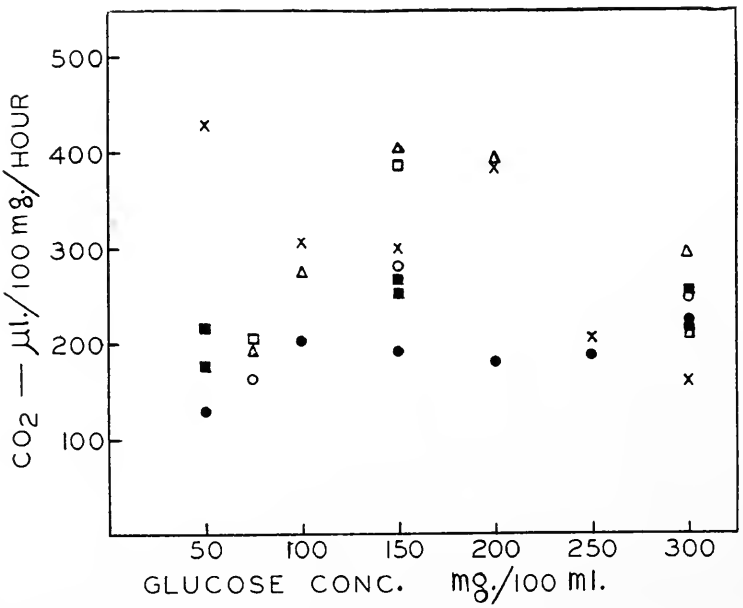

FIGURE 2. The rate of anaerobic glycolysis at different glucose concentrations. Media as described under Methods. Temp. 30.1° C. Each set of symbols represents the data obtained in any one experiment.

In order to make data of the type presented in Table III truly comparable the results can be expressed in terms of 100 mg. of tissue so as to rule out an inequality in the amount of tissue present in the two vessels of any one set. Such calculations, however, do not significantly alter the over-all pattern of the results. In a series of six experiments of the type given in Table III, where glucose was added and the results expressed in terms of 100 mg. of tissue, the following average values were obtained: 92% of the CO_2 release accounted for by lactic acid production; 85% of the glucose consumption accounted for by lactic acid production. In a series of five experiments where glucose was not added the corresponding average values were 80 and 74%, respectively.

In experiments where glucose was not added and the total changes are therefore small the results are quite variable as the data of Table III indicate, the two

experiments given in this table representing the extreme values of five such runs. From the average initial glucose in the vessels of these five experiments it may be calculated that 0.105 mg. of glucose was present for each 100 mg. of tissue, or roughly a value of 105 mg./100 ml. The average initial lactate concentration was

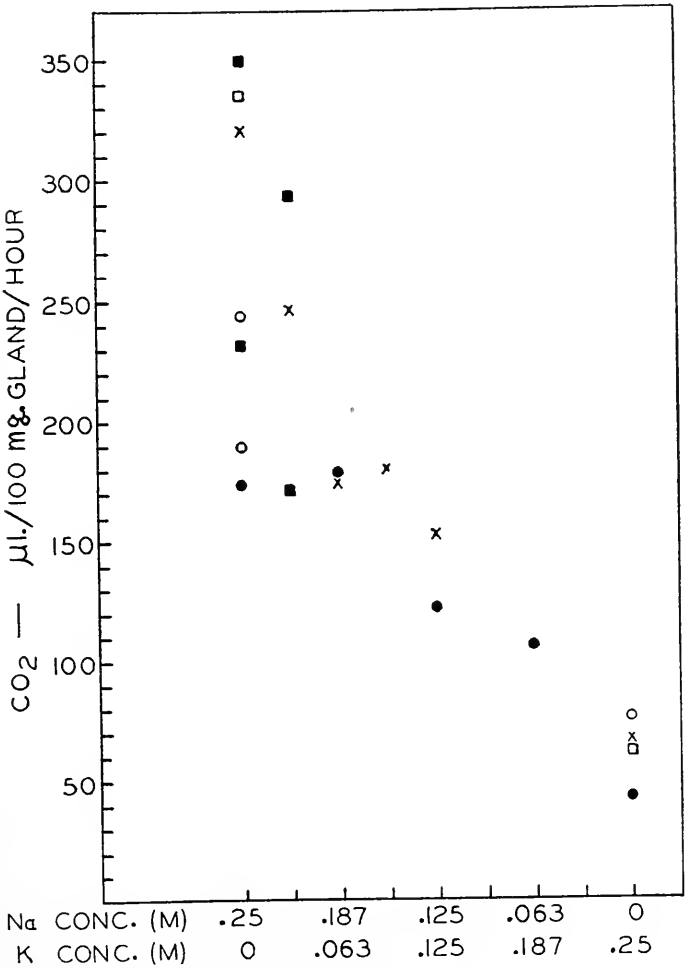

FIGURE 3. The effect of sodium and potassium on anaerobic glycolysis rates. The all-sodium medium was prepared as described under Methods. The all-potassium medium was prepared in the same manner except that the potassium salts were used instead of sodium. These two media were mixed in varying proportions to give media with varying ratios of the two cations. Glucose present in all cases to give an initial concentration of 150 mg. per 100 ml. Temp. 30.1° C.

0.060 mg. per 100 mg. of tissue. If it is assumed that the initial lactic acid was produced from glucose during the interval between removal of the gland from the fish and the time of analysis, then a total initial glucose value of 165 mg./100 ml. might be expected. Since this is much higher than the average value of

53 mg./100 ml. reported by Gray and Hall (1930) for the blood sugar of the scup, it suggests that glycogen breakdown is occurring. Dr. C. T. Teng kindly analyzed for us the glycogen content of the gas gland immediately after its removal from three species of fish. Expressed in terms of glucose per 100 mg. of tissue, he found: scup 0.097 mg., sea robin 0.102 mg., sea bass (*Centropristus striatus*) 0.070 mg. If these are representative values, it indicates that the glucose reserves of the gas gland are small in relation to its glycolytic capacity. Similar values are reported by Fänge (1953). He found the glycogen content of the gas gland in *Gadus callarias* to range from 0.1 to 0.3% of the wet weight.

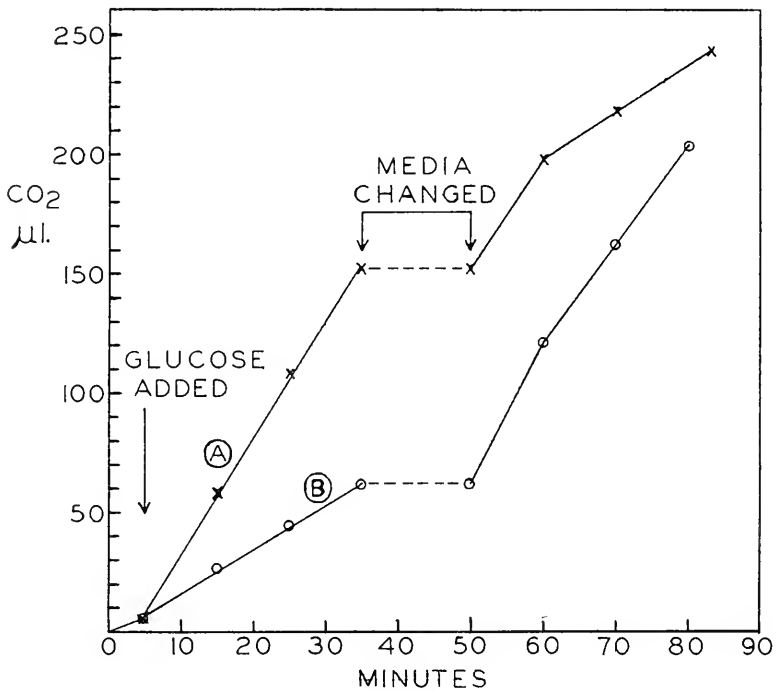

FIGURE 4. The effect upon anaerobic glycolysis rate of changing the gas gland from an all-sodium medium to an all-potassium medium or vice versa. Conditions as described for the data presented in Figure 3. Glucose added to both flasks at the point indicated by the arrow. Weight of Gland A 153 mg.; Gland B 147 mg.

A study of the effect of the concentration of glucose in the medium on the rate of glycolysis is portrayed in the data presented in Figure 2. There is a suggestion of a trend towards maximum glycolytic activity when the glucose concentration is 150 to 200 mg./100 ml., but the rate of glycolysis varies considerably from one gland to another at any one glucose concentration.

The cation composition of the medium appears to have a marked effect upon the rate of glycolysis. In Figure 3 are plotted the results of a series of experiments where the ratio of sodium to potassium in the medium was the only variable.

It can be seen that maximum rates are obtained in a medium containing all sodium ions while minimum rates occur when potassium is the single cation. Rates intermediate to these two extremes are obtained with media containing a mixture of Na and K. In these experiments there is on the average a four-fold increase in rate in changing from an all-potassium medium to an all-sodium one.

Changes in the cation composition of the medium appear to be rapidly reflected in the glycolysis rate as the results of the experiment plotted in Figure 4 illustrate. In this experiment two glands of approximately the same weight were placed respectively in an all-sodium and an all-potassium medium. The vessels were gassed with the 5% CO_2—95% N_2 mixture and allowed to come to temperature in the bath. The rate of glycolysis was then measured for five minutes and found to be approximately the same in both flasks, namely 48 μl. CO_2 evolved per hour per 100 mg. of tissue. Glucose was then tipped into both vessels and the rate of CO_2 evolution measured for 30 minutes. In the vessel containing gland A and sodium as the cation the rate was 190 μl. of CO_2 evolved per hour per 100 mg. of tissue; in the vessel containing gland B and potassium the comparable rate was 76 μl. Both vessels were then dismounted, the gland was removed from each and washed by immersion in a 0.25 *M* solution of the chloride salt of the cation opposite to that from which it had been removed. The glands were then returned to the opposite vessel from which they had been removed, the vessels gassed and allowed to come to temperature. The transfer process required approximately 15 minutes. The production of CO_2 was then followed for another 30-minute period. Gland B, previously immersed in the potassium medium, attained a rate of glycolysis of 194 μl. of CO_2 per 100 mg. of tissue per hour in the sodium medium. This is a value 2½ times that for the same gland in the potassium medium and is almost the same rate found for gland A in a sodium medium. On the other hand, it can be seen that the rate of glycolysis of gland A is slowed down by transfer from a sodium medium to a potassium medium. In the potassium medium the rate of glycolysis of gland A was 117 μl. of CO_2 per mg. of tissue per hour. It should be noted, however, that in the case of gland A the effect of its transfer to another medium becomes definitely apparent only after the first ten-minute reading and that during the last twenty minutes its rate of glycolysis is comparable to that of gland B in the same medium.

This effect of cation composition of the medium upon glycolysis rate prompted us to investigate what effect some other changes in the medium might have. The addition to a sodium medium of Ca and Mg salts, either alone or together, to give a 0.01 *M* final concentration of each of these salts had no appreciable effect upon the glycolysis rate. Inorganic phosphate added to a sodium medium to yield concentrations of 1, 4 or 10 mg. of P per 100 ml. also was without effect. Isotonic sea water (diluted 1 : 1) made 0.025 *M* in $NaHCO_3$ was as good a medium as an all-sodium one.

Variations in the molarity of an all-sodium medium within the range of 0.25 *M* to 0.0625 *M* had no appreciable effect upon the glycolysis rate.

The pH of the medium was varied by altering its $NaHCO_3$ content and holding the CO_2 tension constant. The results of a series of experiments are shown in Table IV. The rate of glycolysis increases as the pH rises. The pH values given are approximate and are calculated from the known values of the CO_2

tension and bicarbonate concentration since facilities were not available for the measurement of pH of solutions in equilibrium with a fixed gas phase.

A comparison of the rates of glycolysis with the four hexoses, glucose, mannose, fructose and galactose, was made. At concentrations of 150 mg./100 ml. the relative rates in the order named for these four sugars were 1.0, 0.86, 0.53 and 0.25. At 75 mg./100 ml. the respective relative rates were 1.0, 0.88, 0.30 and 0.14. These experiments were run in the standard sodium medium with one half of the same gland run in glucose and the other half run with the sugar under comparison. The relative rates reported by Christensen *et al.* (1949) for the action of the hexokinase of rat red blood cells upon glucose, mannose and fructose were 1.0, 0.77 and 0.36. Galactose was unreactive in their experiments. The purity of the sugars used in the experiments reported here is unknown to us.

TABLE IV

The effect of pH upon anaerobic glycolysis rate

$NaHCO_3$ content of medium	Calculated pH	$\mu l.$ CO_2/100 mg./hr.
12.25 mM	7.0	128*
		175
		138
		122
25.0 mM	7.3	273
		288
		327
		251†
50.0 mM	7.6	506
		315*
		397†
		303

*, † One half of the same gland was used in these experiments. Total molarity of medium in all cases made 0.25 *M* by appropriate addition of NaCl. Gas phase 5% CO_2–95% N_2, Temp. 30.1° C.

They were all commercial samples (Eastman Kodak Co.) and were used without any purification.

The glycolysis experiments reported so far have all been performed under anaerobic conditions (5% CO_2–95% N_2) in order to simplify the manometric calculations. Since the gland *in vivo,* especially in deep water fishes, must be in contact with oxygen at relatively high pressures, it seemed important to observe what effect the presence of oxygen might have upon the glycolysis rate. The results of experiments carried out under aerobic conditions (5% CO_2–95% O_2) are shown in Table V. These experiments were carried out in the same manner as those described for the anaerobic experiments reported in Table III. Here, however, the gas exchange values are of course complicated by the fact that oxygen consumption is proceeding at the same time that CO_2 evolution is occurring, both from metabolic oxidations and its release from bicarbonate in the medium by acid production. However, if the RQ of the respiration process is close to 1,

then respiratory CO_2 production will cancel out oxygen consumption manometrically and in the presence of glucose, at any rate, the manometric changes will primarily reflect the glycolysis process. In any case, the total oxygen consumption of the gland (*cf.* Table I) amounts on the average to 1.3 μmols per 100 mg. of gland per hour. This is approximately 20% of the amount of CO_2 produced in the experiments of Table V where glucose is present. The results reported in Table V clearly indicate that glycolysis proceeds readily under conditions where the dissolved oxygen is about equal to that found in water in equilibrium with air under five atmospheres pressure. Indeed the average lactate production, as

TABLE V

Aerobic glycolysis

	Experiment I				Experiment II			
	No glucose added		Glucose added to give 75 mg./100 ml.		Glucose added to give 75 mg./100 ml.			
Vessel	1	2	3	4	1	2	3	4
Gland wt., mg.	84	102	124*	133*	95	102	60	87
Initial glucose, μmols	0.00	—	11.34	—	11.61	—	11.83	—
Initial lactate, μmols	0.65	—	0.75	—	0.89	—	0.60	—
Final glucose, μmols	—	0.00	—	7.83	—	6.73	—	8.17
Final lactate, μmols	—	0.68	—	6.46	—	8.83	—	5.83
Gas consumed (−) or evolved (+), μmols	—	−0.23	—	+6.03	—	+8.58	—	+5.75
Total glucose consumed, μmols	—		3.51		4.88		3.66	
Total lactic acid produced, μmols	—		5.71		7.94		5.23	
CO_2 release accounted for by lactic acid production	—		95%		93%		91%	
Glucose consumption accounted for by lactic acid production	—		81%		81%		71%	

* One gland was cut approximately in half and used for these two vessels. Gas phase 5% CO_2–95% O_2. Temp. 30.1° C.

measured chemically, for four experiments run under aerobic conditions and with glucose added to give 75 mg./100 ml. was 6.04 μmols per 100 mg. of tissue per hour. Under anaerobic conditions at the same glucose concentration and temperature the average lactate production for three experiments was 5.81 μmols per 100 mg. per hour.

A few experiments were performed on homogenates of glands. Glycolysis does not proceed readily in homogenates. In an experiment where lactate production was measured chemically a value of 0.60 μmols per 100 mg. of tissue per hour was found. In a companion run where ATP was added a value of 0.67

μmols was found. These values are roughly 10% of those found for the intact gland. The addition of magnesium does not alter the glycolytic behavior of homogenates. Factors other than these have not been studied.

Discussion

It is of interest that Bohr in 1894 studied the "disengagement of gases in the air bladder of fishes." Ten years later, working on mammalian blood with Hasselbach and Krogh (1904), he described the effect which now bears his name, namely that the oxygen affinity of hemoglobin is markedly affected by the CO_2 tension, or acidity of the blood. One can find no mention that Bohr was aware that the two phenomena he had studied might be related. Some 15 years later Bohr's student Krogh (Krogh and Leitch, 1919), showed that fish blood showed an exceptionally marked Bohr effect. As far as we can ascertain, Hall (1924) was the first individual to investigate the possibility that a relationship might exist between the Bohr effect and oxygen secretion into the swim bladder. Hall placed freshly excised intact glands of the yellow perch in five ml. of distilled water and determined colorimetrically the pH of the water after an interval of usually 30 minutes and reported an average value of 7.05. He did the same for glands removed from fishes whose glands had been previously stimulated by withdrawal of gas and found on the average a pH value of 6.38. Hall states (p. 112) "acidity is evidently produced in the epithelial cells and acid dialyzes into the capillaries. . . . Just what the nature of the effective acid is can not be stated at present."

Since then the concept that the Bohr effect plays a role in the formation of gases in the swim bladder has received considerable attention. The results of Hall were repeated by Akita (1936). Black (1946) studied the chloride content of the gland in order to learn whether HCl might be the acid involved. The role of carbon dioxide and carbonic anhydrase in the process has been implicated by Leiner (1940) and investigated by many workers (*cf.* Fänge, 1953). However, to the best of our knowledge lactic acid has not heretofore been considered as the acidifying agent.

The ability of the gas gland to convert glucose into lactic acid is such that this tissue must be rated along with those like retina as outstanding in this capacity. Expressed in terms of $Q_G^{N_2}$, μl. CO_2 expelled from bicarbonate per dry weight of tissue per hour, the gas gland of scup, as studied here, has had a range of values from 12 to 23 at 30° C. For fish retina at 30° C. Nakashima (1928) reports an average value of 29. However, it is interesting to note that in the presence of 95% O_2 this same worker reports that the value drops to 1.0. This effect of oxygen upon glycolysis is one repeatedly observed for many tissues and has received the name of the Pasteur effect. (*cf.* Lipmann, 1942 and Burk, 1935). It is therefore of particular interest that in our studies the rate of glycolysis of the gas gland has been found to be unaffected by the presence of oxygen. Such an absence of the Pasteur effect in the gas gland would, of course, be essential if lactic acid production were to play a role in the secretion of oxygen, especially when it is considered that in deep sea fishes oxygen tensions in the swim bladder may reach values in the range of 100 atmospheres.

A schematic representation of events leading to the introduction of gas into the swim bladder has been made in Figure 5 based on the assumption that lactic acid production plays a central role in the process. The entering arterial blood is portrayed as carrying in oxyhemoglobin matched against some cation (*e.g.* potassium), glucose and $NaHCO_3$. On reaching the gland glucose diffuses into it and is converted into lactic acid. The latter acidifies the gland and in turn the blood. This acidification of the blood causes a dissociation of the oxyhemoglobin with the release of O_2 and also converts some $NaHCO_3$ into CO_2. The tensions of both these gases in the blood are thus increased and they tend to diffuse across the glandular structures and into the swim bladder. The venous blood is also, however, rich in these dissolved gases and a large part of the O_2 and CO_2 would

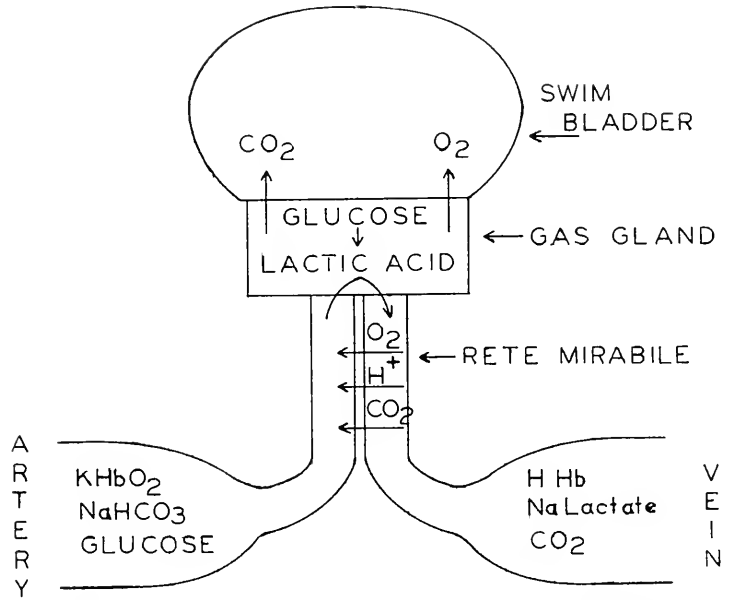

FIGURE 5. A schematic representation of the metabolism of the gas gland in relation to gas production and the role of the *rete mirabile*.

probably be carried away in solution in the blood if it were not for the *rete mirabile*. This amazing network of closely interwoven arterial and venous capillaries is portrayed to function as an exchange device along the lines suggested by earlier workers (*cf.* Jacobs, 1930). Here the outgoing venous blood equilibrates its diffusible components against the incoming blood. Thus O_2 and CO_2 tend to diffuse into the arterial blood and to presaturate the entering blood with these physically dissolved gases. The liberation of the bound O_2 and CO_2 by acidification as this presaturated blood reaches the gland thus further raises the pressure of these gases in the gland. As this cycle continues, higher and higher pressures will be built up which will facilitate the escape of gas into the swim bladder. It seems likely that there also will be a tendency for the H^+ concentration to equilibrate. There is thus also portrayed in Figure 5 the passage of H^+ ions from venous

to arterial blood in the *rete mirabile* which would cause a pre-acidification of the blood reaching the gland. Two other components whose concentration will differ in the venous and arterial blood are lactate and glucose. The latter is perhaps the more important since its equilibration would tend to lower the available supply of glucose to the gland. However, since the passage of glucose across the capillary wall of the rete may not be a simple diffusion process, no further consideration of this factor will be made at this time.

It is perhaps important to point out that such an acidification mechanism could account for the accumulation of both O_2 and CO_2 in the swim bladder. The CO_2 content of the swim bladder appears to be highest during periods just after the gland has been very active (Jacobs, 1930). We are not prepared at this time, however, to state categorically that such a mechanism alone is able to account for the very high gas pressures found in the swim bladders of fish taken at great depths (*cf.* Scholander *et al.*, 1951, 1953). It is of interest to note, however, that Jones and Marshall (1953) have pointed out that both the gas gland and the *rete mirabile* are much more highly developed in bathypelagic teleosts than in shallow water forms. The marked increase in the length of the rete of the deeper water forms as shown in their drawings is indeed most striking and suggests that the equilibration of venous and arterial blood plays an important role in the attainment of high gas pressures.

In the case of those fishes which also appear to concentrate nitrogen to some extent in their swim bladder (*cf.* Scholander and van Dam, 1953), some mechansim in addition to that presented would seem to be required. One could postulate that the hemoglobin of such fishes has the ability to combine slightly with N_2 as well as O_2 and that acidification releases both gases in a constant ratio depending on the species as found by Scholander and van Dam (1953). This would not, however, explain the unusual situation found in *Coregonus* where the nitrogen content of the swim bladder gases reaches nearly 100% (*cf.* Scholander and van Dam, 1953).

Assuming that a mechanism of the type proposed is in operation, a calculation is possible of the order of magnitude for the rate of oxygen production by the gas gland *in vivo*. The average blood sugar of 20 scup is reported by Gray and Hall (1930) to be 53 mg. per 100 ml. The average rate of glycolysis *in vitro* of the gas gland as determined here at a glucose concentration of 75 mg. per 100 ml. may be taken as 4.0 μmols or 0.72 mg. of glucose per hour per 100 mg. of tissue (*cf.* Table V). Thus, if the gas gland utilizes glucose at the same rate *in vivo*, it would require that at the minimum $0.72/53.0 \times 100$ ml., or 1.3 to 1.4 ml. of blood be supplied to 100 mg. of gland per hour. This assumes that all the glucose in the blood will be utilized, which seems doubtful. Let us assume, therefore, that the blood flow is twice this minimum value or 2.8 ml. per hour per 100 mg. of gland. The mammalian kidney has a blood flow of the order of 9.0 ml. per 100 mg. of tissue per hour (Best and Taylor, 1945). If we take the value given by Root (1931) for the O_2 content of scup blood as 7.3 vol.%, it is possible to calculate that 0.2 ml. of O_2 per hour is supplied to each 100 mg. of gland when it has a blood flow of 2.8 ml. per hour. The oxygen consumption of 100 mg. of gland *in vitro* is of the order of 0.03 ml. per hour (*cf.* Table I). There would thus remain 0.17 ml. of O_2 per hour to supply the swim bladder.

The time required to fill a swim bladder with oxygen on this basis can also

be calculated. Expressed as the average of eight determinations, we have found in the scup that the bladder volume is 4.6% of the body weight (*cf.* Jones and Marshall, 1953) and there are 9.1 ml. of bladder volume per 100 mg. of gas gland tissue. Thus, with an oxygen supply of 0.17 ml. per hour per 100 mg. of gland it would require 9.1/0.17 or about 54 hours for the scup to fill its swim bladder with 100% oxygen at one atmosphere of pressure.

These calculations are based on metabolism studies at 30° C. and are subject to criticism on this point as well as on many others. They do, however, furnish some clues as to the rate that might be expected if such a mechanism is in operation. A comparison with some *in vivo* data given in the literature is possible. Jacobs (1930) reports data on *Perca fluviatilis* which indicates that 2–4 days are required for this species to completely refill its swim bladder after it has been emptied by puncture. The gas in the swim bladder at this time was approximately 50% oxygen. Bohr (1894), working with *Gadus callarias*, found that replenishment after puncture was complete in 24–48 hours with the O_2 reaching, in some cases, 80% of the total re-formed gas. The value of 54 hours calculated above to completely fill the swim bladder in the scup with 100% oxygen is thus not very far out of line with these *in vivo* observations.

A similar set of calculations may be made for the amount of lactic acid poured into the blood by the gland, using the same premises as employed for the oxygen calculations. The result is that about 3 μmols of acid are added to each ml. of blood that passes through the gland. Scup blood contains about 4 μmols of bicarbonate per ml. (Root, 1931). The magnitudes of the other buffering components of scup blood are not known to us so a calculation of the expected pH shift in the blood is not possible at this time.[3]

Summary

1. Metabolic studies have been carried out on the gas gland of the swim bladder of the scup, *Stenotomus chrysops*, at 30° C.

2. The intact gland has a $Q_{O_2} = 2.0$ which is not appreciably altered by the addition of glucose. Homogenates of the gland have a much lower rate of oxygen consumption.

3. The intact gland converts glucose to lactic acid at a rapid rate. This conversion has been followed by measurement of glucose disappearance, lactic acid appearance and the release of CO_2 from a bicarbonate medium. Values of $Q_{N_2}^{CO_2}$

[3] Since submitting this manuscript for publication there have appeared two interesting articles bearing on the subject discussed here. In one, Scholander and van Dam (1954) present data which indicate that oxygen remains combined with some fish hemoglobins at high oxygen pressures even when the blood pH is lowered by the addition of lactic acid to a value of 5.7, or CO_2 tensions are increased. The effect of a combination of low pH and high CO_2 tension, such as might be encountered *in vivo*, is not reported. They interpret their results as throwing serious doubt on the participation of the Bohr effect in the accumulation of oxygen in the swim bladder. In the other, Scholander (1954) discusses in detail the possible function of the *rete mirabile* and by means of a mathematical analysis shows that very great pressures could be built up in the swim bladder if the rete acts as an exchange device. By the same token it would seem possible that a very marked acidification of the blood reaching the gland could also be achieved if both CO_2 and hydrogen ions were exchanged between the venous and arterial blood. It is obvious that data are sorely needed on the oxygen content of blood entering and leaving an active gland.

ranging from 12–23 have been found. Lactic acid appearance accounts for 70–90% of the glucose that disappears. The gland does not show a Pasteur effect since similar values are obtained under either aerobic or anaerobic conditions.

4. The rate of anaerobic glycolysis is greatest at an alkaline pH and is markedly influenced by the cation composition of the medium. Activities in an all-sodium medium are 3–4 times those in an all-potassium medium.

5. A schematic diagram of the possible role of lactic acid production in the events leading to the accumulation of gas in the swim bladder is presented and calculations on the rates of oxygen production to be expected under such circumstances are made.

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A NOTE CONCERNING THE DISTRIBUTION OF POLYSACCHARIDES IN THE EARLY DEVELOPMENT OF THE HYDROMEDUSAN PHIALIDIUM GREGARIUM [1, 2]

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Since many organisms are actually supported by polysaccharides, that is, they form the framework for the architecture of the plant or animal, it was thought that it would be most interesting to follow the distribution of polysaccharides during the course of development, from egg to polyp, in the hydromedusan *Phialidium*. Fortunately, there is a simple and dependable histochemical technique to demonstrate these substances, namely, the periodic acid-Schiff method and, as will be shown, the non-starch polysaccharides are present in large amounts throughout the development and no special region of secretion of the external chitinous perisarc can be seen. On the other hand, there is a most striking and curious pattern associated with the attachment process of the planula larva.

Methods

The jellyfish were collected in the evening at a night light off the dock of the Laboratory of the University of Washington, at Friday Harbor, in Puget Sound. They were immediately placed in fresh sea water in finger bowls (100 mm. diameter) on the water table and kept at approximately 13° C. Six to a dozen jellyfish were placed in each bowl and these were removed the next day so that the eggs which had been shed could develop. For the attachment stages, two per cent agar in sea water was prepared to cover the bottom of clean finger bowls. Early planulae were transferred to these dishes, and when they attached to the agar, they could be easily removed without being damaged and transferred to the fixative.

The embryos were fixed in Bouin's fluid at 2° C., dehydrated in a series of graded alcohols, and embedded in paraffin. Polysaccharides were demonstrated by the periodic acid-Schiff technique (Gomori, 1952). The best results were obtained when a three-hour period of salivary digestion, which removes all the glycogen, preceded the staining. Control slides were run without the oxidation which showed an evenly distributed, very faint pink tinge.

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and J. T. Bonner, NR 164–274.

[2] It is possible that some of these observations were made on individuals of the closely related species, *P. hemisphericum*, for it is likely that the two species are both present in Puget Sound. Specific characters include the numbers of tentacles and marginal vesicles, and since these structures vary within rather wide limits, certain identification rests upon the examination of large numbers of individuals and the rearing of the hydroid stages.

I am indebted to Dr. D. L. Ray of the University of Washington for this information and for her kindness in reading the manuscript.

Results

As can be seen from Figure 1, in the egg there is a fairly even distribution of non-starch polysaccharide located primarily in large granules and this same pattern persists throughout cleavage as well as in the blastula stage. Even in the beginning of gastrulation, as the future endoderm cells wander into the blastocoel at one pole, there is no major difference among the cells in their staining intensity.

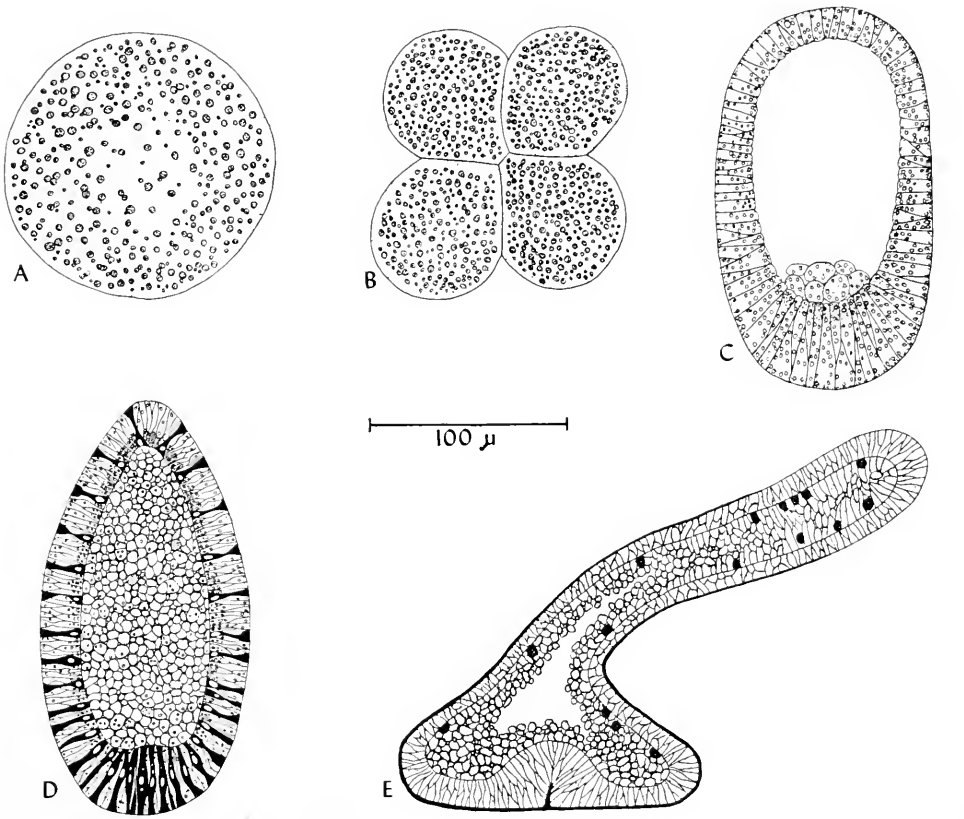

Figure 1. Drawings showing the distribution of non-starch polysaccharide in the development of *Phialidium*. The intensity of the black reflects the concentration of the polysaccharide. A, egg; B, 4-cell stage; C, 23 hours after fertilization showing the beginning of gastrulation by polar ingression (note that in this drawing and the next, the anterior end which attaches to the substratum is pointing downward); D, a late planula larva 60 hours after fertilization; E, a young attached larva just prior to the appearance of annulations in the chitinous perisarc.

It is only well after the gastrulation is complete and the motile planula shows contractile worm-like movements and ciliary activity, that there is a beautiful pattern of intensely stained goblet cells distributed within the entire ectodermal layer.

These cells are clearly more concentrated in the blunt anterior pole, in fact, in some larvae they appear so dense in that region that they almost lie side by side in a solid mass. The tapering, posterior end (which will later become the

hydranth end of the developing polyp) has few or none of these cells and there is a gradient of increasing goblet cell concentration as one passes anteriorly. In a surface view it is striking to see that these trumpet-shaped cells are spaced in an orderly fashion, each occupying the position farthest removed from all its neighbors. At this stage there are no especially intense staining regions in the endoderm.

Upon fixation the planula attaches its anterior end to the substratum and proceeds rapidly to flatten like a pancake. Almost immediately, hard chitinous material is secreted about this flattened mass and then some hours later a small nipple appears at the location that was the posterior end. This nipple, which is of a fixed diameter, continues to elongate (probably largely by mass cell migration or morphogenetic movement), showing annulations in the perisarc as it proceeds, and this is the stem or coenosarc of the future primary hydranth that eventually appears at the apical end.

Once fixation was accomplished and the first chitinous material surrounded the flattened embryo, there was no evidence of the intensely stained trumpet-shaped cells at all. They had apparently given off all their material in the fixation process, and we might assume from this that these gland cells have during the course of evolution been perfected in the planula solely for the function of fixation. The fact that planulae possess gland cells is well known and described in the early literature (see, for example, Wulfert, 1902) but their orderly distribution over the surface, their activity in relation to fixation and the production of polysaccharides was never specifically examined.

It is particularly surprising that the rounded tip of the young coenosarc which rises upward, does not have a special zone of secretion, for it is there that the chitinous wall is primarily deposited. The cells of this rising nipple appear evenly stained and, therefore, are high in polysaccharide content but they have no special gland cells nor any clearly defined region of deposition. In the endoderm, lying near the mesoglea, there are round masses of more intensely stained material, but because of their internal location, it is hard to understand what function they might have.

Summary

The distribution of non-starch polysaccharides was studied from egg to attached polyp in the hydromedusan *Phialidium gregarium* and it was found that the polysaccharides were fairly evenly distributed throughout the development except in the late planula. There a number of gland cells richly supplied with polysaccharide appear in the ectoderm and show a gradient—sparse in the posterior end and dense in the anterior attachment region. These cells are apparently concerned with fixation, for after the larva has attached and secretes a hard chitinous covering, these ectodermal concentrations of polysaccharide are no longer visible.

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THE EFFECT OF RADIATION FROM SMALL AMOUNTS OF P^{32}, S^{35} AND K^{42} ON THE DEVELOPMENT OF ARBACIA EGGS [1]

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In the recent extensive use of isotopes in biological research, little concern has been manifested for the effects of trace amounts of radioactive isotopes on cellular processes. Radioactive tracer studies in whole animals are usually not controlled at the level they seek to elucidate and few studies involving single cells have been designed to determine minimum levels of radioactivity which affect synthetic and developmental processes. Kamen (1947) states (p. 125), "It must be emphasized that tracer radiations in themselves constitute an ever present hazard in research on metabolism because marked physiological effects invalidating conclusions can arise if high tracer concentrations are used." Unfortunately, as he points out, little systematic information on the effects of low radiation dosage is available and what knowledge exists indicates marked variability in sensitivity in different forms. The dose range to produce various biological effects varies from 10 to 10^6 roentgens (Patt, 1953).

Thus, although our knowledge of radiation effects is large (see Henshaw, 1944; Lea, 1947; and Gray, 1952), little attention has been devoted to a study of the effects of ionizing radiations emitted from trace amounts of radioactive isotopes upon single cells. That small amounts of radiation may have significant effects on *Paramecium* has been demonstrated for x-radiation by Hance and Clark (1925), and more recently for β-radiation by Daniel and Park (1953). Packard (1916) reported acceleration of cleavage in *Arbacia* treated with short exposures to radium.

Mullins (1939), working with *Nitella*, found that concentrations of Na^{24} above one microcurie (μC) per ml. decreased the amount of ion penetration into these cells. Brooks (1943) determined the uptake of P^{32} and Na^{24} in *Arbacia* and *Asterias* eggs. His P^{32} concentrations ranged from 0.045 to 0.29 μC per ml. and because the amount of β-radiation emitted by the P^{32} was less than that observed by Mullins (1939) to affect *Nitella* permeability, he assumed that the radiation was not a factor in his measurements. In a later study (Brooks and Chambers, 1948) the P^{32} uptake in several species of Pacific coast sea urchins was investigated using concentrations of 0.001 to 0.3 μC per ml.

The present study was undertaken to determine the dosage levels of P^{32}, S^{35} and K^{42} upon eggs or sperm which would have an effect on the cleavage and subsequent development of *Arbacia* eggs. The results indicate that radiation from

[1] This project was supported in part by a grant from the United States Atomic Energy Commission, Contract AT(30-1)1069.

P^{32}, S^{35} and K^{42} at dose levels ranging from 0.25 to 50 μC per ml. (which includes the range used by others for permeability studies) has an accelerating or retarding effect on the first cleavage, depending upon the dose, and also retards subsequent development of the plutei.

Methods

Eggs and sperm of *Arbacia punctulata* were prepared in the manner described by Blum and Price (1950).

The isotopes used in this study were S^{35}, P^{32} and K^{42}.[2] Sea water solutions of each of these were prepared by evaporating carrier-free isotope solutions to dryness and dissolving the isotope residue in sea water. The pH of such solutions was the same as that of sea water. Stock solutions of S^{35} were prepared weekly in concentrations of 0.5, 5.0 and 50 μC per ml. P^{32} solutions were prepared in the same concentrations and used within two days. The K^{42} solutions were prepared in a concentration of one μC per ml. and used immediately.

The experiments were performed in the following manner: four 25-mm. stender dishes were placed in running sea water to a depth of one cm. to keep the contents at the temperature of the sea water. To dish one was added one ml. of filtered sea water; to dishes two, three and four were added one ml. of filtered sea water containing isotope concentrations of 0.5, 5.0 and 50 μC per ml., respectively.

When the object of the experiment was the irradiation of sperm, one or two drops of dilute sperm suspension were added to each of the dishes. Generally after two hours, one ml. of freshly washed eggs was then added, the time noted, and samples of the cleaving eggs taken at two-minute intervals for 14 minutes after cleavage began in the control. One-drop samples were preserved in 5 per cent formalin in sea water in shell vials for later counting. The per cent cleavage for a given time interval was determined by counting 100–200 eggs in each sample period. Egg irradiation experiments were performed in the same manner as the sperm experiments. One ml. of a dilute suspension of freshly washed eggs was added to each dish. Such additions diluted the isotope concentrations by one half so that eggs were exposed to 0.25, 2.5 and 25 μC of radioactivity per ml. Doubling the concentration of the eggs had no effect on the results. All experiments were carried out in duplicate. The temperatures of the control and experimental solutions varied from 22° to 24° C. but did not vary more than one half degree C. during any one experiment.

Irradiated sperm were always fertilized with non-irradiated eggs and irradiated eggs with non-irradiated sperm. No experiments were performed in which irradiated eggs were fertilized with irradiated sperm. After preserving samples of the first cleavage, the remaining zygotes in the dishes were permitted to develop. When control forms had reached the pluteus stage, usually after 48–72 hours, samples of all dishes were preserved in formalin sea water for comparison. It should be noted that after fertilization the developing larva remained in the same isotope environment as the irradiated gametes.

[2] The isotopes used in the study were obtained on allocation from the Isotopes Division, United States Atomic Energy Commission. The authors would like to acknowledge the helpful assistance of Mr. S. Berman in preparation of the isotope solutions.

Results

Preliminary experiments utilizing P^{32} had been previously carried out and reported upon (Green and Roth, 1950), but the data reported here were obtained during the summer of 1953.

Figure 1 is a plot of the per cent first cleavage against time of *Arbacia* eggs fertilized with sperm which had been irradiated for two hours prior to fertilization by three dose levels of S^{35}. The graph shows that the time of cleavage is delayed

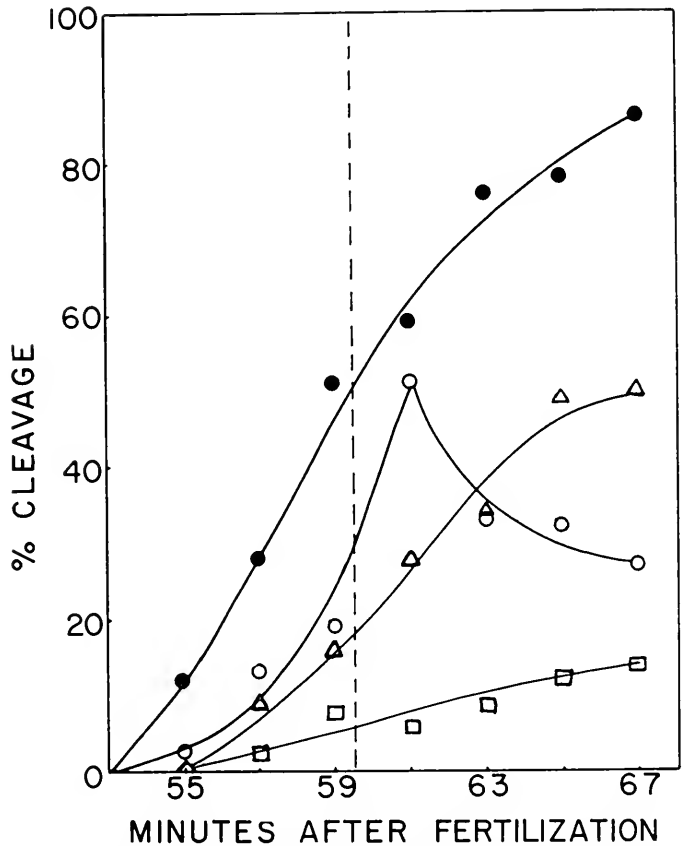

Figure 1. Plot of per cent first cleavage against time when sperm were irradiated for two hours prior to fertilization of non-irradiated eggs. ● = non-irradiated; ○ = 0.5 μC per ml. S^{35}; △ = 5.0 μC per ml. S^{35}; □ = 50 μC per ml. S^{35}.

by prior irradiation of the sperm, roughly in proportion to the dosage of radioactivity. The decrease in per cent cleavage in the case of the 0.5-μC dose after 61 minutes does not represent an error in counting but is probably attributed to experimental variation.

Figure 2 is a plot similar to Figure 1 and shows the results of irradiation of eggs for two hours with three dose levels of S^{35} prior to fertilizing with non-

irradiated sperm. Although the dose levels shown in Figure 2 are different from those of Figure 1, there is some overlapping and one can readily see that egg irradiation prior to fertilization has a less marked retarding effect on subsequent cleavage than does the irradiation of sperm. Figure 2 shows that the lowest dose level had an accelerating effect on cleavage which may not be too significant. The vertical dotted line appearing in both Figures 1 and 2 indicates the per cent

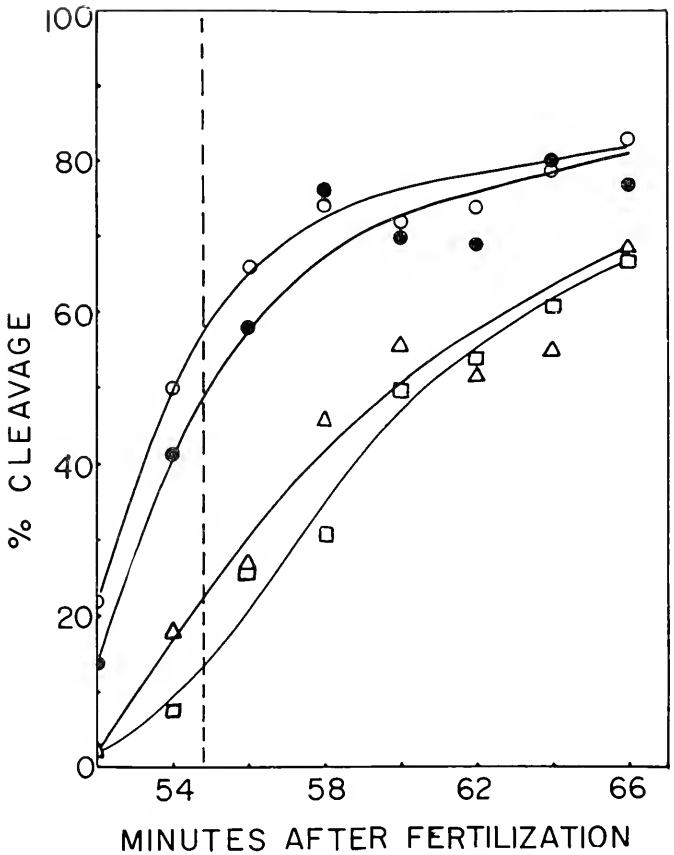

FIGURE 2. Plot of per cent first cleavage against time when eggs were irradiated for two hours prior to fertilization with non-irradiated sperm. ● = non-irradiated; ○ = 0.25 μC per ml. S^{35}; △ = 2.5 μC per ml. S^{35}; □ = 25.0 μC per ml. S^{35}.

cleavage attained by the experimental cells when the control cells had reached 50 per cent cleavage.

The data presented in Table I were obtained from graphing the results of a number of experiments in the manner of Figures 1 and 2. The amount of retardation or acceleration of cleavage by the irradiation of unfertilized gametes is presented as the per cent cleavage of the experimentally treated eggs at that time when 50 per cent of the control eggs had cleaved. This method of expressing the results was chosen because most of the egg suspensions treated with the higher

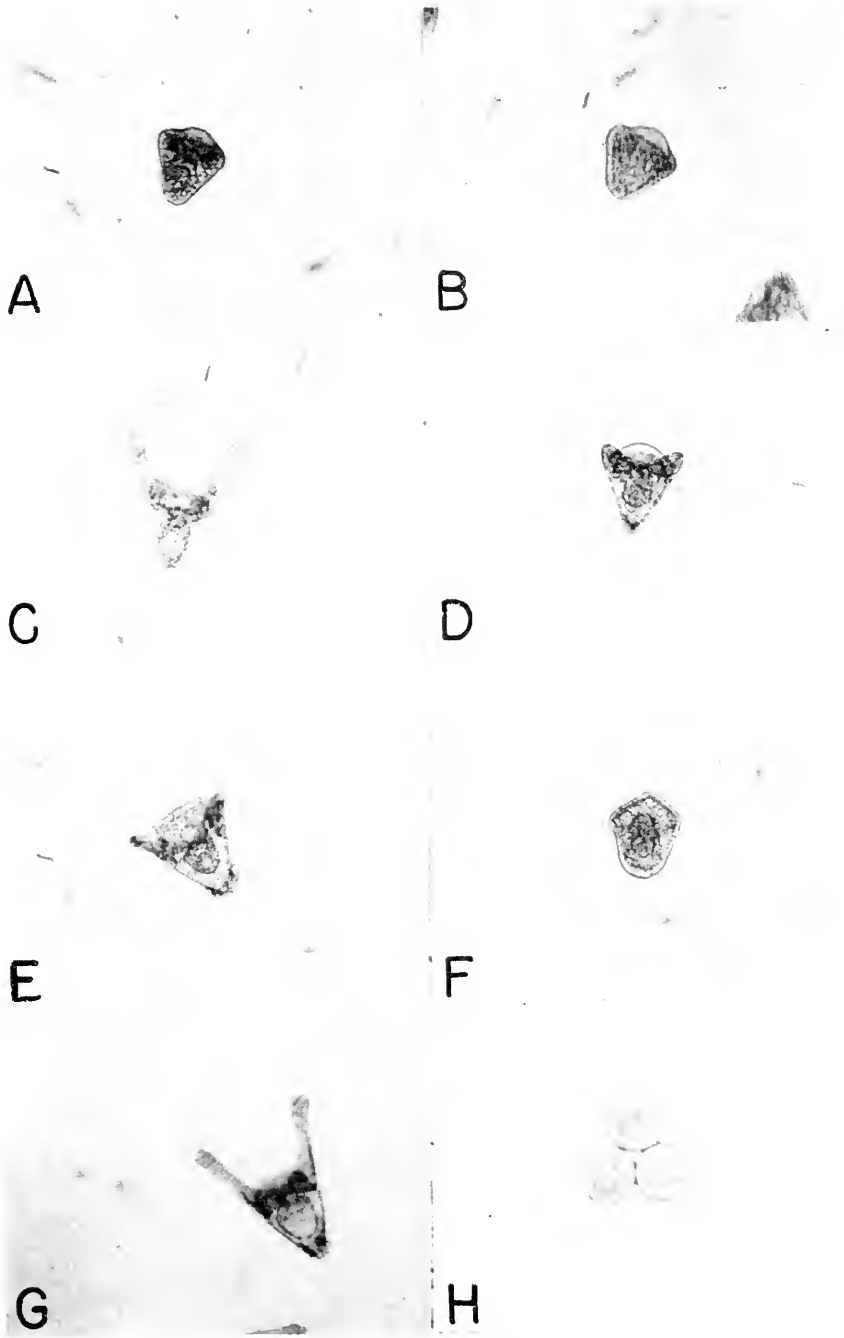

FIGURE 3. For explanation see text under "Results."

radiation doses did not attain 50 per cent cleavage during the period of observation. In general, duplicate experiments with eggs and sperm from different female and male *Arbacia* are given in the table. And, although the results from such experiments show variation, the direction and order of magnitude of the effects were frequently duplicated.

Figure 3 shows a series of photographs of developing larvae of *Arbacia* in which the effects of sperm and egg irradiation are compared. Photographs A, C, E and G are of non-irradiated cells and should be matched with photographs B, D, F and H, respectively, of corresponding irradiated cells. Egg irradiation is shown by photograph B (0.25 μC per ml. of S^{35}) and F (0.25 μC per ml. of P^{32}). In neither case are the plutei strikingly different from their corresponding

TABLE I

Effect of ionizing radiations on Arbacia egg cleavage

Date	Exp. No.	Isotope	Gamete irrad.	Prefert. expos. hrs.	Per cent of experimental eggs cleaved when 50% of controls have cleaved						
					Exposure dosage—microcuries/ml.						
					0.25	0.5	1.0	2.5	5.0	25.0	50.0
8/29/53	14A	P^{32}	Egg	2	60			43		27	
8/29/53	14B	P^{32}	Egg	2	55			50		15	
8/28/53	13	P^{32}	Sperm	2		14			0		0
7/22/53	5A	S^{35}	Egg	0	61			40		74	
7/13/53	2A	S^{35}	Egg	1	55			47		40	
7/13/53	2B	S^{35}	Egg	1	40			40		25	
7/17/53	4A	S^{35}	Egg	2	60			21		15	
7/17/53	4B	S^{35}	Egg	2	67			60		63	
7/24/53	6A	S^{35}	Sperm	2		25			15		5
7/24/53	6B	S^{35}	Sperm	2		32			13		6
8/22/53	9B	K^{42}	Egg	2		30					
8/21/53	10A	K^{42}	Sperm	2			10				
8/21/53	10B	K^{42}	Sperm	2			14				

controls. However, the effect of P^{32} was somewhat greater, as might be expected from the greater energy of its β-radiation. Sperm irradiation is shown by photographs D (0.5 μC per ml. of S^{35}) and H (0.5 μC per ml. of P^{32}). Both photographs indicate an appreciable retardation in growth as compared with corresponding controls (photographs C and G). These pictures support the data in Table I in showing that egg irradiation prior to fertilization has a less marked effect on subsequent cleavage and development than sperm irradiation.

DISCUSSION

It is rather difficult to calculate the exact exposure in terms of roentgens or equivalent roentgens (e.r.) of an individual egg or spermatozoan. The total

radiation dose in the solution during two hours, when radiation equilibrium obtains, may be calculated by the methods of Marinelli, Quimby and Hine (1948). For concentrations of 0.5, 5.0 and 50 μC of P^{32} per ml. this would be 1.74, 17.4 and 174 e.r., respectively, and for S^{35}, 0.139, 1.39 and 13.9 e.r., respectively. However, because of the small volume and thickness of solution used, the condition of "radiation equilibrium" was not fulfilled (especially in the case of P^{32}) and therefore the doses actually received by the eggs or the sperm were considerably less than those given above. This is particularly true in the case of the eggs, which lay at the bottom of the dishes and consequently were irradiated essentially from one side. In the case of the developing larvae in the presence of the higher concentrations of isotope for from 48 to 72 hours, the radiation dose might be appreciable, however. Daniel and Park (1953) found that solutions of S^{35} delivering from 0.77 to 3.13 e.r. per hour under radiation equilibrium conditions, stimulated *Paramecium* division. Their dose levels are well within the range of activities observed by us to be stimulatory to the *Arbacia* egg.

The date reported by Table I clearly show that cleavage delay results on irradiation of sperm with low levels of β-radiation. This finding is supported by the work of Mitueo *et al.* (1939) on eggs and sperm of *Pseudocentrotus depressus* exposed to β-rays from radium emanation, and was found to be true for *Arbacia* gametes irradiated with x-rays (Henshaw and Francis, 1936). Both Henshaw and Mituo irradiated testicular mashes and their results are subject to possible effects of so-called "necrohormones" (Heilbrunn and Young, 1935). Our studies were performed, however, on quite dilute sperm suspensions so that the indirect effects observed by Heilbrunn are not applicable.

The effects of low dose levels of β-radiation upon *Arbacia* zygotes and developing embryos cannot be determined by the results of Table I or Figure 3. The retardation of cleavage from sperm irradiation is continued into the pluteus stage but as all zygotes (egg-irradiated ones also) remained in the radiation environment of the gametes, the effects of gamete irradiation and zygote irradiation cannot be separated. One possible exception to this is the experiment performed in which *Arbacia* plutei were obtained after K^{42} irradiation of gametes. In this case because of the short life of this isotope, radiation levels could be expected to fall well below injury levels before plutei were formed. Egg irradiation had no effect on plutei formation in this experiment, while sperm irradiation resulted in the development of no plutei, although some initial cleavage occurred.

The knowledge that trace dose levels of these isotopes may cause biologically abnormal effects in *Arbacia* cannot be readily transferred to other types of cells. But this knowledge does carry the implication that rapidly dividing cells in the presence of radioisotopes, or, more important, cells which accumulate isotopes, may be modified in a subtle manner by the radiation emitted by these isotopes, even though the particular process studied by the investigator does not appear to be altered. A greater awareness of potential cell damage from radioactive tracers should lead to further investigations and to more systematic knowledge of limitations of these tools in biological research.

The authors would like to acknowledge the helpful assistance of Miss Mary Hodge in performing the egg counts.

Summary

Arbacia gametes (eggs or sperm), exposed to radiation from S^{35}, P^{32} and K^{42} at dose levels ranging from 0.25 to 5.0 μC per ml. and subsequently fertilized with non-irradiated gametes, formed zygotes whose first cleavage was accelerated or retarded, depending upon the dose, and whose further development was slowed at the higher dose levels. Some experimental exceptions to these findings are reported and discussed. The implications of the results for biological tracer methodology are stated.

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SYNTHESIS OF DESOXYRIBONUCLEIC ACID IN LETHAL AMPHIBIAN HYBRIDS [1]

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It has been found that by cross-fertilization between certain combinations of related amphibian species lethal hybrids arise (Baltzer, 1934; Brachet, 1944; Moore, 1946). These lethal hybrids stop developing at a stage corresponding morphologically to early gastrula. Microscopical examination has shown that various abnormalities occur in the hybrid embryos, *e.g.* irregular mitoses and pycnosis (Schönmann, 1938; Brachet, 1944). When the controls have reached stage 13 (early neurula) all the cells, except those in the blastoporal region, have died (Schönmann, 1938; Moore, 1948).

The results of Hoff-Jørgensen and Zeuthen (1952; *cf.* also Løvtrup, 1954) have disclosed that no increase in total DNA occurs during the early development, up to a late blastula stage. This can only mean that the DNA used in the formation of new nuclei during the early stages of development must be present as a reserve in the mature oocyte. Hoff-Jørgensen and Zeuthen could demonstrate that the size of this store corresponds to the DNA content in about 2700 diploid nuclei in *Rana platyrrhinus*, and that it, to a major extent, is localized in the cytoplasm. It is a quite obvious suggestion that the developmental block observed in hybrids may reflect the lack of ability to synthesize substances necessary for continued development. On the basis of the observations mentioned above it would seem possible that DNA might be one of these substances. In that case one might expect the hybrid embryos to develop until the DNA reserves were exhausted. Although there is a discrepancy as to the morphological stage at which the DNA exhaustion and the developmental block occur, it was still considered worth while to test this possibility.

A necessary condition for doing this work was that the DNA analyses were carried out by the microbiological assay of DNA (desoxyribosides) worked out by Hoff-Jørgensen (1951), as the other available methods of DNA determination seem to be too unspecific or too insensitive to estimate DNA when present in such low concentrations as in early amphibian embryos (*cf.* the discussion by Løvtrup, 1954).

Material and Methods

Embryos

Normal *Rana pipiens* embryos, and *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrid embryos obtained by stripping *R. pipiens* eggs directly from the same female

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donors of control eggs into suspensions of *R. sylvatica* sperm, were reared in 10% Ringer's solution, without bicarbonate or phosphate, at a temperature of 15° C.

Preparation of dry samples

A single tube of dry material was prepared as follows. Jelly was removed with forceps from 25 embryos, which were then pipetted into a 10 × 75 mm. test tube. Excess medium was removed, and the tube nearly filled with acetone. The embryos were then crushed thoroughly with a ball-tipped glass rod. The tube was then centrifuged, the supernatant decanted, the precipitate re-suspended in acetone, re-centrifuged, decanted again, and left in a desiccator over calcium chloride for 24 hours. The open end of the tube was then drawn out and sealed off, and the tube stored until analyses for DNA could be made.

Hybrid and control embryos were prepared simultaneously. Each tube was labelled with the developmental stage number (Shumway, 1940) of the controls and with a designation of the time (in hours) elapsing since fertilization.

Determination of DNA [3]

The principle of the microbiological DNA assay is that a certain strain of lactic acid bacteria, *Thermobacterium acidophilus* R 26, is unable to grow in the absence of desoxyribosides. The extent of growth is almost proportional to the concentration of these substances in the culture medium. By measuring the growth obtained after adding an aliquot of the biological material to a culture medium free of desoxyribosides, it is possible to estimate the content of DNA. Before the microbiological assay is carried out the DNA in the samples is first hydrolyzed by the following procedure: To each tube of dry material is added 0.5 ml. of 0.5 N NaOH, and the tube placed in a boiling water bath for 15 minutes. After this is added 2.5 ml. buffer-activator solution (0.06 *M* maleic acid; 0.01 *M* $MgSO_4$), and the pH is checked and adjusted to 6.5–7.0 if necessary. Then 0.1 mg. crystalline desoxyribonuclease, contained in 0.1 ml. is added, and the tubes incubated 20 hours at 37° C. After this the sample is transferred to a volumetric flask, the volume made up to 5 ml., and DNA assayed as described by Hoff-Jørgensen (1951).

It may be argued that this method is not specific for DNA, as it determines the sum of DNA and free desoxyribosides. It should be noticed, however, that determinations on extracts not treated with desoxyribonuclease generally give negligible values (*cf.* the discussion following the paper by Hoff-Jørgensen, 1954). This observation thus leads to the not surprising conclusion that free desoxyribonucleosides are rarely found in the cells, even in embryos. It seems reasonable, therefore, to apply the name "DNA" to the substances determined by the present method, namely, those which liberate desoxyribonucleosides upon treatment with desoxyribonuclease. This does not imply anything with respect to the degree of polymerization of the substances, about which nothing is known. However, there is certainly no reason to believe that it is present in a high-polymeric form, ready for incorporation in the nuclei arising during segmentation. From a genetical point of view it would rather seem required that of the DNA incorporated during

[3] All the determinations of DNA were carried out in the laboratory of Dr. E. Hoff-Jørgensen. We gladly acknowledge our gratitude for this assistance.

the early development at least 50 per cent, *viz.* the DNA in the male chromosomes, be synthesized from rather low-molecular substances. The constancy of DNA during segmentation should not, therefore, be taken to demonstrate that no DNA synthesis occurs. Rather it indicates that DNA is synthesized at the expense of preformed desoxyribosides, and that no *total* synthesis of DNA occurs.

Results

The results are shown in Figure 1. The DNA content at the beginning of development is 0.063 ± 0.002 μg (standard deviation: ± 0.005 μg). This value is calculated as the average of five determinations (only three of these are shown

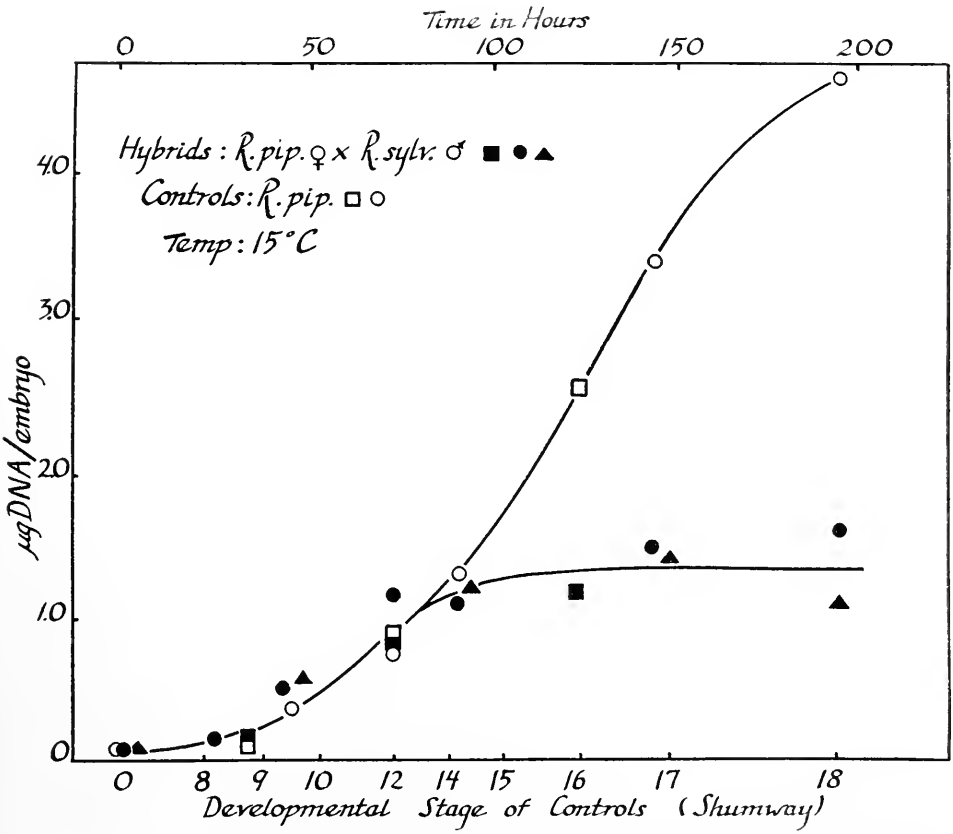

Figure 1. Desoxyribonucleic acid content of normal and hybrid embryos at various stages of development.

in the figure) on eggs from 2 to 4 hours after fertilization (2–8 cells). The content is the same in hybrids (0.065; 0.065) and in controls (0.068; 0.059; 0.058). According to Sze (1953) the DNA content of a diploid nucleus in *R. pipiens* is 10.4×10^{-6} μg. The reserve would thus correspond to about 6000 nuclei.

At the end of segmentation the content of DNA begins to rise in both hybrids and controls, the rate of synthesis increasing gradually for about 50 hours, *i.e.*, until the controls have reached stage 14 (Shumway). At this time the synthesis of DNA ceases in the hybrids, the final content calculated as the average of the last five values being 1.31 ± 0.08 μg DNA (standard deviation ± 0.17 μg). In the controls synthesis continues at constant rate (linear increase) until between stages 17 and 18, when a decrease in rate of synthesis is observed. There are rather few points on the control curve, but the shape of the curve is exactly the same as found in *R. platyrrhinus* (Hoff-Jørgensen and Zeuthen, 1952; Løvtrup, 1954). It should also be mentioned that the absolute amounts are approximately the same in the two species; thus the content in the oocyte of the European species is about 0.065 μg according to the former authors. As the DNA content per nucleus is almost twice as high in *R. platyrrhinus* nuclei, the reserve supply corresponds to that of only 2700 nuclei. The highest value found in *R. pipiens* is 4.62 μg at stage 18, and in *R. platyrrhinus* a value close to 5 μg was found (Hoff-Jørgensen and Zeuthen, 1952; Løvtrup, 1954).

Discussion

It is seen right away that the possibility mentioned in the introduction has been disproved by the results, *i.e.*, the hybrid embryos are able to synthesize DNA, and do so at normal speed until the early neurula stages have been reached.

The results obtained thus clearly demonstrate a dissociation between DNA synthesis and morphological development. The next question to consider is whether there is a dissociation between DNA synthesis and cell division (formation of new nuclei). During development the DNA of the hybrids is increased about 20-fold, corresponding to 120,000 cells. Were cell division lagging behind DNA synthesis to any appreciable extent, an accumulation of DNA would occur. No indication of such accumulation has been observed (*cf.* Schönmann, 1938; Brachet, 1952), but this unfortunately cannot be considered decisive evidence, as DNA may not always be detectable by cytochemical methods. Thus the DNA reserve in the amphibian oocyte cannot be demonstrated by the Feulgen reaction (*cf.* Brachet, 1952, and the discussion in Løvtrup, 1954).

The decrease in cell size which takes place during early amphibian development seems, according to Schönmann, to occur also in the hybrids. Without settling this question definitely, this observation seems to suggest that cell division proceeds normally, in spite of the abnormal mitoses.

It has been observed that the animal (blastoporal) region survives longer than other parts of the hybrid embryos (Baltzer, 1934). It has been shown by transplantation that both inductive power and capacity for further differentiation are not lost (Lüthi, 1938; Brachet, 1944; Moore, 1947, 1948). As the DNA synthesis proceeds normally in the hybrids until the early neurula stages, it would seem reasonable to suggest that this synthesis corresponds to cell divisions localized in the blastoporal (animal) region. The major part of the cells formed here will in the normal embryo invaginate and form the mesoderm. If this interpretation is correct, it seems possible from our results to divide the pre-larval DNA synthesis (cell division) into two phases, the first of which (mesodermal) is found in both hybrids and controls. The initiation of the second (linear) phase ap-

parently is conditioned by a normal gastrulation. It would seem that this second (ectodermal) phase to some extent is associated with the development of the neural tissues, epidermis, etc. It is interesting in this connection to note that a third phase of DNA synthesis, correlated at least partly with the development of the endoderm, has been found to begin during early larval development (*cf.* Løvtrup, 1954).

Much work has already been devoted to the study of the physiology and biochemistry of hybrid development. Thus the results of Steinert (1951) indicate that RNA also is synthesized in hybrid embryos, although to a lesser extent than in the controls. This finding is in agreement with the fact that very little RNA is synthesized before neurulation. In normal embryos a considerable increase in respiratory rate occurs during gastrulation. Contrary to this, only a very slight increase has been found in the hybrids (Barth, 1946; Chen, 1952). In agreement with this is the observation that carbohydrate is consumed at a very low rate in the hybrid embryos (Gregg, 1948).

It is difficult to correlate these various findings, but it seems warranted at least to conclude that only a minor fraction of the total energy consumed during gastrulation is used for DNA synthesis and cell division.

Summary

1. The DNA contents of normal *R. pipiens* embryos and *R. pipiens* ♀ × *R. sylvatica* ♂ embryos at various developmental stages have been estimated by the method of Hoff-Jørgensen (1951).

2. The DNA content of *R. pipiens* embryos remains constant from fertilization until near the end of segmentation, at which point it increases at a constant rate until shortly before muscular movements begin.

3. The DNA content of hybrid embryos (which do not gastrulate) is identical with that of *R. pipiens* embryos until the latter reach the neural fold stage, at which point it does not increase further.

4. The significance of these results is discussed briefly.

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THE COMPOSITION OF THE BASIC PROTEINS OF ECHINODERM SPERM

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Protein with strongly basic properties has been found in the nuclei of most cells in association with desoxyribonucleic acid. In the sperm of some species of fish these proteins are simple in composition (protamines), containing only 7 or 8 amino acids and of these arginine accounts for about 90 per cent of the protein nitrogen (Hamer and Woodhouse, 1949; Felix, 1953). It has also been claimed that protamines are found in other animals, for example in fowl sperm (*Gallus domesticus*) (Fischer, 1954). However, in the sperm of cod (Stedman and Stedman, 1951) and mammals (Dallam and Thomas, 1953) proteins of histone type have been reported and these differ from protamines in having 16 amino acids. Arginine is again the principal component (20–30 per cent protein N) and, along with lysine and histidine, gives the protein its characteristic basic properties (Hamer, 1951, 1953a).

These basic proteins are believed to form some part of the structure of the chromosomes of the cells and so it is particularly interesting to know if they show species differences in composition. With this in view, basic proteins have been isolated from the spermatozoa of three echinoderms, the sea urchin, sand dollar and starfish, and the amino acid compositions determined. A similar protein isolated from the sperm of a mollusc, the squid, was also examined. The only previous chemical examination of the basic proteins of the sperm of invertebrates has been by Hultin (1947) and Hultin and Herne (1948). These workers reported that the compositions of sea urchin and mollusc basic sperm proteins were intermediate between protamines and histones, containing in all 11 amino acids. There was a considerable difference between the mollusc and sea urchin proteins while the basic proteins from different species of sea urchin were more similar in composition.

The results presented here show that in the species examined the sperm contain histone-type basic proteins and that there are significant species differences in composition. Two preliminary reports have included some of the results presented here (Hamer, 1953b, 1954).

Materials and Methods

Sperm from sea urchins (*Arbacia punctulata*) and sand dollars (*Echinarachnius parma*) were obtained by injection of 0.5 *M* KCl and the sperm collected in sea water. Ripe testes of starfish (*Asterias forbesi*) and squid (*Loligo pealii*) were removed, cut into small pieces and the sperm allowed to shed into sea water. The sperm were collected by centrifugation, washed first with sea water and then several times with a saline-citrate solution (0.14 *M* NaCl, 0.02 *M* citric acid).

Sperm nuclei so obtained were next extracted directly with 0.2 *M* HCl for 24 hours and then, after centrifuging, the extract was dialyzed against distilled water. All these operations were carried out in the cold. This extract now contained the protein or group of proteins classified as basic nuclear proteins (protamines and histones) since they are of nuclear origin, are extracted by acid, and remain soluble after removal of the acid by dialysis (Hamer, 1953a). It was found that the basic protein in the dialyzed solution could not be precipitated by addition of small amounts of ammonia (unlike mammalian histones) or sodium hydroxide and so picric acid was added and the protein picrate collected. This was then dissolved in acetone and any insoluble material centrifuged off. The solubility of basic protein picrates in acetone has previously been used for fractionation by

TABLE I

Composition of basic proteins

	Sea urchin*	Sea star	Sand dollar	Squid
Alanine	9.8	9.85	9.3	4.2
Ammonia	3.8	3.9	3.7	4.0
Arginine	28.0	23.8	34.1	47.7
Aspartic acid	2.7	2.9	1.9	3.4
Glutamic acid	3.9	4.0	3.0	3.7
Glycine	5.7	4.4	4.85	3.6
Histidine	2.1	2.9	1.0	2.0
Leucine	7.2	5.8	6.1	5.8
Lysine	17.1	19.0	13.9	8.2
Phenylalanine	1.0	0.9	0.9	1.0
Proline	4.4	4.25	3.4	2.6
Serine	4.0	3.8	5.3	4.8
Threonine	3.45	3.1	4.2	2.1
Tyrosine	1.1	1.1	1.1	1.9
Valine	3.7	3.9	4.2	2.8
Methionine	1.0	1.4	x	x

Amino acid nitrogen expressed as percentage of protein nitrogen.

* Cystine and tryptophane estimation were carried out only on the sea urchin basic protein. Neither was present in significant amount (less than 0.1 and 0.06%, respectively). The nitrogen content of this protein sulphate was 14.4 per cent.

x Not estimated.

Rasmussen (1934) and Hultin (1947). Sulphuric acid was added dropwise until precipitation of the protein sulphate was complete. The sulphate was dissolved in a small amount of water and re-precipitated by addition of acetone, to remove any remaining picric acid. After washing again with acetone the white protein sulphate was dried *in vacuo*. It may be noted here that similar techniques were applied for the preparation of basic fractions from echinoderm eggs following direct extraction of the eggs with acid. Small amounts of proteins were obtained but these were insufficient for quantitative analysis.

The analytical methods used were those previously applied to thymus histones (Hamer, 1951, 1953a). The protein was hydrolyzed for 18 hours with 6 N HCl under reflux. After repeated evaporation to remove acid, aliquots were analyzed by the starch column chromatographic technique of Stein and Moore (1949).

Separation of leucine and isoleucine was not carried out and these results are reported together as leucine. The identity of all components was confirmed separately by paper chromatography. Methionine, cystine and tryptophane were tested for independently when sufficient material was available by the following methods: methionine by the nitroprusside reaction (Horn, Jones and Blum, 1946); tryptophane using p-dimethylaminobenzaldehyde in sulphuric acid on unhydrolyzed protein (Spies and Chambers, 1949); cystine by Shinohara's modification of the Folin-Winterstein reaction (Block and Bolling, 1951). Nitrogen was determined by a micro-Kjeldahl method and phosphorus by Holman's (1943) colorimetric method after perchloric acid combustion. Recoveries of protein nitrogen in terms of amino acid nitrogen and ammonia ranged from 95–99 per cent.

Results

The results of the amino acid analyses are given in the table. It will be seen that all the basic proteins examined have the general type of composition found in the histone group. In each case, the basic amino acids arginine, lysine, and histidine together account for about 50 per cent of the protein nitrogen. Only small amounts of the aromatic amino acids tyrosine and phenylalanine are present and, in the case of the sea urchin specimen, analysis showed there was no significant amount of tryptophane or cystine. Glutamic and aspartic acids represent 7–8 per cent of the whole. Compared with histones from mammalian cells, the echinoderm sperm histones contain appreciably more alanine and lysine but less leucine, phenylalanine and tyrosine. The protein from the squid sperm contains considerably more arginine than is found in the specimens from sea urchin and starfish by colorimetric estimation though this was not sufficient to be detected chromatographically on the starch columns.

From estimations of the nitrogen and phosphorus contents of sea urchin sperm nuclei before and after extraction with acid it was estimated that about 27 per cent of the dry weight of the nuclei was nucleic acid, 21 per cent histone extractable by acid and the remaining 52 per cent acid-insoluble protein. The nature of this protein has not been investigated though in the case of mammalian tissues it has been found to be similar in composition to the basic protein but to contain, in addition, measurable amounts of cystine and tryptophane.

Discussion

The results presented above differ from those of Hultin and Herne (1948) in that 16 amino acids were found in the basic proteins of all the species examined whereas only 11 had previously been reported for related species (*e.g., Arbacia lixula, Brissopsis lyrifera*). It would seem unlikely that this is due to a species difference but is more likely to arise from differences in analytical methods. For qualitative analysis Hultin and Herne carried out a preliminary fractionation of the hydrolysates on carbon and other adsorbents. Possibly the small amounts of tyrosine, phenylalanine, and methionine present were lost in this treatment but the failure to detect glutamic acid and aspartic acid is difficult to account for.

Significant species differences were found between the three echinoderms examined. Considerable variations occur in the lysine and arginine contents while there are minor variations in the amounts of other amino acids, for example

leucine and glycine. The molluscs, as represented by the squid specimen, show more considerable changes in composition but the basic protein is still of the histone-type. At the moment there seems no rational way of predicting whether the sperm nuclei of a particular species will contain a protamine or a histone as the basic component of the desoxyribonucleo-protein complex. A number of fish and fowl sperm contain protamines while the sperm of some mammals and invertebrates contain histones. At present there are insufficient data to justify a claim that protamines occur widely in sperm nuclei (Fischer, 1954).

The results quoted for the relative amounts of histone, nucleic acid and acid-insoluble material are in agreement with those obtained by Bernstein and Mazia (1953) using sperm nucleoprotein of the sea urchin *Strongylocentrotus purpuratus*. There is certainly a considerable amount of protein associated with the nucleic acid in the sperm nuclei which is not "histone" in the sense that it is not readily extracted by acid. This has also been found in studies on mammalian somatic cells, and in discussing the possible arrangement of these fractions it has been suggested that the histone may be held by simple ionic forces while at least part of the acid-insoluble protein is held by chemical links to the nucleic acid (Hamer, 1953a, 1954). The work of Barton (1952a, 1952b) on the fractionation of invertebrate sperm with acid and nucleases lends some support to such a structural arrangement.

The materials studied in this work were prepared at the Marine Biological Laboratory, Woods Hole during the tenure of a Lalor Foundation Fellowship. It is a pleasure to acknowledge the help of the Lalor Committee in making my work possible. The analytical investigations were completed in the laboratories of the Birmingham Branch of the British Empire Cancer Campaign, England.

Summary

1. Basic proteins have been extracted from sperm of the sea urchin, sand dollar and starfish, and also from the squid. The amino acid composition of these proteins has been determined.

2. All the basic proteins were of histone type and were similar in general properties and composition to mammalian histones.

3. There are significant species differences among the echinoderms examined, particularly in the arginine and lysine contents. The basic protein of squid sperm contains much more arginine and less lysine than the echinoderm specimens.

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THE RELATIONSHIP BETWEEN CORPORA ALLATA AND REPRODUCTIVE ORGANS IN STARVED FEMALE LEUCOPHAEA MADERAE (BLATTARIA)

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It has long been known that hormonal regulation plays an important role in insect reproduction. Aside from the medial neurosecretory cells of the protocerebrum whose action on egg development has been demonstrated in Calliphora (Thomsen, 1952), the corpora allata are necessary for the normal function of the ovary and the accessory sex glands of most insect species investigated (see review: Scharrer, 1953). In the absence of the corpora allata, the oocytes remain in an immature state characteristic of the onset of a given reproductive cycle, and the accessory sex glands stay inactive.

Another important factor in insect reproduction is nutrition. Egg production does not occur in many insect species, when food in adequate quantity and quality is lacking. The question arises, whether the inability of starved insect females to reproduce mature ova constitutes a primary effect, or whether it is due to an influence of inanition on the endocrine system. A previous study indicated that the latter alternative applies to the activity of the corpus allatum of the milkweed bug, *Oncopeltus fasciatus* (Johansson, 1954). In the present paper this problem is studied in a different species belonging to another order of insects.

Material and Methods

Leucophaea maderae is an ovoviviparous cockroach which has been bred in the laboratory for years (*cf.* Scharrer, 1946a, 1946b). Freshly emerged adults which were to serve as donors and hosts, were taken from the stock colonies and isolated in glass jars of pint size. The food consisted of dogchow and apples. The specimens which were used as hosts were starved from the day of emergence and only given water. All specimens were kept at room temperature, *i.e.*, at 26–33° C.

Besides Leucophaea, also females of *Oncopeltus fasciatus,* were used as donors. They were taken from a laboratory colony fed on dried seeds of the milkweed.

Organs to be implanted were dissected out in insect Ringer, taken up in a fine glass capillary connected with a plunger device, and implanted into the abdomen of the host.

The corpora allata of Leucophaea are paired organs. The implantation of one corpus allatum, therefore, means that only half of the organ present in a single individual was used. When three or four corpora allata were implanted, they were taken from two individuals of the same sex and adult age. In Oncopeltus

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the corpus allatum is unpaired. Thus the number of corpora allata implanted in these cases is equivalent to the number of specimens used as donors. Abdominal ganglia of Leucophaea were implanted as controls.

The hosts were killed at various intervals after the implantation and examined. In a few cases they died before examination took place.

Results

In newly emerged females of Leucophaea the ovaries are small, the longest oocytes being about 1 to 1.4 mm in length. No yolk is present. The accessory

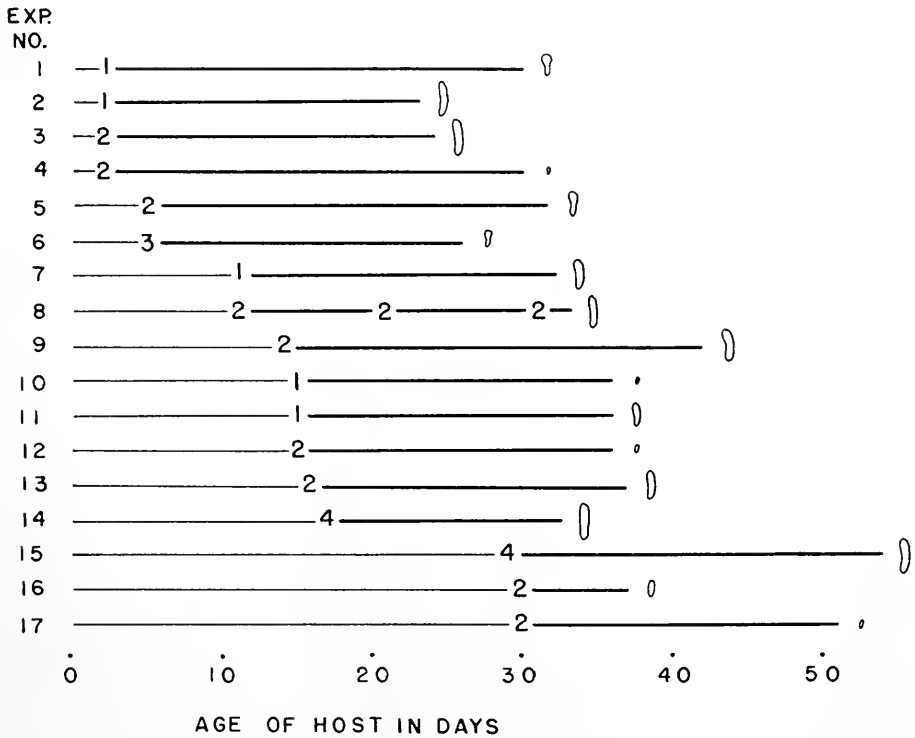

Figure 1. Diagram showing the result of implantations of corpora allata from fed females of *Leucophaea maderae* to starved female hosts. The number of corpora allata implanted and the day of implantation are given in the breaks of the lines. The right end of the heavy lines marks the day on which the host was examined. A schematic drawing in mm. of the largest oocyte observed is shown on the right.

sex glands are inconspicuous and do not show signs of secretion (Scharrer, 1946b). In fed females the oocytes grow and reach their maximal size of about 5 mm. after four to five weeks. During this period yolk is deposited in the oocytes. The accessory glands increase in size and obtain a bluish color due to the accumulation of their secretory product. The corpora allata are necessary, both for the growth of the oocytes and for the secretory activity of the accessory sex glands (Scharrer,

1946b). The corpora allata of Leucophaea do not show any appreciable increase in size during this period of activity.

Starvation of the females of this species prevents egg maturation (Scharrer, 1943). The accessory glands may show some secretory activity, but this does not seem to reach the level characteristic of normally fed specimens. Starved animals could be kept alive up to 74 days, but in none of the 16 specimens examined had the oocytes grown, and no yolk was deposited.

Starvation does not seem to influence the size of the corpora allata of Leucophaea.

Altogether 27 experiments were carried out in which Leucophaea served as donors. In 17 cases corpora allata from normally fed female Leucophaea were used (Fig. 1), whereas in 10 cases corpora allata from fed males were used (Fig. 2). Of the 17 cases with female donors, four did not show any growth of the

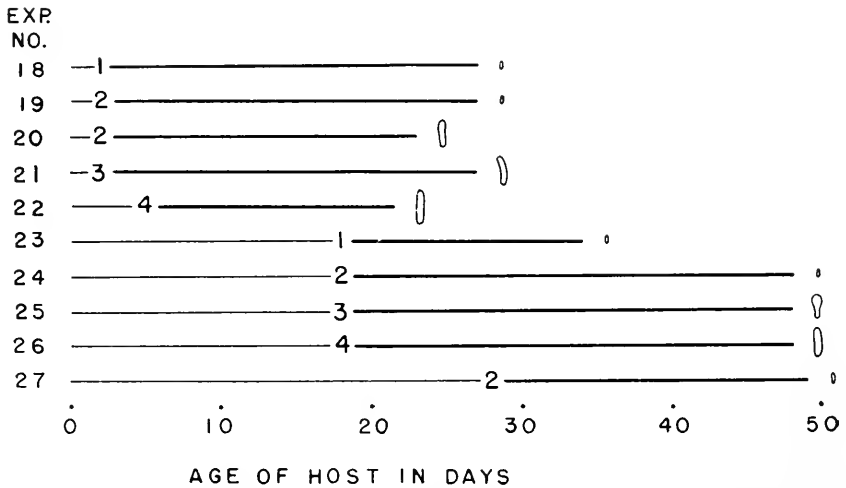

FIGURE 2. Diagram showing the result of implantations of corpora allata from fed male Leucophaea to starved females. For explanation see Figure 1.

ovaries when examined later (Expts. No. 4, 10, 12, 17). In five of the 10 cases with male donors, no development of the ovaries was observed (Expts. No. 18, 19, 23, 24, 27). In the rest of these experiments the implantation of corpora allata into starved hosts resulted in deposition of yolk and growth of the oocytes. As to the negative results, it ought to be mentioned that even in fed females sometimes no eggs develop. The number of grown oocytes observed in these implantation experiments ranged from one (Expts. No. 6, 7) up to normal numbers (Expts. No. 16, 26), *i.e.*, up to 40 (Scharrer, 1946b). In a few cases the shape of the grown oocytes was somewhat irregular. In two cases (Expts. No. 14, 22) eggs were deposited. Premature deposition of eggs is occasionally observed in normal females under laboratory conditions.

The adult age of female donors ranged from one (Expts. No. 7, 13) up to 54 days (Expt. No. 12). In the majority of the cases, the females were from 18 to 38 days old; nearly all had growing oocytes. The female which was 54

days old, had already deposited her eggs when used as a donor. The age of the male donors ranged from 18 to 35 days. It is seen that in the case of female donors even one single corpus allatum from a one-day old specimen can give positive results (Expt. No. 7). It seems that when males are used as donors, two or three corpora allata are necessary to be effective.

Growth of the oocytes took place even when the host had starved up to 30 days before implantation (Expt. No. 16).

The accessory sex glands also responded to the corpus allatum implants. In the experiments in which no growth of the oocytes took place, the accessory sex glands did not seem to increase their activity beyond a level which may be observed also in starved females. In cases with grown oocytes, the accessory sex glands seemed to show an increase in secretory activity beyond the starvation level, and in some cases reached the maximal size characteristic of fed females.

In none of eight experiments, in which one to six corpora allata from fed, egg-laying females of Oncopeltus had been implanted, had the oocytes grown.

In 12 starved females abdominal ganglia from Leucophaea were implanted as controls. No effect was found on the oocytes or the accessory sex glands.

Discussion

It has previously been found that in the milkweed bug, *Oncopeltus fasciatus* implants of physiologically active corpora allata into starved females may induce egg production (Johansson, 1954). The present paper shows that the same is true also in a different species belonging to another order of insects, *i.e.*, in the roach *Leucophaea maderae*. The ovaries of these two species are of different types. Oncopeltus has an acrotrophic type of egg tube, whereas the Blattaria have panoistic ovarioles. In both species starvation apparently prevents egg development by acting on the corpus allatum. In Oncopeltus this is seen in the lack of normal growth of the corpus allatum in starved specimens. In Leucophaea, where the corpora allata do not show any visible growth in fed specimens following emergence, no difference in the size of the corpora allata of fed and starved females could be observed. Also in this species, however, the corpora allata of starved females appear to be physiologically less active than those of fed ones.

No qualitative differences could be found between the action of corpora allata from males and females of Leucophaea, but it seems that quantitative differences exist, the male corpora allata being the less active. The negative results with implants from Oncopeltus may also have a quantitative rather than a qualitative explanation.

The presence of corpora allata has been found necessary for the normal activity of the accessory sex glands. In allatectomized females of Leucophaea the accessory sex glands do not secrete (Scharrer, 1946b). Since these glands in starved females sometimes show a limited secretory activity, it would seem that they have a lower threshold of response to the corpus allatum hormone than the ovaries, and that starvation does not totally prevent the secretion of corpus allatum hormone. A similar phenomenon is observed in Melanoplus where secretion in the oviduct may take place under conditions which prevent yolk production, and Pfeiffer (1945) suggests that the oviducts are able to respond to the corpus allatum hormone before the ovaries react with yolk production.

The author wishes to express his gratitude to Dr. B. Scharrer for supplying the material of Leucophaea and for helpful suggestions.

Summary

1. In starved females of *Leucophaea maderae* whose ovaries remain immature, egg development can be induced by the implantation of corpora allata from fed donors. Implants from adult males have the same qualitative effect as those from females, but they are by comparison less active. Thus in Leucophaea as well as in Oncopeltus (Johansson, 1954), the inhibition of ovarian development caused by starvation is not a direct effect, but is mediated through the corpora allata.

2. While the oocytes of female Leucophaea, starved from emergence, remain undeveloped, their accessory sex glands may show some measure of secretory activity. The result indicates that these glands whose activity depends on the corpus allatum have a lower threshold of response than the ovaries, and that starvation does not totally prevent the secretion of corpus allatum hormone.

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ADENOSINETRIPHOSPHATASE ACTIVITY OF AMERICAN COCKROACH [1] AND WOODROACH [2] THORACIC MUSCLE [3]

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The enzymes of mammalian tissues, particularly muscle and liver, which split inorganic phosphate from adenosinetriphosphate (ATP) have received considerable study during the past several years. Du Bois and Potter (1943) developed a method for the quantitative determination of the adenosinetriphosphatase (ATPase) of tissue homogenates and showed that it was greater in cardiac and skeletal muscles than in liver, lung, kidney, submaxillary gland, spleen, pancreas, brain, and smooth muscle of the normal rat. The ATPase in the corpora lutea of rat ovaries changes little during pregnancy and lactation (Biddulph *et al.*, 1946). More recent studies have been concerned with the kinds of ATPase found in liver and the particles with which it is associated. Kielley and Kielley (1951, 1953) and Novikoff *et al.* (1952) showed that liver mitochondria dephosphorylate ATP. A "soluble" ATPase was obtained from muscle (Kielley and Meyerhof, 1948), and from the supernatant fraction of liver (Swanson, 1951). The ATPase of yeast has been purified by Meyerhof and Ohlmeyer (1952), and that of liver by Swanson (1952). Heppel and Hilmoe (1953) separated and partially purified three different ATPases from bull seminal plasma and studied their mechanism of action on ATP. The ATPase of extracts of rabbit muscle was studied by Humphrey and Humphrey (1950). The kinetics of the dephosphorylation of ATP by rabbit myosin was investigated by Ouellet, Laidler and Morales (1952), and the mechanism of hydrolysis of ATP by lobster muscle was studied by Koshland and Clarke (1953).

The presence of ATP in the tissues of *Drosophila melanogaster* was reported by Albaum and Kletzkin (1948), and in insect muscle by Calaby (1951). The major part of the work on the enzymatic dephosphorylation of ATP by insect tissues has been done with insect muscle. Gilmour (1948) found a soluble Mg-activated enzyme in myosin extracts of grasshopper muscle which split both high energy phosphate bonds of ATP. In a further study of muscle of locusts (*Locusta migratoria* and *Castrimargus musicus*) Gilmour and Calaby (1952) demonstrated that myokinase or adenylate kinase was not responsible for the removal of the second phosphate from ATP and that the apyrase could utilize adenosine di-

[1] *Periplaneta americana.*

[2] *Leucophaea maderae.* In a previous paper the common name "woodroach" was used for this species, following the usage suggested by Scharrer (1951). Taxonomists, however, generally use the common name of "Madeira roach" for this species, as pointed out by Gurney (1953), in order to avoid confusion with native species of the genus *Parablatta,* also referred to commonly as "woodroaches."

[3] This investigation was supported in part by a grant from funds supplied by the Wisconsin Alumni Research Foundation.

phosphate (ADP), inosine triphosphate (ITP), and inosine diphosphate (IDP) as substrates. Sacktor (1953) found that the ATPase of mitochondria from the thoraces of the house fly, *Musca domestica,* is activated by Mg and Mn but not by Ca ions. A Ca-activated ATPase was found in the muscle fibrils, and adenylate kinase was present in the mitochondria. Sacktor *et al.* (1953) studied the dephosphorylation of ATP by several tissues of the American cockroach, *Periplaneta americana.* These tissues were rated in the following order of decreasing activity: muscle, fat body, Malphighian tubes, nerve cord, brain, hindgut, foregut, and midgut. Mg was more effective than Ca ions in activating the ATPase of these tissues. The activity of the muscle and hindgut of the female was greater than that in these tissues of the male.

Since the catalytic breakdown of ATP by ATPase presumably supplies energy for cellular functions, it was of interest to study the ATPase system in the thoracic muscle of the American cockroach, *Periplaneta americana,* and to determine the relation of the activity of this enzyme to the age and sex of the roach. Results of preliminary studies of the thoracic muscle of the woodroach, *Leucophaea maderae,* are also reported.

Experimental Materials and Methods

The chemicals used were reagent grade and the cofactors and substrates were obtained from different sources.[4] The sodium salts of these compounds were adjusted to pH 7.4 for experimental purposes.

The roaches from which tissue was obtained were isolated at one day of age so that insects of known ages would be available for experimental purposes. A few experiments were done, however, with tissue from insects of unknown ages. The roaches were kept in glass jars and were given water and food at regular intervals.

The meso- and metathoracic muscles were removed immediately after the roaches were killed. The technique of dissecting out these tissues was described in detail in a previous paper (McShan, Kramer and Schlegel, 1954). The muscle was placed in a sharp-pointed glass homogenizer in an ice bath and homogenized in sufficient water to give a one per cent homogenate (weight of tissue in grams multiplied by 99 gives the required ml. of water).

The ATPase activity of the muscle homogenates was determined by the method reported by DuBois and Potter (1943). Three amounts of tissue were used in each determination. The controls used were the complete system without tissue, and without ATP, enabling us to make correction for the inorganic phosphate present in the reaction medium, in addition to that released by the action of the ATPase.

The constituents of the system were measured into small tubes and heated to

[4] Adenosinetriphosphate (ATP), adenosinediphosphate (ADP) and adenosine-5′-phosphate (AMP) were obtained from the Pabst Laboratories, Milwaukee, Wisconsin; glucose-1-phosphate, fructose-1-phosphate and fructose-1,6-disphosphate from the Schwartz Laboratories, Inc., New York; sodium beta-glycerophosphate and sodium phosphoglycerate from National Biochemicals, Inc., Chagrin Falls, Ohio; disodium phenylphosphate and sodium phenolphthalein phosphate from Paul-Lewis Laboratories, Inc., Milwaukee, Wisconsin; paranitrophenylphosphate from the Sigma Chemical Company, St. Louis, Missouri; and a sample of sodium phenolphosphate purified in our laboratory.

38° C. in a water bath. The proper amounts of tissue were then added to the tubes and incubation was continued for 15 minutes at which time the reaction was stopped by addition of 0.2 ml. of 50 per cent cold trichloracetic acid (TCA). The tubes were centrifuged to remove the precipitate and the amount of inorganic phosphorus in 0.3 ml. of the supernatant was determined by the method of Fiske and Subbarrow (1925) as a measure of the ATPase activity of the muscle

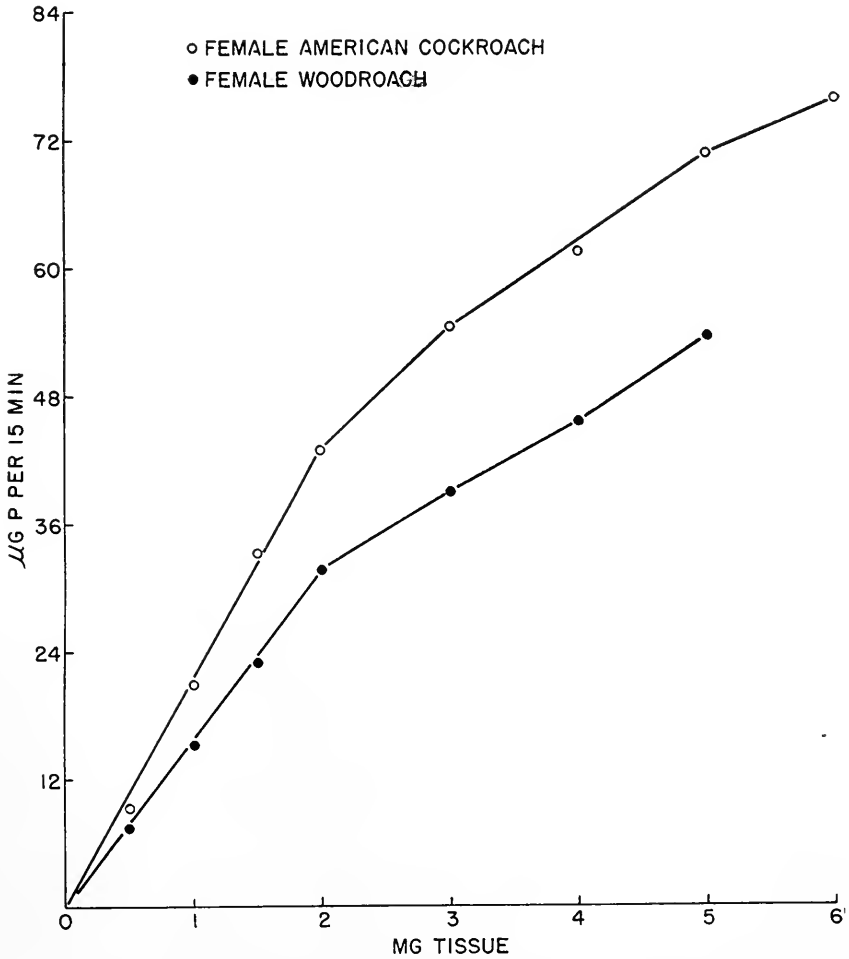

FIGURE 1. Curves showing the relations between the μg. of inorganic phosphorus released from 2.8×10^{-3} *M* ATP by different amounts of muscle tissue. The relation is linear with 0.5 mg. to 2.0 mg. of fresh tissue.

homogenate. Known amounts of phosphorus in the form of KH_2PO_4 were run with each determination as standards to serve as a basis for calculating the phosphorus content of the TCA supernatants.

One unit of ATPase activity is defined as the amount of enzyme required to release 1 μg. of inorganic phosphorus from ATP in 15 minutes at 37° C. (Du Bois

and Potter, 1943). The μg. of phosphorus released in 15 minutes by the different amounts of tissue were used for calculating the μg. of phosphorus hydrolyzed by 1 mg. of tissue. The values used for preparing the graphs and given in the tables are expressed in terms of the μg. of phosphorus released by 1 mg. of muscle in 15 minutes. These values represent the number of ATPase units per mg. of muscle.

Results

Relation of ATP concentration to enzyme activity

Maximum ATPase activity was obtained when 2 mg. of muscle tissue were incubated with as low as 1.4×10^{-3} *M* ATP. In order to insure an excess of substrate a final concentration of 2.8×10^{-3} *M* ATP was used in the system for the routine determinations.

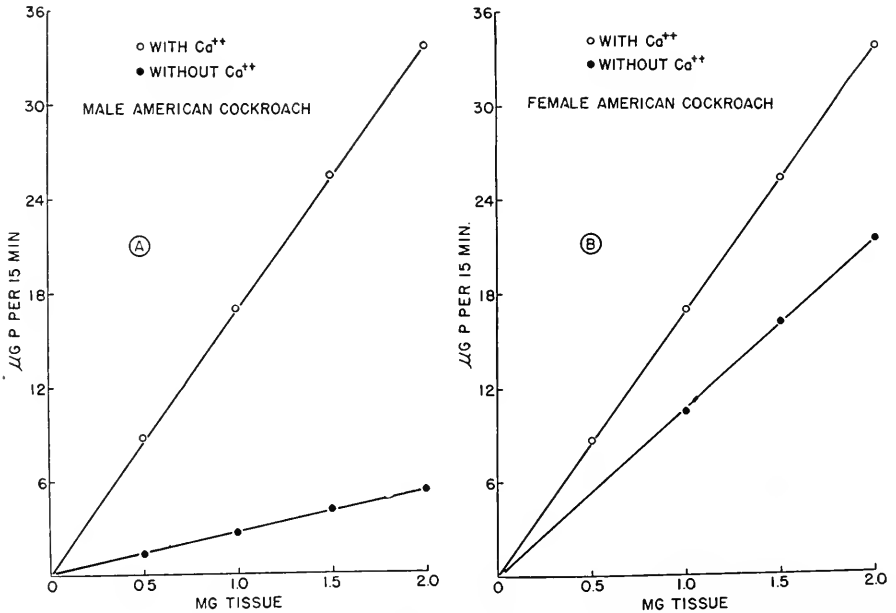

Figure 2. Effect of Ca ions on the release of inorganic phosphorus from ATP by the ATPase of muscle from the male and female cockroach.

Effect of time and enzyme concentration

The rate at which inorganic phosphorus was released from ATP by different amounts of tissue was not constant with time. This decrease in ATPase activity with time has been reported for liver homogenates (Novikoff *et al.*, 1952), for purified yeast ATPase (Meyerhof and Ohlmeyer, 1952), and for mitochondria of flies (Sacktor, 1953).

The results given graphically in Figure 1 for the muscle of the female cockroach and female woodroach show that the amounts of inorganic phosphorus released were linearly related to the enzyme concentration when 0.5, 1.0, 1.5 and 2.0 mg. of

tissue were used. This linear relation was obtained when the incubations were continued for 10, 20 and 30 minutes.

Effect of activating ions

Bivalent cations such as Ca, Mg and Mn are known to activate the ATPase of mammalian, yeast and certain insect preparations. Results represented in Figure 2A and B show that the ATPase in the thoracic muscle of the female cockroach is activated much less (58 per cent increase in activity) by Ca ions than is the enzyme of the male muscle (533 per cent increase in activity). It is also

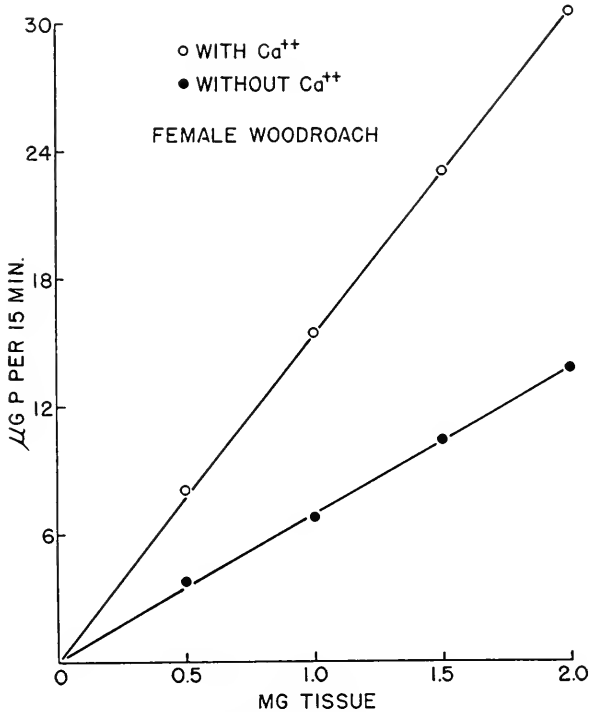

FIGURE 3. Effect of Ca ions on the release of inorganic phosphorus from ATP by the ATPase of muscle from the female woodroach.

of interest that the activity of the ATPase of muscle from the female cockroach is much greater in the absence of added Ca ions than is this enzyme in the muscle of the male. Sacktor (1953) reported results of this kind for the muscle and hindgut of male and female cockroaches. In the light of these results the question arises as to whether the greater ATPase activity of the female muscle without added Ca ions may not be due to a greater concentration of these ions in this muscle than in the muscle of the male roach.

The activation of the ATPase of muscle from the female woodroach is shown in Figure 3. The degree of Ca activation in this case is less than was found for the male but greater than that obtained for the female American cockroach.

As has been found for the ATPase of other kinds of tissue Mg and Mn ions were also found to activate the ATPase of muscle from the male and female cockroach. The degree of activation with different concentrations of Ca, Mg and Mn ions is shown in Figure 4. The ATPase activity was more nearly the same when the concentration of each of the ions was 2.8×10^{-3} *M*. Mg ions were more effective in activating the system than Ca and Mn ions at a concentration of 1.4×10^{-3} *M*. Similarly, Sacktor (1953) found that Mg ions were more effective than Ca ions at a concentration of 1×10^{-3} *M*. Ca ions were more effective in activating the ATPase of cockroach muscle at concentrations greater than 2.8×10^{-3} *M*.

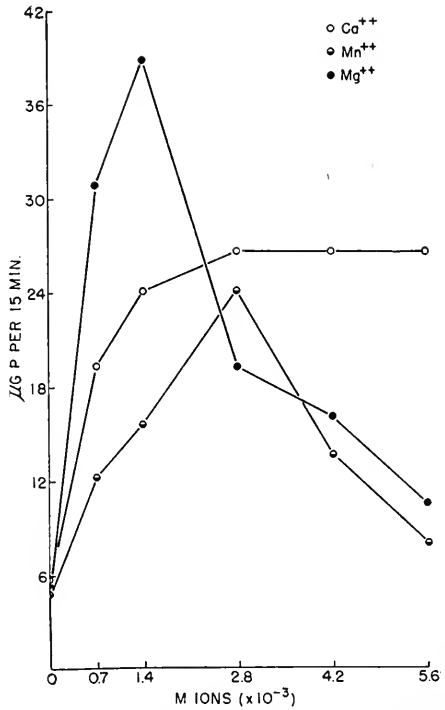

FIGURE 4. Activation of the ATPase of cockroach muscle by increasing concentrations of Ca, Mn and Mg ions.

On the basis of the above results 0.05 ml. of 0.04 *M* $CaCl_2 \cdot 2H_2O$ and 0.2 ml. of 0.01 *M* ATP (2.8×10^{-3} *M* final concentration of each) were used in the system for the determination of the ATPase of cockroach and woodroach muscle. The amounts of the other reagents used in the system were: 0.15 ml. of 0.5 *M* sodium diethylbarbiturate buffer of pH 7.4 and, as indicated above, 0.05, 0.10 and 0.15 ml. of one per cent homogenate plus sufficient water to give a total volume of 0.7 ml. for incubation. Three amounts of tissue were used for each determination to demonstrate that the phosphate released from the ATP was linearly related to the ATPase of these amounts of tissue.

TABLE I

Comparison of ATP and other phosphate esters as substrates for ATPase of male American cockroach muscle

Substrate*	ATPase Units**
Adenosinetriphosphate	15.1
Adenosinediphosphate	0.6
Fructose-6-phosphate	1.2
Glucose-1-phosphate	0.6
beta-Glycerophosphate	0.0
Phenylphosphate	0.9
Fructose-1,6-diphosphate	0.0
Adenosine-5′-phosphate	0.0
para-Nitrophenylphosphate	0.0
Phosphoglycerate	0.0
Phenolphthaleinphosphate	0.9

* The substrates were used in the form of the sodium salts and in a final concentration 0.003 *M*.

** Units per milligram of fresh muscle. Average value of three experiments using three different amounts of tissue for each experiment.

Effect of muscle homogenate on different substrates

The results given in Table I indicate that under the conditions used in these experiments the ATPase of male cockroach muscle is specific for ATP. The small amount of inorganic phosphorus released from certain of the other compounds used as substrates might be due to low activity of ATPase for these compounds, or to the action of another phosphatase.

Relation of age and sex to ATPase activity of cockroach muscle

The ATPase activity of the muscle of the American female cockroach appears to be greater than that of the male at 10, 20 and 30 days of age (Table II). This is in agreement with the results of Sacktor *et al.* (1953) who reported that the ATPase activity of female roach muscle is significantly greater than that of the male. The results of Table II also suggest that for both sexes there is a trend

TABLE II

Adenosinetriphosphatase of thoracic muscle from the American cockroach

Sex	Age, days	ATPase units*
Male	10	19.2
	20	16.9
	30	15.7
Female	10	21.6
	20	19.5
	30	18.1

* Units per milligram of fresh tissue. The values are averages of two to five determinations in which three different amounts of tissue were used. The time of incubation was 15 minutes. Ca ions were present in a concentration of 2.8×10^{-3} *M*.

toward a decrease in ATPase activity with an increase in age from 10 to 30 days. A definite conclusion on this age-ATPase activity relation is not justified, however, until determinations are made using a larger number of roaches over a wider age range.

Results of preliminary experiments on the thoracic muscle of the woodroach of various ages suggest that the sex difference in ATPase activity of the American cockroach does not exist in the woodroach, *Leucophaea maderae*. In this connection it should be mentioned that the succinoxidase activity of muscle from male woodroaches is essentially the same as the activity of muscle from the female, whereas the activity of this system in male American cockroach muscle is three to four times that of the female.

Lawrie (1952), in a study of the biochemical differences of red (high in myoglobin content) and white skeletal muscle (low in myoglobin content) of various vertebrates, indicated that in general high myoglobin content in muscle is associated with high enzymatic activity for succinic dehydrogenase, succinoxidase and cytochrome oxidase. ATPase activity, on the other hand, decreases with an increase in myoglobin content. It is interesting to note that the male American cockroach has red thoracic muscles and shows higher succinoxidase activity and lower ATPase activity than these muscles in the female which are white. There is no evidence for the presence of myoglobin in insect muscle. Sacktor *et al.* (1953), however, have suggested that in insects the difference in color may be due to the cytochrome content. Whatever the color difference may be due to, it should be noted that in the woodroach, *Leucophaea maderae*, in which both sexes have red muscles, succinoxidase and ATPase activity are apparently equivalent in both sexes.

Summary

1. The adenosinetriphosphatase (ATPase) activity of the thoracic muscle of the American cockroach, *Periplaneta americana*, and the woodroach, *Leucophaea maderae*, was studied. The optimum conditions for eliciting maximum ATPase activity of the muscle of these two species are: 0.2 ml. of 0.01 *M* ATP, 0.05 ml. of 0.04 *M* $CaCl_2 \cdot 2H_2O$, 0.15 ml. of 0.5 *M* diethylbarbiturate buffer of pH 7.4, and 0.05, 0.10 and 0.15 ml. of one per cent homogenate.

2. The ATPase activity of the muscle from females was activated less by calcium than was that of the male American cockroach. The degree of calcium activation of the ATPase of the female woodroach muscle was intermediate between that of the male and female cockroach muscle.

3. The ATPase activity of muscle from female American cockroaches 10 to 30 days of age was greater than that of male roaches of the same ages. Present results suggest that there is not a difference in the ATPase activity of muscle from male and female woodroaches.

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THE EFFECT OF COLCHICINE ON RECONSTITUTIONAL DEVELOPMENT IN DUGESIA DOROTOCEPHALA [1]

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Many investigators have used the regeneration phenomenon observed in planarians in an attempt to understand the basic mechanisms which control developmental processes. Most of these studies have been made at the organismic level. However, as early as 1904, Bardeen and Baetjer demonstrated that the regenerative powers of *Planaria maculata* and *P. lugubris* could be destroyed by exposing animals to the action of x-rays before section. When pieces were exposed to the radiations only a small blastema developed. Histological observations showed an absence of mitosis in all irradiated pieces while controls showed parenchymal cells in division. Because of the differentiation of imperfect eyes in one case following treatment of the entire animal, these authors suggested that x-rays affected growth processes more than differentiation. Later work with x-rays (Curtis, 1928) and radium (Wiegand, 1930) confirmed the work of Bardeen and Baetjer and showed that large doses selectively destroyed parenchymal cells.

Wolff and Dubois (1948) demonstrated cellular migration in regeneration by grafting a piece of normal tissue into the irradiated anterior 2/5 of the *Euplanaria lugubris*. Donor pigment and cells were found to have migrated through the irradiated host tissues and formed a blastema. The duration of migration varied directly with the distance covered to the regenerating surface.

Curtis and Schulze (1934) made direct counts of the parenchymal cells in species capable of regeneration (*Euplanaria agilis, P. maculata*) and in others possessing a limited capacity to regenerate (*Procotyla fluviatilis*). The only cells observed to be in mitosis in this study were the free parenchymal amoebocytes. Their observations indicated that the capacity to reconstitute is correlated with the number of these cells which they suggest are present as a persistent embryonic stock. Hyman (1951), however, holds that this does not explain the capacity of a post-pharyngeal piece of *P. fluviatilis* to regenerate a tail and not a head.

Colchicine has been used to study cellular changes in limb regeneration in larval urodeles. Thornton (1943) treated larvae of *Amblystoma punctatum* and *A. opacum* with colchicine solutions and found that limb stumps of larvae kept in 1 : 1,500 continuously, from the time of amputation, completely failed to regenerate. While the mechanism of colchicine inhibition of regenerating limbs was not investi-

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gated in this work the author suggested that it was no doubt due to the inhibition of mitosis.

Since work on planarian regeneration has shown that cells migrate from the general parenchyma to the site of injury and contribute to the restoration of the lost parts by division, it appeared that colchicine might be useful in the study of planarian reconstitution. Because colchicine effectively inhibits cells in division (and possibly in migration) in other organisms, the present work was undertaken in an attempt to answer, at least in part, some of the following questions concerned with colchicine effects on planarian reconstitution: (1) Does colchicine inhibit regeneration of planarian pieces? (2) If so, how does this affect the head frequency gradient found in normal regeneration? (3) Is the process of fission altered by colchicine? (4) Are the effects of colchicine treatment reversible? (5) What effect, if any, does colchicine have on physiological dominance in reconstituting pieces of planarians? (6) How does colchicine affect differentiation?

Materials and Methods

The material used in this investigation was the flatworm, *Dugesia dorotocephala*. The stocks were taken from spring-fed streams leading into the Fox River at Cary, Ill. and the Des Plaines River at Schiller Park, Ill. During the course of this work large stocks were successfully maintained in the laboratory. Tap water, that had been aerated for at least 24 hours, was used exclusively. Beef liver was fed approximately twice a week. Only animals that had become adjusted to laboratory conditions and were starved for 7–10 days were used in the final experiments. Unless otherwise stated the animals were 14–16 mm. in length in all experiments. Control and experimental animals were always taken from the same stock.

Studies were made on pieces of various lengths. After sectioning in water, groups of similar pieces were placed in aerated tap water (control) and into various test solutions (prepared with tap water) of colchicine. During the fall, winter and spring the animals were allowed to reconstitute at room temperatures (21–23° C.). During the summer the bowls containing the control and experimental pieces were kept on the water table where fluctuations in temperature were not excessive (17–20° C.).

Preliminary tests showed that solutions of $M/1{,}000$ to $M/4{,}000$ colchicine were excessively toxic and that pieces or entire animals exposed to these concentrations cytolyzed within a few days. Similar tests also showed that $M/15{,}000$ was at the lower limit of concentrations yielding colchicine effects. Thus, concentrations ranging from $M/5{,}000$ to $M/15{,}000$ were used in all experiments reported in this work.

Experimental

1. *Effects of colchicine on regeneration in transverse pieces from different body levels*

Pieces, approximately 1/8th the length of 14–16 mm. animals, taken from different body levels were placed directly into $M/5{,}000$ and $M/10{,}000$ colchicine

for periods of 2, 5, 7 and 10 days after which they were returned to water. Daily observations were made until the 10th day. This series included 250 animals (1250 pieces).

Control pieces differed in degree of head regeneration at various levels. Anterior (A) pieces developed almost 100 per cent normal heads while pieces from more posterior levels showed various degrees of head inhibition. At the most

TABLE I

Designation of regulation values

Type	Assigned regulation value	Control
I	100	Old tissue shows regulation to typical form and proportion of new individual. Head types vary according to level of origin of piece.
		Experimental—Colchicine treatment
II	85	Old tissue shows slightly less than normal regulation to new form and proportion; normal head and tail develop.
III	70	Old tissue shows little regulation to new form and proportion; normal or near normal head and tail develop.
IV	55	Old tissue shows no apparent regulation to new form and proportion; small anterior blastema with eyes develops and a posterior blastema may or may not develop.
V	40	Old tissue shows no apparent regulation to new form and proportion; small anterior blastema without eyes develops and a posterior blastema may or may not develop.
VI	25	Old tissue shows no regulation to new form and proportion; no blastema develops but eyes appear in the anterior cut surface.
VII	10	Old tissue shows no regulation to new form and proportion; pieces remain unchanged since time of cut.

posterior levels (E) almost all were headless.[3] Although many of the control pieces showed head inhibition almost all showed regulation in general body form. That is, the regenerating piece decreased in width and extended in length when compared to the original section cut from the parent.

[3] Head inhibition in short transverse pieces has been described many times (Child, 1911, 1912, 1914, 1920; Child and Watanabe, 1935; Watanabe, 1935; Rulon, 1936; Watanabe, 1941). Such inhibition appears to be the result of two factors, namely, the level from which the piece was taken and the intensity of factors originating from the posterior cut surface (Buchanan, 1922).

It was immediately noted that pieces treated with colchicine could not be compared with the controls on the basis of head inhibition since extended treatment, with the higher concentrations, resulted in little or no new tissue. When such pieces were removed to water those surviving regulated (after 2–3 weeks)

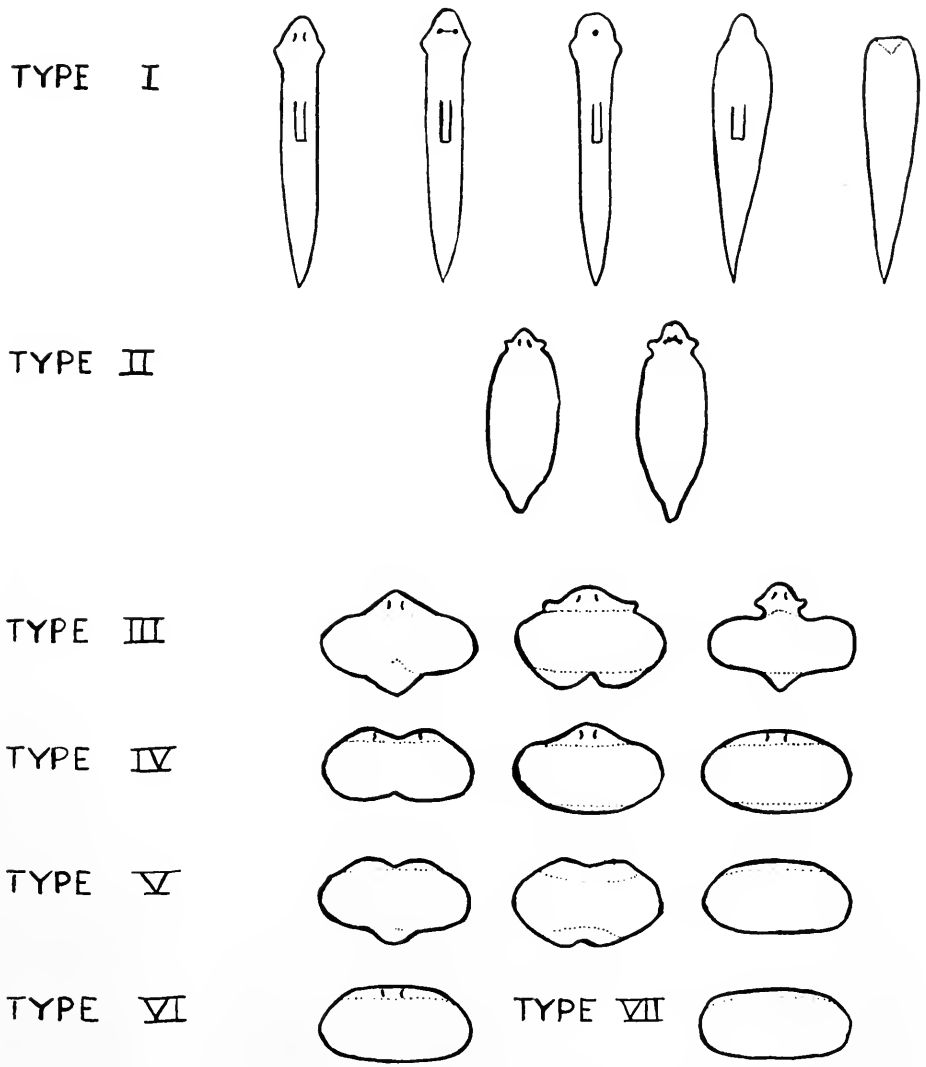

FIGURE 1. Classification of types of regenerates obtained subsequent to colchicine treatment.

to normal body form and regenerated almost 100 per cent typical heads with normal or supernumerary eyes. Since the present experiments were based on observations made 10 days after section, the pieces were classified according to the degree of regulation at that time (Table I, Fig. 1). All regenerates were

classified as to one of 7 types. Type I was the control and was classified as 100 per cent regulated. Types II to VII included those ranging from slightly less than normal regulation to a total lack of change from the time of section. In order to compare data from different experiments (levels, concentrations, etc.) a *Regulation Index* was devised by assigning each type a numerical value which roughly indicated the degree of regulation. Through the use of the assigned value and the number of forms of each type the Index was calculated as follows:

$$\frac{100n + 85n + 70n + 55n + 40n + 25n + 10n}{\text{number of surviving pieces}} = \text{R I (Regulation Index)}$$

One-eighth pieces in *M*/5,000 colchicine for 10 days developed no blastemae and retained the shape and size they had at the time of section (Type VI–VII). All pieces surviving from A and B levels developed eyes buried below the overlying epithelium (Type VI), while at C, D and E levels high percentages remained unchanged (Type VII). After 10 days in *M*/10,000 colchicine all levels showed greater regulation than in *M*/5,000. All surviving A, B and C pieces had small anterior blastemae with eyes (Type IV), while D and E showed a sizable percentage (40 and 36) with small blastemae lacking eyes (Type V). These data then indicate that A and B levels have a greater capacity for eye differentiation than have more posterior pieces. The regulation indices roughly indicate the inhibition effected by colchicine at the different levels.

With two days initial exposure to *M*/5,000 colchicine, regulation was high (by the 10th day) with the indices being above 82 at all levels. With *M*/10,000, the indices were above 94 at all levels. With 5 days exposure to *M*/5,000 there was no regulation of form at any level. However, the anterior pieces showed 100 per cent differentiation of eyes (Type IV), while many posterior pieces failed to show such development. Regulation was greater in all pieces treated with *M*/10,000 but best at posterior levels (D and E). With a 7-day exposure to *M*/5,000 anterior pieces showed the same results as a 5-day exposure but posterior pieces showed a greater capacity to regulate. With *M*/10,000, regulation at 7 days was little different from that of 5 days with the exception of the B level where it was considerably less. All of these data, as well as those for continuous exposure, show that *M*/5,000 colchicine is more inhibitory at all exposure periods than is *M*/10,000. These data also show that anterior pieces (A and B) exposed for 5, 7 and 10 day periods to *M*/5,000, while lacking the capacity for regulation of form and growth, give 100 per cent differentiated eye spots.

The consistent appearance of eye spots at anterior, but not at posterior levels, is in conformity with the gradient work of Child (1911). It must be pointed out, however, that these are not in differentiated heads but in completely unregulated pieces without blastemae. With the exception of a 5-day initial and a 10-day continuous exposure to the high concentrations, posterior pieces showed greater regulation and less susceptibility to the toxic effects of colchicine than did anterior pieces.

In general, these experiments have shown that posterior pieces have greater capacity for blastema formation and viability while anterior pieces have a greater capacity for differentiation of eye spots.

2. A critical period in reconstitution as determined by the use of colchicine

Since the data on transverse pieces, in high concentrations of colchicine, showed that 5 days' exposure was more lethal and permitted less regulation than 7 days (pieces D and E in *M*/5,000 colchicine) a critical period in development, centering around 5 days, was indicated. In order to identify this period more accurately

TABLE II

Comparison of Regulation Indices of 1/6th pieces when treated with M/5,000 and M/7,000 colchicine for 1–9 days after section. (Data in per cent.)

Level	1 day		2 days		3 days		4 days		5 days	
	Dead	Index	Dead	Index	Dead	Index	Dead	Index	Dead	Index
A	0	100								
B	0	100								
Control C	0	100								
D	0	100								
A	0	91.1	0	97	48	82.6	38	48.5	30	55.6
B	0	94.6	2	78	62	85	72	35.6	62	43.9
M/5,000 C	16	98.9	0	90.4	48	83.2	40	36.5	60	70
D	0	100	0	94.6	18	75.3	11	22.3	22	63.2
A	0	100	0	100	0	100	0	53.2	0	100
B	0	100	0	98.8	44	95.9	0	52.6	32	79.8
M/7,000 C	0	100	0	98.8	20	100	0	40	0	94
D	0	100	0	100	0	91.6	0	55	0	96.8

Level	6 days		7 days		8 days		9 days			
	Dead	Index	Dead	Index	Dead	Index	Dead	Index		
A										
B										
Control C										
D										
A	40	17.5	80	25	80	15	100	10		
B	100	16.5	98	25	100	11.1	100	10		
M/5,000 C	80	25.6	96	13	80	10.6	100	13		
D	46	64.7	96	20	100	13	100	13		
A	64	65	84	12.4	72	14.2	96	25		
B	84	55.5	92	25	92	25	96	10		
M/7,000 C	60	59.5	96	16	92	12.3	100			
D	56	90	60	37.8	92	10	96	25		

(if such actually existed) a further study was made in which slightly larger pieces were exposed to colchicine for more closely graded intervals.

In these experiments, pieces approximately 1/6th the post-cephalic length were used. These pieces were exposed to *M*/5,000 and *M*/7,000 colchicine for 1, 2, 3, . . . 9 days. At the end of each period the pieces were returned to water.

Records were made on the tenth day. An additional analysis was undertaken by treating the pieces with *M*/5,000 colchicine beginning at different time intervals after section. The data, showing treatment with colchicine immediately after cutting, are shown in Table II while those showing treatment at delayed intervals are shown in Table III.

Because so many data are involved (1,075 animals or 4,300 pieces) only Regulation Indices and viability for the different levels are given. It is shown in Table II that exposure for 4 days (not 5, as suggested in the previous section) is more effective in inhibiting growth, and regulation, in both *M*/5,000 and *M*/7,000, than either 3 or 5 days. This difference, relative to exposure time, could not be found in viability (as shown for 1/8th D and E pieces) possibly because the pieces in the present experiment were slightly larger.

The critical period for regeneration and regulation was not shown when pieces were placed into *M*/5,000 colchicine on the 4th day after section (Table III).

TABLE III

Regulation Indices of 1/6th pieces when kept in water for 1–6 days after section and placed into M/5,000 colchicine for the remainder of a 10-day reconstitutional period. (*Data in per cent.*)

Level	1 day		2 days		3 days		4 days		5 days		6 days	
	Dead	Index	Dead	Index	Dead	Index	Dead	Index	Dead	Index	Dead	Index
A	0	100										
B	0	100										
Control C	0	100										
D	0	100										
A	2	54.6	2	54.3	32	54.5	40	56.3	28	62.5	42	64.3
B	26	52.5	42	55	44	53.3	56	60.5	42	64.6	38	55
M/5,000 C	8	48.7	26	47.2	48	48	56	55.7	22	51.1	22	68
D	0	49.3	0	57.2	30	51.5	34	56.3	14	74.8	30	70.4

However, these data show that 4-day regenerates from all levels are more sensitive to the lethal effects of colchicine than are pieces which have regenerated for 3 or 5 days.

Sensitivity of developmental processes to inhibiting agents has been shown to be associated with cleavage and differentiation. Rulon (1950) demonstrated that the fertilized eggs of *Dendraster* were more sensitive to thiourea treatment than were blastulae. Blastulae tolerated high concentrations that were lethal to newly fertilized eggs. Changes associated with reconstitution appear to include an initial adjustment to the effect of section and perhaps dedifferentiation, followed by reorganization including cell proliferation with the final return to species character. It is suggested that the critical period noted here, with its counterpart in normal development, is associated with that time in development when reorganization and cell proliferation are occurring in the reconstitutional process. Histological studies, to be reported later, should verify this proposal.

3. The effect of colchicine on regeneration when whole animlas are treated prior to section

The preceding work showed that planarian pieces were inhibited in both reconstitution and regulation of form when exposed to certain concentrations of colchicine. Obviously the colchicine was exerting its effects on tissue which had been stimulated to regenerate by cutting. The problem which then presented itself dealt with the effect of colchicine on unactivated tissue. To test this, uninjured entire animals were exposed to various concentrations of the agent for 2–6 days after which they were sectioned into 1/6th pieces and permitted to reconstitute in water. Because these experiments were performed in summer, all intact animals and pieces were kept on the water table at 17–19° C. The data were taken on the tenth day following section. This experiment included 200 animals. In general, pieces from treated animals behave much the same way as if they, themselves, had been exposed to the agent. That is, there was prolonged inhibition of regulation and blastema formation in the higher concentrations. The most

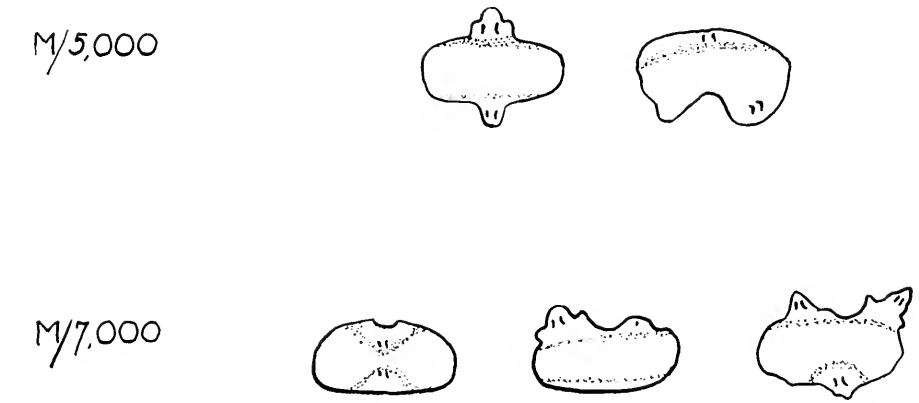

FIGURE 2. Types of heteromorphic forms obtained from 1/6th pieces of 14–16 mm. animals pre-treated with colchicine different lengths of time (*M*/5,000 for 4 days; *M*/7,000 for 6 days). Whole animals and pieces maintained at 17–19° C.

important difference between the regenerates obtained here and those from treatment following section occurred in the development of a high percentage (36 per cent at D level) of heteromorphic forms when the whole animals were treated for 4 days with *M*/5,000 and 6 days with *M*/7,000 colchicine. Regenerates classified as heteromorphic included: (1) simple bipolars, (2) unregulated bipolars (with or without blastemae), (3) poorly regulated forms with two heads at the anterior end, and (4) poorly regulated forms which have regenerated two heads anteriorly and one posteriorly (Fig. 2).

A slightly different series was prepared in which the intact animals were kept at room temperature (21–23° C.) in *M*/2,000, *M*/5,000 and *M*/10,000 colchicine for only two days. After this exposure the animals were cut into 1/8th instead of 1/6th and permitted to reconstitute in water, also at room temperature. This experiment included 225 animals.

This second series showed that increased temperature, with a reduction in

exposure time and size of piece, resulted in considerably less regulation when whole animals were treated with *M*/5,000 prior to section. When treated with *M* 10,000, under these changed conditions, regulation was also decreased, but it was noted that approximately 50 per cent of those pieces classified as Type V (small blastemae, no eyes) had two separate blastemae at each end of the piece.

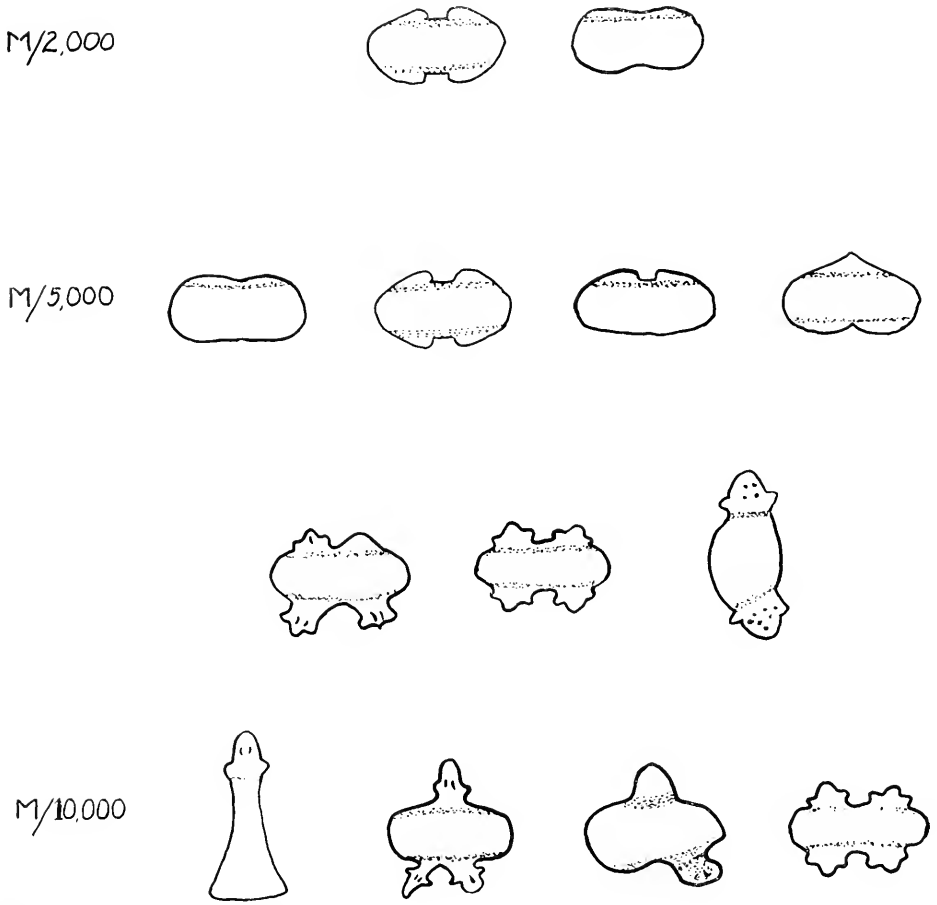

FIGURE 3. Types of regenerates obtained from 1/8th pieces of 14–16 mm. animals pre-treated with colchicine for two days. Whole animals and pieces maintained at 21–23° C.

High percentages (28–44) of heteromorphic forms also appeared. In general, these were similar to those found in the first series but with the addition of a 5th type which had regenerated two heads at each end of the piece (Fig. 3). At D level more than 50 per cent of these forms had 3 or 4 heads.

DISCUSSION AND CONCLUSIONS

This work has shown that certain concentrations of colchicine markedly inhibit regeneration and regulation to normal body form in planarians. It has shown that the degree of inhibition was roughly proportional to the concentration

and that the character of the regenerate showed a high degree of consistency. In all experiments using solutions below the toxic limits it was observed that pieces which survived early treatment with colchicine developed normal heads and attained normal body form and proportion by 10–15 days after return to water. The head frequency gradient (Child, 1913, 1914; Buchanan, 1922; Rustia, 1925; Rulon, 1936, 1938; Watanabe, 1935, 1941) was, therefore, obliterated by this agent. In tap water, short transverse pieces from D and E levels regenerate largely into headless, or near headless forms. It has been found (Buchanan, 1922; Rulon, 1948 and others) that factors inhibiting normal head regeneration arise at the site of posterior section and that these factors may be blocked by nerve anaesthetics and other agents. Such head-inhibiting factors are of short duration since a 24-hour delay in making a posterior cut will permit the development of 100 per cent normal heads (Child and Watanabe, 1935). The present experiments indicate that since colchicine prevents regenerative changes in the piece over an extended period, the head-inhibiting posterior effects have subsided by the time the pieces are returned to water.

The gradient of eye-spot formation, however, was still apparent after 10 days since exposure to high concentrations for this period of time resulted in 100 per cent eye-spots in pieces from anterior levels while no more than 56 per cent of the posterior pieces showed such development. Even though growth and regulation of these pieces was totally inhibited, differentiation was expressed in the appearance of eyes in pieces otherwise unchanged since the time of isolation. This indicates that while colchicine inhibits regeneration it does not at the same time totally inhibit differentiation.

Many investigations have shown a correlation between the presence of nerve tissue and the capacity to reconstitute to normal. Olmsted (1922) has shown that certain polyclads can restore missing parts only if cephalic ganglia are left intact. Silber and Hamburger (1939) and Beyer and Child (1930) observed head development on the medial surface of lateral pieces and suggested that the nervous system plays a role in the localization of head regeneration through its influence on the condition of the cells near it. In the present experiments it is possible that the appearance of eyes below the epithelium at the anterior cut surface of a piece is also correlated with the localizing influence of the nerve cords.

The occurrence of regenerates possessing 3–4 blastemae or 3–4 heads may find its explanation in the obliteration of the medio-lateral differential or gradient. All head formation in normal reconstitution is initiated at the medial point of a cut surface and progresses laterally. The occurrence of two blastemae or two heads at a cut surface (Figs. 2, 3) indicates that the medio-lateral gradient is reduced. A high percentage of pieces taken from animals treated with colchicine before section showed the formation of paired blastemae or heads at both cut surfaces. Apparently colchicine effectively destroys the medio-lateral differential and in so doing frees each side of the piece for independent differentiation. The localizing influence of the nerve cords may be operative here also, as it appears to be in eye spot differentiation after treatment with colchicine.

Summary

1. Colchicine was shown to inhibit regeneration in pieces of *Dugesia dorotocephala*. In addition it was shown to inhibit regulation to normal form.

2. Within certain limits of concentration, the inhibitory effects of colchicine were reversible. Two to three weeks after return to water pieces from all levels developed normal heads (some with supernumerary eyes). Thus, through the use of colchicine it was possible to obliterate the head frequency gradient characteristic of this species.

3. While colchicine was found to exert a marked inhibition of blastema formation in the reconstitution of planarian pieces it did not have the same effect on eye-spot differentiation. Pieces treated with higher concentrations of the agent failed to show any new tissue at the anterior and posterior cut surfaces by 10 days after section. Eye-spots, however, differentiated beneath the covering epithelium in anterior pieces.

4. Heteromorphic forms with 3–4 heads developed from pieces taken from whole animals exposed to colchicine. This indicates that the medio-lateral differential was reduced subsequent to colchicine treatment and that the two lateral halves of the piece underwent independent differentiation.

5. With the use of colchicine evidence was obtained that the 4th day of reconstitution represents a critical period in development. That is, pieces treated with *M*/5,000 colchicine for 4 days, and then returned to water, showed more inhibition than pieces treated for 3 or 5 days.

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CHEMORECEPTION IN INSECTS AND THE ACTION OF DDT [1]

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The unstabilizing action of DDT on many excitable tissues has been firmly established by work in several laboratories (see Roeder and Weiant, 1948, 1951 for references). However, as Roeder and Weiant demonstrated, not all irritable tissues are equally sensitive. At high concentrations of DDT they observed multiple discharges (impulse trains) in cockroach motor nerves, accounting for the tremors observed in isolated legs. At much lower concentrations, motor nerves were unaffected, but certain proprioceptors were selectively unstabilized, also firing short trains or sequences of impulses in place of single spikes. These barrages of sensory input were taken to account for the hyperexcitability and chaotic behavior of insects in the early stages of DDT poisoning.

If the selective action of DDT extends also to different types of receptor, DDT might be a useful tool for the separation and study of the sense modalities mediated by the many varieties of sensilla scattered over the cuticle of insects. Furthermore, since there are strains of housefly highly resistant to DDT, a comparison in different strains of a sense modality affected by DDT might provide information about the sense concerned and the mechanism of resistance to DDT.

With this in mind the action of DDT on chemoreceptors is considered in this paper. An attempt is made to correlate information on receptor function which has been obtained by electrophysiological methods with that derived from behavioral studies.

1. Electrophysiological studies relating chemical stimulation and DDT action

In the course of experiments on chemoreception (Roys, 1954) the vapors of volatile substances such as benzene and toluene were applied in increasing measured concentrations to intact roaches (*Periplaneta americana* L.) and to isolated legs and antennae. It was found that a concentration range in which the free insect appeared to show mild awareness of the chemical also caused a steady increase in the number of afferent nerve impulses recorded from electrodes in the isolated appendage. At higher concentrations, which the animal tried to avoid, electrical activity in the nerves of detached legs changed from a steady high level to a fluctuating pattern of great bursts alternating with periods of relative inactivity. This, and the fact that DDT is capable of producing abnormal impulse trains in certain proprioceptors of the insect leg, suggested an experiment in which the actions of benzene or toluene and DDT were combined.

[1] The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army, and Tufts College. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

Isolated roach or fly legs or segments of legs were pierced by hot-drawn tungsten electrodes. Afferent electrical activity in the leg was amplified and examined with the aid of an oscillograph and loud speaker. Each experiment was also recorded on magnetic recording tape for later study.

To minimize mechanical disturbance of the preparations the chemicals were applied as vapors. DDT was volatilized by heating the crystals in a glass tube and gently blowing the vapor over the preparation, taking care not to overheat the leg. Typical DDT trains recurred quite regularly and were unaffected by bending large spines. However, toluene vapor from an olfactometer had a well defined effect on the DDT trains, increasing their duration and frequency.

The effect is illustrated in Figure 1. The metathoracic femur of a housefly (*Musca domestica* L.) was mounted with an electrode in each cut end and covered with vaseline. The vaseline blanket limited the number of receptors responding to external stimulation, and provided a relatively simple pattern of afferent discharge. In the untreated preparation (1A) a single fiber was firing somewhat irregularly. Exposure to the vapor of toluene (1B) brought in several other fibers and increased the frequency of discharge, which had not quite returned to the resting level after the toluene had been removed (1C). Three minutes after a brief exposure to DDT vapor (1D) a sequence of well-defined DDT trains appeared, taking the place of the sequence of single spikes. However, the number of exposed receptors had been so reduced by the vaseline coating that the DDT trains were relatively infrequent in the absence of toluene. Exposure of the DDT-treated preparation to toluene (1E) was found to influence the repetition frequency of whole trains in a manner similar to the way in which it affected the single action potentials before DDT treatment. Also, several previously inactive fibers, also firing in trains, were brought by toluene to the discharge level so that the total number of impulses in unit time for a given toluene concentration was much greater after treatment with DDT. Removal of the benzene (1F) led to a return to a condition in which, once more, only occasional trains appeared. The effects of toluene can be obtained and removed many times on the same preparation, but the effects of DDT are irreversible under these conditions. Similar results were obtained from the tarsi of roaches.

This gives weight to the suggestion (Roeder and Weiant, 1948) that DDT in itself does not stimulate receptors, but changes their pattern of response to normal stimuli from discharge of single spikes to repeated high frequency trains of impulses. As with proprioception, one might expect DDT poisoning to produce excessive reflex behavior due to the increased numbers of impulses delivered by each chemoreceptor at a given level of stimulation. Behavioral confirmation of this is described in the next section.

2. DDT and contact chemoreceptor thresholds

When the tarsi of various insects come in contact with certain "acceptable" compounds, the insects respond by extending the proboscis and attempting to feed. This reflex has been the basis for numerous studies of tarsal chemoreception, notably by Minnich, Frings, and Dethier (see Dethier and Chadwick, 1948, for references). Modifications from their techniques were used in the studies described here.

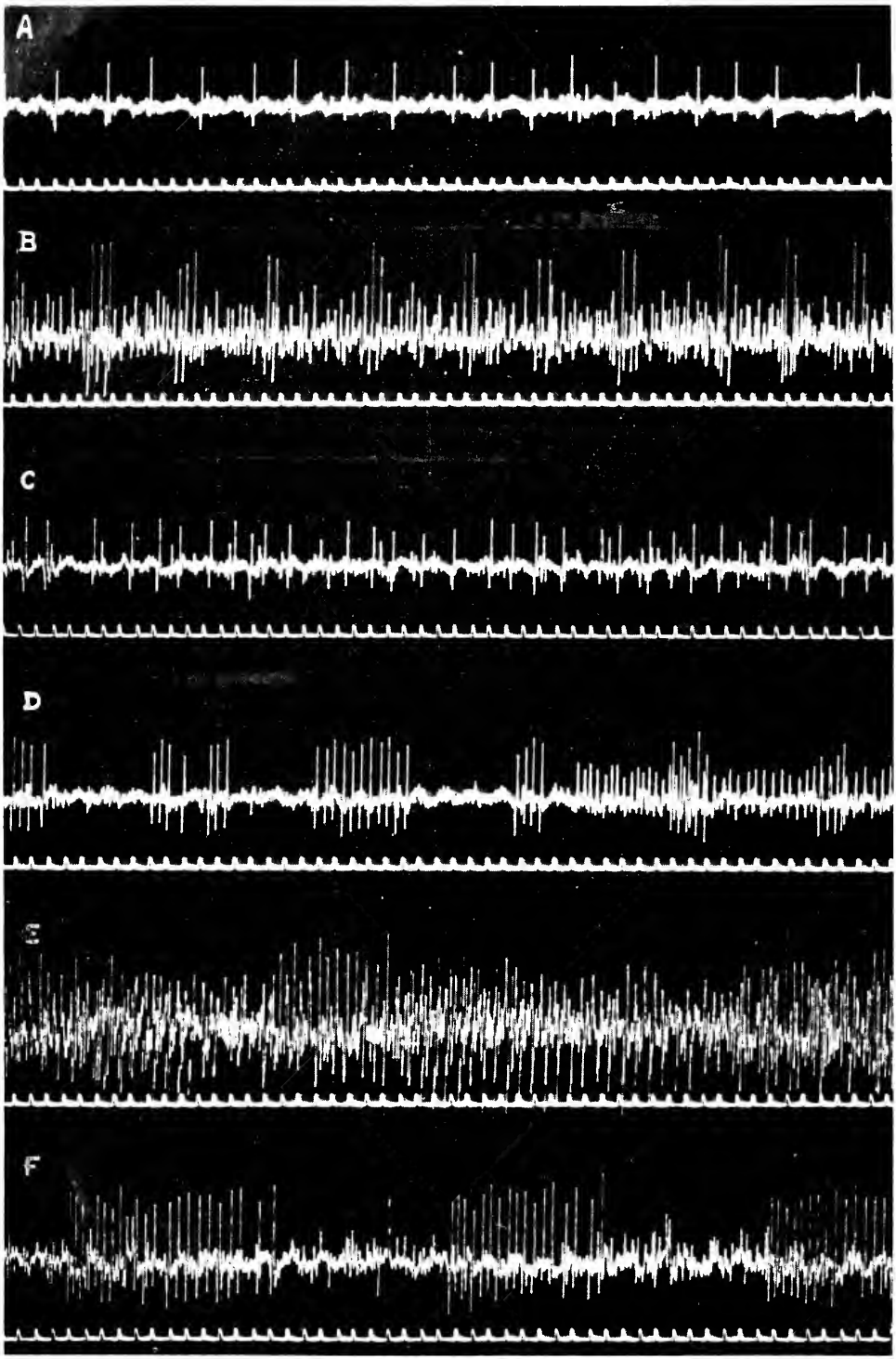

FIGURE 1.

Tarsal thresholds were determined for two strains of houseflies (*Musca domestica* L.), one DDT-sensitive, the other DDT-resistant, obtained from Dr. L. E. Chadwick of the Army Chemical Center, and for DDT-sensitive blowflies (*Phormia regina* Meigen) obtained from Professor V. G. Dethier of the Johns Hopkins University.

The larvae were reared on a milk-yeast-agar medium at 30° C. Pupae and adults were kept at room temperature (21–25° C.). Emerging flies were removed daily and given access to a supply of 0.1 *M* sucrose. One day later flies to be used were anesthetized with carbon dioxide and attached by their wings to paraffined sticks. When volatile substances were to be tested, olfactory receptors (antennae, palpi and labellum) were extirpated at this time.

Thresholds were determined the following day by one of several procedures. For sucrose acceptance thresholds the flies were first allowed to drink distilled water to repletion. Each fly was then held for two seconds with its tarsi in one of a series of sucrose concentrations. Proboscis extension within this time constituted a positive response. If the fly failed to respond, it was tested on 0.1 *M* sucrose, a concentration to which all normal flies should respond. Response here was taken to indicate that the fly was normal in so far as its feeding response was concerned, and it was scored as negative to the previously tested solution. Flies not responding to 0.1 *M* sucrose were considered to be injured or grossly abnormal and data on them were discarded.

Rejection thresholds for NaCl and ethanol were determined in two ways. The various concentrations of the compound to be tested could be made up in 0.1 *M* sucrose, in which case the rejection threshold was the salt or alcohol concentration just preventing the normal positive response to sugar. Or, having denied the flies water before testing, the solutions could be made up with distilled water (no sucrose), the rejection threshold obtained being relative to the thirstiness of the flies. Thresholds against water were much more difficult to obtain than thresholds against sugar, but a few were determined to show whether the action of DDT on sugar receptors masked an effect of DDT on "rejection" chemoreceptors. In either case the flies were given ten seconds to reject the test solution. Extension through the ten second period constituted a positive response (sub-threshold salt or alcohol). Flies failing to respond, or retracting within ten seconds, were checked for two seconds on 0.1 *M* sucrose or distilled water, depending on the type of experiment. Flies responding here were classed as negative; those not responding at all were discarded. In no case was a fly allowed to drink a test solution.

The usual method of DDT application was to place the tarsi of the mounted flies in contact with a DDT-coated glass plate for five minutes immediately before testing. Sensitive houseflies developed symptoms of poisoning seven to eight minutes after exposure at the concentration used—0.01 milligram DDT per square

FIGURE 1. The effect of toluene vapor on electrical activity in afferent fibers of the housefly femur before and after treatment with DDT. A. Untreated preparation. B. Increased activity upon exposure to toluene vapor. C. Toluene vapor withdrawn. D. Three minutes after exposure to DDT vapor. E. Effect of toluene vapor on the DDT-treated preparation. F. Toluene vapor withdrawn, leaving only DDT trains. Time marks, 10 millisecond intervals. For further explanation see text.

centimeter (approx. 9.3 mg./sq. ft.) deposited from acetone solution. To determine whether the cuticular surface of the sensitive tarsal chemoreceptor was the site of selective action by DDT, the insecticide was also applied to another area. In these experiments the meso- and metathoracic legs were immobilized in paraffin ("two-leg flies") and a small droplet of DDT in mineral oil (one milligram per milliliter) was applied to the tip of the abdomen. To answer the question of whether cuticular impermeability barred DDT from the receptors of resistant flies sucrose thresholds were determined for flies injected with DDT. Sensitive as well as resistant flies were run as a check on the method. One per cent DDT in tri-ethylene glycol was serially diluted with Pringle's insect Ringer to 10^{-5} for

TABLE I

Tarsal sucrose acceptance thresholds of Musca and Phormia with and without various DDT treatments

Species	DDT sensitivity	Treatment	Median molar threshold	Log median threshold	Standard error log threshold	Regression response on concentration	Standard error regression	Total flies tested
a. Musca	sensitive	control	.0067	−2.176	±.039	2.00	±.16	479
		tarsal DDT	.00074	−3.129	.029	1.37	.24	201
b. Musca	resistant	control	.0043	−2.365	.068	1.59	.21	204
		tarsal DDT	.0041	−2.389	.063	1.79	.25	204
c. Phormia	sensitive	control	.0020	−2.689	.053	2.16	.28	192
		tarsal DDT	.00043	−3.363	.083	1.50	.22	162
d. Phormia (2-leg)	sensitive	control	.013	−1.887	.046	3.80	.77	66
		tarsal DDT	.0026	−2.585	.055	3.29	.77	64
		abdominal DDT	.0029	−2.536	.059	2.90	.72	63
e. Musca	sensitive	tarsal DDT	.0047	−2.331	.075	1.24	.19	216
		injected DDT	.012	−1.913	.053	2.34	.32	216
f. Musca	resistant	tarsal DDT	.0054	−2.271	.055	2.35	.30	132
		injected DDT	.0064	−2.191	.058	2.98	.37	132

resistant flies and 10^{-6} for sensitive flies; 0.004 milliliter of this was injected into the thorax, giving doses of 0.04 and 0.004 microgram per fly, respectively.

Whenever there were sufficient data, a probit analysis of the results was made (Finney, 1952). In other cases median thresholds were estimated by graphic interpolation.

The tarsal chemical thresholds obtained are listed in Tables I and II. DDT lowered the tarsal sucrose threshold of DDT-sensitive houseflies by a factor of nine, but did not affect the response threshold of resistant flies (I,a,b). The sucrose threshold of blowflies was also strikingly lower following exposure to DDT (I,c).

The method of application was unimportant; DDT picked up by the tarsi, applied in mineral oil to the abdomen (I,d) or injected in saline suspension (I,e) all increased the sensitivity to sucrose. The sucrose thresholds (I,e) of sensitive houseflies following tarsal and internal application of DDT differ by a factor of

three, and at first sight suggest that injected DDT was only half as effective as externally applied DDT in lowering the sucrose threshold. However, this difference can be accounted for by the fact that thresholds of the injected flies varied over a wide range. Only about half of the injected flies appeared to be effectively treated, their thresholds and variance being similar to those of the tarsally poisoned flies in this group. The remaining flies had thresholds scattered about a concentration some nine times higher, but with a similar variance. Possibly the large amount of solution injected interfered with circulation, preventing the transport of DDT to the receptors. The thresholds of flies in I,e and I,f cannot be

TABLE II

Tarsal salt and alcohol rejection thresholds of Musca and Phormia tested against water or sucrose solutions, with and without tarsal application of DDT

Species	DDT sensitivity	Treatment	Median molar threshold	Log median threshold	Standard error log threshold	Total flies tested
NaCl against 0.1 M sucrose						
a. Musca	sensitive	control	1.7	0.22		125
		tarsal DDT	1.7	0.23		70
b. Phormia	sensitive	control	1.4	0.161	±.019	110
		tarsal DDT	1.3	0.130	.018	110
NaCl against water						
c. Musca	sensitive	control	1.0	0.00		30
		tarsal DDT	1.2	0.12		51
Ethanol against 0.1 M sucrose						
d. Musca	sensitive	control	6.8	0.83		59
		tarsal DDT	4.4	0.65		34
e. Musca	resistant	control	3.5	0.55		20
		tarsal DDT	2.5	0.40		21
f. Phormia	sensitive	control	1.7	0.22		44
		tarsal DDT	1.8	0.25		44
Ethanol against water						
g. Musca	sensitive	control	1.1	0.05		15
		tarsal DDT	1.1	0.05		13

compared with those in I,a and I,b, it being a common experience in such tests that thresholds vary widely in different experimental groups.

The threshold of resistant flies was unchanged (I,f), even when DDT was injected in amounts sufficient to poison some individuals. In no case did DDT treatment significantly affect a salt or alcohol threshold, either tested against sucrose or against water (Table II).

It should be pointed out that absolute threshold values changed from time to time over a considerable range, even though an attempt was made to maintain standard rearing and test conditions. The comparisons indicated in Tables I and II represent data collected in concurrent experiments where alternate flies in the

same population were given the various treatments. Some of the data represent lumping of results from duplicate experiments run within a period of a few days. In all these cases the individual experiments were consistent with each other, although slight displacements of the absolute thresholds increase the standard errors. Considerable other data not suitable for inclusion in the tables substantiate the results reported.

In spite of an intensive search in several laboratories there is no electrophysiological evidence of afferent nerve impulses from chemoreceptors concerned with detection of sugar or other substances producing proboscis extension by flies. Nevertheless, perception of such stimuli can be understood only in terms of increased or decreased receptor activity. Because DDT in general increases the activity of irritable units if it affects them at all, and because it also increases the fly's sensitivity to sugar, the simplest explanation of these observations is that there must be chemoreceptors which respond to acceptable compounds by sending nerve impulses more frequently to the central nervous system. DDT would then act by converting each single impulse to a group or train of impulses (Fig. 1), increasing the message rate relative to the supply of information. Chances for central summation, either spatial or temporal, would be increased, and so therefore the likelihood of response. In other words, assuming a steady central excitatory state, if proboscis extension be elicited by a sensory input consisting of a certain number of impulses in a given time interval from a specific group of receptors, then the tendency to high frequency repetition caused by DDT should make possible the requisite input from a smaller number of receptors, and hence from a lower concentration of stimulant.

A somewhat more complicated but also plausible explanation is that the chemoreceptors detecting acceptable compounds are relatively DDT-insensitive. The sensitizing action of DDT would then have to be on some other receptor system, activity in which would facilitate the proboscis response. This idea will be referred to again.

Although salt and alcohol are representatives of different human taste modalities and may also be different to the insect, both are rejected and they can be discussed together here. In no instance was a rejection threshold affected by DDT treatment. Yet, the preceding electrophysiological observations indicate that DDT increases the afferent response to chemical stimulation. Moreover, as demonstrated by the rejection thresholds against sugar compared with those against water, rejection thresholds depend on the intensity of the opposing acceptable stimuli, high sugar concentrations raising the rejection thresholds. And, sugar is a more effective stimulus following DDT treatment. Therefore, DDT must increase the activity of "rejection" receptors to an extent that balances its effect on sugar receptors or their adjuncts. Furthermore, acceptable and unacceptable compounds must act primarily on different receptor units and not in opposite manner on one receptor type. Otherwise one would have to attribute to DDT simultaneous stabilizing and unstabilizing actions on the same cell in order that the rejection threshold remain unchanged. This confirms Dethier's (1953) conclusion that there must be at least two varieties of chemoreceptors on fly tarsi.

DDT applied to the surface of the abdomen or injected into DDT-sensitive houseflies and blowflies lowered sucrose thresholds just as it did when applied to the tarsi. Because neither externally applied nor injected DDT affected the

thresholds of DDT-resistant flies, it seems reasonable to say that cuticular impermeability is not solely responsible for the resistance of this strain. Rather, resistance must include some intrinsic mechanism by which the receptors are protected from the unstabilizing action of DDT. Pratt and Babers (1953) reached a similar conclusion for the thoracic ganglion after studying its DDT-sensitivity in susceptible and resistant houseflies. Although they believe that enzymatic detoxification of DDT is not rapid enough to account for the difference observed between the two strains, it seems possible that a strategic deployment of the protective enzymes to the primary sites of DDT action could account for such resistance.

3. Surface texture and the proboscis extension reflex

In the course of tarsal chemoreception tests on *Phormia* and *Musca* it was observed that an occasional fly would attempt to feed when placed on a clean, smooth surface, especially during the earliest stages of DDT poisoning. Using surfaces at hand it was found that proboscis extension occurred when the tarsi were placed on smooth glass, Lucite, celluloid, polished metals and glazed kymograph paper, but not on newsprint, rough wood, frosted glass or the investigator's finger or clothing. Proboscis extension being the customary criterion of effective stimulation of the "acceptance" receptors, this behavior seemed worthy of further investigation.

Additional observations were made on 338 houseflies mounted on sticks in the manner previously described. After 24 to 40 hours of starvation (until a good response to water was obtained), they were tested in the following manner. Each fly was held so that its tarsi were in contact with either the smooth or rough side of a piece of frosted glass for five seconds, then transferred to the other surface. This was repeated, and if no response was evoked the fly was tested on distilled water. When a positive response was obtained with a dry surface, the fly was repeatedly tested for five-second intervals on the two surfaces.

Of 177 flies responding positively to distilled water, one invariably responded to both surfaces, 155 to neither, and 21 discriminated between the two, responding only to the smooth side. None responded preferentially to the rough. A few *Phormia* were later observed to respond to a clean, smooth surface even though satiated with distilled water. Feeding dry powdered sugar one hour before testing did not alter the proportion of flies responding to surface texture. DDT applied in the manner described above increased the level of response to almost all stimuli.

Like most other behavior patterns the proboscis reflex is not solely the product of a single chemical releasing stimulus, but depends on the balance between facilitating and inhibitory influences from a number of sources, *e.g.,* other chemical stimuli, nutritional state, water balance and mechanical excitation. If these secondary stimuli were to produce a sufficiently favorable state of central excitation, it is possible that spontaneous activity from the chemoreceptors could exceed the threshold for proboscis extension. A background of summating activity from many mechanoreceptors stimulated in unison as by a smooth surface might well contribute to a favorable central state. Rough surfaces should stimulate in a more erratic fashion.

DDT could increase the likelihood of feeding response either by amplifying the activity of the chemoreceptors, or by increasing the number of mechanorecep-

tors active at any one time. Although DDT eventually increases the irritability of certain mechanoreceptors and chemoreceptors, the magnitude of its effect on the sugar threshold is probably indicative of an early action on sugar receptors.

One wonders why flies should be more prone to feed on a smooth surface. Perhaps the vacuum-cleaner type mouth part functions more efficiently here. Also, the resemblance between smooth surfaces and a water surface may be great to a fly.

4. Perception of DDT by houseflies

The fact having been established that DDT can increase the sensitivity of various receptor systems to their appropriate stimuli, there remained an interesting question as to whether the insect can detect DDT itself. Ability to do so would make possible a behavioral type of resistance through selection of strains avoiding the chemical. A rather surprising answer to this question was obtained by

TABLE III

Distribution of houseflies in petri dishes between chemically impregnated and control (acetone-treated) papers

Compound tested	DDT sensitivity	Flies on treated paper	Flies on control paper	Chi-square	Probability	Total flies on paper	Total possible flies on paper
DDT	sensitive	1336	1136	16.2	.01	2,472	12,100
DDT	resistant	792	808	0.16	.5–.7	1,600	9,040
DDT	sensitive	611	506	9.86	.01	1,117	4,800
DDE	sensitive	558	547	0.110	.7–.8	1,105	4,800
1,1-bis(p-chloro-phenyl) ethane	sensitive	564	575	0.106	.7–.8	1,139	4,800

confining flies in chambers with two pieces of paper, one containing DDT, and recording periodically the distribution of the insects.

The procedure was as follows. Seven-centimeter filter papers were prepared with 0.01 milligram DDT or other test compound per square centimeter deposited from acetone solution. After complete evaporation of the solvent the papers were cut in half and paired with acetone-treated control half circles in the bottoms of clean petri dishes. To keep them in place, the papers were dampened with distilled water. Ten flies were placed in each arena and counts of the number of flies on each of the two test half-circles in each petri dish were made every minute from the eleventh through the twentieth minute after the beginning of the experiment. Clean dishes and fresh papers were used for each test.

Under these conditions sensitive flies exposed to DDT in the course of the experiment did not begin to show overt signs of DDT poisoning until thirty minutes or more after introduction into the dishes. Each dish contained one test and one acetone control paper. Both papers and dishes were arranged according to a Latin square design in order to minimize the influence of external factors such as light and temperature gradients, and bias on the part of the observer.

The results are shown in Table III. In both series of tests the data clearly show that DDT-sensitive flies have a predilection for the DDT-treated surface. DDT-resistant flies show no such preference. However, DDT-sensitive flies fail to discriminate between surfaces treated with DDE (1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene) or 1,1-bis (p-chlorophenyl) ethane and acetone controls. This suggests that the factor in the DDT-treated paper which is the basis for preference is connected with the specific physiological activity of DDT rather than with general physical or chemical properties which it presumably shares with the two relatively non-toxic DDT analogs tested.

Proboscis extension and withdrawal are not the only responses to chemical stimulation of the tarsi. When one of the tarsi touches an acceptable substance the fly turns toward that point and begins feeding. Avoidance of noxious chemicals also occurs. Thus stimulation of contact chemoreceptors initiates orientation behavior related to feeding as well as the feeding reflex itself. Probably, the same receptors control both activities. Since it is apparent that there are different groups of receptors responding to acceptable and unacceptable compounds, the preference of DDT-sensitive flies for DDT could be taken to show that DDT selectively potentiates (by causing impulse trains) the sensory input from "acceptance" receptors during the early stages of poisoning, "rejection" receptors being either less sensitive or slower to respond. On this basis a DDT-treated surface could have an illusory attractiveness to flies when first walking on it. This would account for the increased time spent on such surfaces in the experiment. DDT-sensitive flies, by choosing to spend additional time in DDT-treated areas in a partially treated environment contribute to their own destruction. Through loss of ability to detect the insecticide, relatively resistant flies will come in contact with less DDT and further improve their chances of survival.

General Discussion

Among the many receptors on the tarsi of flies several types responding to different modalities of stimulation control the feeding response. These include at least two types of contact chemoreceptor (see also Dethier, 1953), activated by acceptable and unacceptable compounds, releasing or inhibiting the feeding reflex and orientation to food. Under conditions of near-starvation a suitable pattern of tactile stimulation can release feeding behavior. Therefore, one may assume that mechanoreceptors modulate the effects of afferent impulses from chemoreceptors, even though touch is not normally the effective stimulus for the proboscis extension reflex.

Such a cooperation between sensory systems should make possible an economy in terms of the number of receptors of each type necessary; a limited amount of cross summation between associated stimulus modalities would increase the effectiveness of each stimulus. A high level of spontaneous activity in unstimulated receptors should increase similarly the effect of stimulation of a portion of the receptor population.

Electrophysiological observations, both those relating to insect mechanoreceptors and those reported here for chemoreceptors, show a background of spontaneous activity. DDT increases the effectiveness of this background by converting single nerve impulses to trains of impulses. The more frequent afferent impulses

which result from normal stimulation are also made repetitive by the action of DDT. Opportunities for summation both within and among modality groups are thus increased.

Observation of DDT-poisoned insects corroborates this interpretation. Shortly after exposure to the insecticide certain response thresholds of DDT-sensitive individuals become lower. A decided hyperexcitability develops, and this gives way to random reflex activity as, presumably, more and more receptors become unstabilized. Uncoordinated locomotor movements and proboscis extension and retraction are characteristic symptoms of this stage of poisoning. In DDT-sensitive flies the effect of DDT on individual receptors is irreversible.

Summary

1. DDT appears not to stimulate sensory endings, but makes them capable of repetitive discharge following stimulation with toluene and benzene.

2. Pretreatment with DDT lowers about nine-fold the sucrose acceptance thresholds of DDT-sensitive houseflies and blowflies. Sucrose thresholds of resistant flies are unaltered.

3. Pretreatment with DDT does not change salt or alcohol rejection thresholds, either against sucrose or water.

4. Under certain conditions clean smooth surfaces can evoke feeding behavior similar to that in response to acceptable chemicals.

5. DDT-sensitive houseflies, given a choice, spend a greater amount of time on DDT-treated areas. Resistant flies do not. Surfaces treated with DDE and 1,1-bis (p-chlorophenyl) ethane are not discriminated from control surfaces.

6. It is concluded that acceptable and unacceptable compounds are perceived through different sets of receptors.

7. The proboscis extension reflex is not controlled by chemoreceptor activity alone, but also by tactile stimuli.

8. The failure of DDT to affect reflex behavior of this strain of DDT-resistant houseflies is not due to failure to penetrate the cuticle, but must be due to some mechanism intrinsic to the nervous system.

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LOSS AND GAIN OF HEAT-TOLERANCE BY THE CRAYFISH

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It has been well established that a fish becomes increasingly tolerant of heat as it becomes acclimatized to higher temperatures within the range of thermal tolerance of its species, and that its acclimatization to low temperature entails a loss of heat-tolerance (Doudoroff, 1942; Brett, 1944, 1946). Inasmuch as aquatic arthropods are exposed to the same fluctuations in environmental temperature as fish, one might reasonably expect to find that they, too, are capable of gaining and losing heat-tolerance. The matter has not been explored as thoroughly for aquatic arthropods as for fish, however, and there are but few instances in which it is clear that individual animals have gained or lost heat-tolerance, *i.e.*, that they have undergone "physiological" acclimatization, as the term is used by Prosser *et al.* (1950). It is true that aquatic arthropods from warm waters have been found to be more heat tolerant than related species, or even members of the same species, inhabiting cooler waters (Mayer, 1914; Huntsman and Sparks, 1924; Fox and Wingfield, 1937; Mason, 1939; Whitney, 1939; Park, 1945; Walshe, 1948; Marlier, 1949; Bovbjerg, 1952), but in most cases the differences in tolerance can be attributed as well to selection as to physiological acclimatization (Fox, 1939).

With one possible exception, the literature bearing directly upon the problem of physiological acclimatization among aquatic arthropods indicates that they do become increasingly tolerant of heat as their environmental temperatures are increased. Huntsman (1924) showed that lobster larvae raised at temperatures between 20 and 25° C. were more heat resistant than those raised at 15°, Edwards and Irving (1943) reported the thermal death point of the sand crab, *Emerita talpoida,* to be about 10° higher in summer than in winter, and Marlier (1949) found indications that the lethal temperature of larvae of the caddis fly, *Hydropsyche angustipennis,* increased from 31° in the spring to 32° in early summer. Furthermore, Bovbjerg (1952) observed that two species of crayfish, *Orconectes propinquus* and *Cambarus fodiens,* became increasingly tolerant of temperatures between 34 and 35° as the advancing season warmed their habitats, or after they had been maintained in warm water in the laboratory for five or six weeks. The possible exception was reported by Whitney (1939), who found nymphs of the mayfly, *Baetis rhodani,* to be no more heat tolerant after 40 hours at 15° than controls maintained between 10 and 11°. In view of Brett's (1946) experience with the goldfish, however, in which it was shown that the development of an increased heat-tolerance required a latent period of from one to seven days, the latent period being longer the lower the acclimatization temperature, it may be that the absence of acclimatization in *Baetis* was more apparent than real. It seems quite possible that more than 40 hours of acclimatization were required to increase the heat-tolerance enough to be detected by the method employed.

Less seems to be known about the loss of heat-tolerance by aquatic arthropods. Aside from the foregoing observations on the sand crab and crayfish, in which the seasonal gain of tolerance suggests a previous seasonal loss, no literature bearing directly upon this matter has been found.

Relatively little appears to have been published about the temperature relations of the crayfish. The mere fact of its occurrence in the northern part of the United States shows, of course, that it can survive over a range of temperatures extending from about 0 to more than 30°, and direct observations by several authors bear this out. Crayfish are active at temperatures between 4 and 6° (Van Deventer, 1937) and under two inches of ice (Bovbjerg, 1952). They will live at 3° in the laboratory (Kyer, 1942), and adult females have been found to survive freezing overnight (Langlois, 1937). Bovbjerg (1952) has recorded that they occur in ponds that reach 30°, and that acclimatized crayfish have remained alive after a period of seven days at 34 to 35° in the laboratory. In connection with the present study it was observed that adult crayfish were abundant in a shallow river of which the surface temperature at any rate reached 32°. In view of its convenient size, longevity, availability and wide range of thermal tolerance, the temperature relations of the crayfish are of considerable interest not only to the ecologist but also to the physiologist concerned with the problem of explaining the mechanism of acclimatization to temperature. The experiments to be described in this paper were undertaken to provide further information on the thermal relations of the crayfish, especially in connection with the loss and gain of heat-tolerance.

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Material and Methods

The crayfish, *Orconectes rusticus* (Girard), were collected in July of 1953 and 1954 from Sugar Creek, a small tributary of the Portage River, which enters western Lake Erie at Port Clinton, Ohio. The stream temperatures at the times of collection varied between 22 and 26° C. The animals were maintained at Stone Institute in shallow tanks of running lake water, the temperature of which ranged betwen 22 and 26° (usually between 23 and 24°), and the pH between 8.4 and 8.7 (usually between 8.5 and 8.6). The crayfish seemed to remain in good health under these conditions—they fed eagerly on mussel, fish, bread and lettuce, many molted, the mortality was low, and those not removed for use in the experiments were alert and lively after about six weeks.

Aside from several dozen adult crayfish used only to test the effects of size on heat-tolerance, the seven or eight hundred specimens used in the course of the study ranged between 17 and 42 mm. in total length (from the tip of the rostrum to the tip of the telson when straightened out), most of them being between 25 and 35 mm. They were sexually immature, and presumably in their first summer of life (Van Deventer, 1937; Tack, 1941). Groups of from 25 to 100 were taken

at random from the stock tanks and maintained in well-aerated glass aquaria at acclimatization temperatures of 30 ± 0.1, 12 ± 0.5 and $4 \pm 0.5°$. Those held at 30° were warmed to this temperature from that of the stock tank over a period of several hours. Those in the low-temperature baths were transferred directly from the stock tank, it having been found unnecessary to cool them slowly. Care was taken that no metal came into contact with the water in the aquaria. The animals at 30 and 12° were fed bread and lettuce; those at 4° did not feed. About a fourth of the water in each aquarium was replaced every day or so with fresh lake water, previously brought to the appropriate acclimatization temperature and aerated. The crayfish remaining in the stock tanks were used as controls.

The heat-tolerance of control and experimental animals was studied by testing their survival for 12 hours or longer at 33, 34, 35, 36 and 37°, it having been ascertained in preliminary tests that this range of temperatures spanned the 12-hour median heat-tolerance limits (Doudoroff, 1942; Brett, 1944) of the several acclimatized groups. The test temperatures were maintained to within 0.1°. The animals were tested singly in 10-oz. wide-mouth bottles or in groups of 5 to 10 in one-liter Erlenmeyer flasks, these vessels being held at the test temperatures in constant temperature baths. Each bath held a number of such vessels, so that it was possible to test as many as 40 crayfish simultaneously at one temperature. In preparation for a test, aerated fresh lake water was brought to the temperature of the acclimatization bath from which the animals were to be taken. One hundred ml. of this water were then poured into each bottle, or one liter into each flask, so that at least 100 ml. of water were provided for each animal being tested. The crayfish were then transferred to the vessels and warmed gradually to the test temperature, the test period being started as soon as this temperature had been reached. Aeration sufficient to maintain an oxygen concentration of at least 80% air-saturation was continued throughout the warming and test periods.

The warming period usually lasted for from 15 to 60 minutes, according to the difference between the acclimatization and test temperatures. This procedure was chosen in preference to that of transferring the crayfish abruptly from the acclimatization temperature to the test temperature because the property under consideration was the ability to survive for 12 hours or longer at the test temperature, rather than that of being able to withstand the shock of a sudden plunge from one temperature extreme to another. A sudden plunge into warm water was found to constitute so massive a shock, especially when the difference between the two temperatures spanned some 25 degrees or more, that the distress of the animals was intense, sometimes enough to cause them to throw off one or both chelae. It is possible, of course, that the period of warming allowed them to gain somewhat in heat-tolerance over that possessed at the acclimatization temperature, but such a gain in tolerance, if it occurred at all, was not great enough to obscure the results. In this connection Brett (1941) has reported that raising the temperature gradually from acclimatization to test temperatures over a period of 15 minutes had no effect on the lethal temperatures of speckled trout.

The condition of each crayfish was observed as it reached the test temperature, and from time to time in the course of the ensuing test period. When no movement of the appendages could be detected, or elicited by prodding with a glass rod, the crayfish was considered to be dead. Death was confirmed in each

case at room temperature after the test, and at this time the length of each animal was measured, the sex determined, and gastroliths sought. Each gastrolith found was measured along its greatest diameter, and its thickness noted, the size serving as an index to the stage of the molt cycle (Scudamore, 1947). As the responses of the crayfish tested in 1954 were practically the same as those of the 1953 collection, the data of the two years have been combined.

Results and Interpretation

1. Heat-tolerance of control animals

Crayfish that had been maintained in the stock tanks for from one to five weeks at temperatures between 22 and 26° survived for not more than 6 hours at 37°, for more than 12 but usually not more than 24 hours at 36°, for at least 10 days at 35°, and for at least 24 days at 34°. Their survival of 12-hour and 24-hour exposures to the three higher temperatures is shown in Table I. Evidently the 12-hour median tolerance limit for these animals was above 36°, but below 37°. When the per cent surviving at each temperature is plotted against the test

Table I

Survival of crayfish taken from temperatures between 22 and 26° and tested at 37, 36 and 35°

Test temperature °C.	Number of tests	Number of crayfish	Crayfish surviving			
			for 12 hours		for 24 hours	
			Number	Per cent	Number	Per cent
37	9	76	0	0	—	—
36	15	109	86	78.9	19	17.4
35	12	78	78	100	73	93.6

temperature (Figure 1), the 12-hour median heat-tolerance limit is seen to have been in the vicinity of 36.4°. That for 24 hours was 35.6°.

Their survival for long periods at 34 and 35° confirms and extends Bovbjerg's (1952) observations. In view of the proximity of these two temperatures to the 24-hour median tolerance limit of the controls, it is of interest that the crayfish molted and fed at 34 and 35°. The small difference between survival- and death-temperatures is quite striking, although not peculiar to the crayfish. It has been observed before in experiments with the sand crab (Edwards and Irving, 1943) and with the greenfish, *Girella nigricans* (Doudoroff, 1942).

2. Loss of heat tolerance

When transferred from the stock tanks to an acclimatization bath at $4 \pm 0.5°$, the crayfish lost the heat-tolerance characteristic of the control group, the loss becoming greater the longer the exposure to low temperature. The 12-hour median heat-tolerance limit fell from 36.4 to 35.3° by the end of the fourth day, to 34.8° by the end of the eighth day, to 34.1° by the end of the twelfth day, and

to 33.5° by the end of the sixteenth day in the cold, as shown in Figure 2. Apparently the loss of tolerance did not begin immediately upon exposure to the cold, however, the tolerance after two and a half days being about the same as the original value. The fact that the minnow, *Pimephales promelas,* shows a similar latent period in the loss of heat-tolerance (Brett, 1944) suggests that this may be a rather general characteristic of animals. The apparent slight increase in tolerance after one day in the cold may have been due to chance, the number of crayfish tested at this point being rather small, but it seems worth noting in this

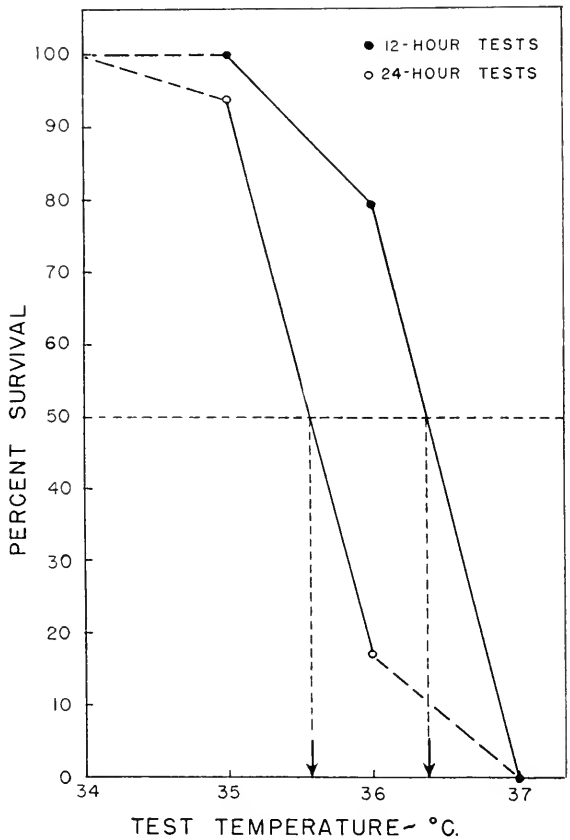

FIGURE 1. Survival at 35, 36 and 37° of crayfish acclimatized to temperatures between 22 and 26° C. The arrows mark the 12- and 24-hour median heat-tolerance limits.

connection that Sumner and Doudoroff (1938) and Doudoroff (1942) have made similar observations on two species of fish. Presumably the crayfish would have continued to lose heat-tolerance, had they been left longer at 4°.

Stock crayfish transferred to 12 ± 0.5° lost their original heat-tolerance in a similar manner, but more slowly. As shown in Figure 2, the 12-hour median tolerance limit had fallen to 35.6° by the end of five and a half days, and to 35.1° by the end of the second week.

3. Gain of heat-tolerance

The heat-tolerance lost during exposure to low temperature was regained quite rapidly when the crayfish were removed from the cold bath. Twenty specimens that had been held at 4 ± 0.5° for thirteen days, long enough for them to have lost their original heat-tolerance by more than two degrees and to have reached a median tolerance limit of about 34° (Fig. 2), were warmed to room temperature (23.5 to 24°) in the course of about two hours and maintained at this temperature for 22 hours. When they were then tested at 36°, 90% lived for 12 hours and 40% survived 24 hours at the test temperature. Evidently their heat-tolerance was at least as great as that of the control animals from the stock tanks, the

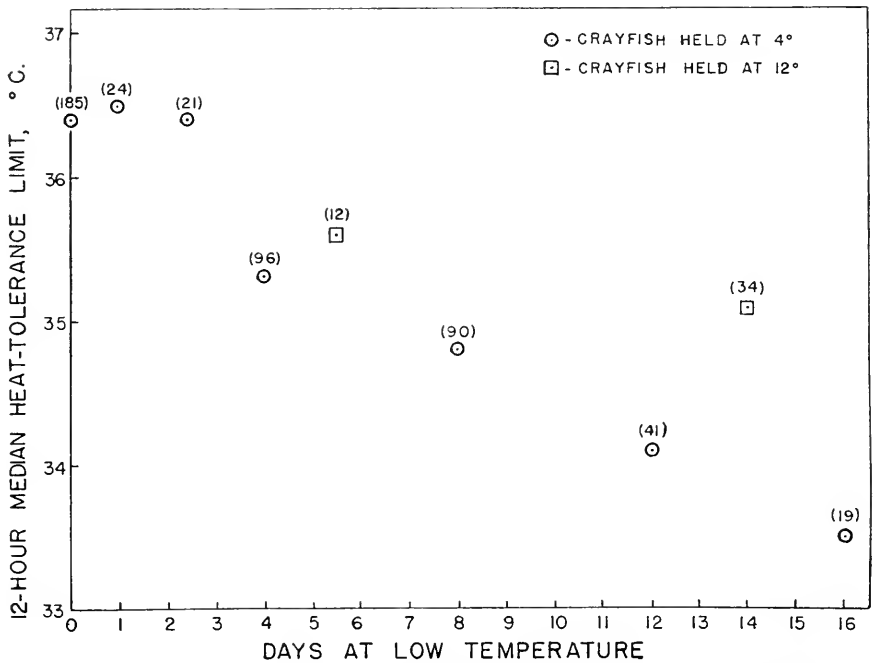

FIGURE 2. Loss of heat-tolerance at 4 ± 0.5 and 12 ± 0.5° C. Numbers of test animals shown in parentheses.

survival of the latter at 36° averaging 78.9% for 12 hours and 17.4% for 24 hours (Table I). Results consistent with these were obtained with six crayfish tested at 35° after one day, and with nine tested at 36° after two days, at room temperature, five of the six and seven of the nine surviving the 12-hour test periods. As in fish (Doudoroff, 1942; Brett, 1944, 1946), the rate of gain of heat-tolerance by the crayfish seems to be considerably higher than the rate of loss, the tolerance lost in about ten days at 4° (after the first two and a half days had elapsed) being recovered in not more than one day.

Upon acclimatization to a higher temperature, the crayfish gained a heat-tolerance somewhat greater than that characteristic of acclimatization to 22–26°.

The 12-hour and 24-hour median heat-tolerance limits of a group of crayfish maintained in an aquarium at 30 ± 0.1° were compared with those of a control group maintained under the same conditions in another aquarium, but at room temperature (between 22 and 25°). Both groups were tested over a three-week period, 20 of each group being tested at the end of each of the first two weeks, and 10 of each group at the end of the third week. The 12-hour median tolerance limit of the 30° animals was 36.6° by the end of the first week and remained at this level for the next two weeks. The corresponding limits for the controls were 36.2, 36.4 and 36.5° after the first, second and third weeks, respectively. The 24-hour limit also was 36.6° at the end of the first week and, like that for 12 hours, did not change from this value during the second and third weeks. The corresponding weekly 24-hour tolerance limits for the controls were 35.5, 35.6 and 35.5°. The slight increase in the 12-hour median tolerance limit is not in itself convincing, but the increase by about one degree in the 24-hour tolerance limit represents a clear-cut gain. The reality of this gain is indicated by the fact that the three tests were consistent, although conducted a week apart. Furthermore, five of the animals from the 30° aquarium lived for more than 12 hours at 37°, one of them, in fact, surviving a 24-hour test at this temperature. It will be recalled that none of the controls taken from temperatures between 22 and 26° lived for more than 6 hours at 37°. It is of interest that continued exposure to 30° did not cause an increase in tolerance over that shown by the end of the first week, and that the limit for 12 hours reached a value no higher than that for 24 hours. These observations suggest the possibility that 36.6° was not far from the maximal heat-tolerance limit attainable by the population from which the collections were made. In view of the high rate of gain of heat-tolerance described earlier, it seems not improbable that acclimatization to 30° was completed in much less than a week.

4. Heat-tolerance in relation to sex, size and the molt cycle

Although all crayfish used in these tests were drawn from the same population and maintained under the same conditions, they were of course not all alike. Males and females were about equally represented, the lengths ranged from 17 to 42 mm., and the animals were at different stages of their molt cycles. About 3% of the specimens had gastroliths large enough to indicate that they were at the molting stage; the rest had smaller gastroliths (about 22%) or none that could be seen macroscopically (75%). As the crayfish were distributed randomly among the tests, it seems unlikely that the results would have been affected by these variables, even if there had existed a relationship between heat-tolerance and sex, size, or the stage of the molt cycle. As a matter of fact, the evidence at hand indicates that no such relationship existed.

Because of the random distribution, males and females, large and small specimens, and representatives of different stages in the molt cycle could be found in each series of tests. The effects of each of these variables could be investigated, therefore, by comparing the animals that survived with those that died at each test temperature. In this manner it was found that as in the sand crab (Edwards and Irving, 1943) and the amphipod, *Hyalella azteca* (Bovee, 1949), sex did not affect the heat-tolerance of the crayfish. It must be borne in mind, of course, that

the specimens were immature. No relationship between size and tolerance could be detected, and the absence of such a relationship is indicated further by the fact that 30 adult specimens, of body lengths between 56 and 82 mm. and with the same history as the control animals in the stock tanks, were neither more nor less heat tolerant than the controls. No inconsistency is seen between these results and reports that larger specimens of the sand crab (Edwards and Irving, 1943) and *Hyalella azteca* (Bovee, 1949) were somewhat more heat resistant than small ones, the methods used in the present study being so different from the others that no direct comparison can be made. Using methods essentially the same as those described in this paper, Doudoroff (1942) and Hart (1947) found no correlation between size and survival time of fish at extreme temperatures. Finally, the stage of the molt cycle evidently had no effect upon heat-tolerance, despite the changes associated with molting and the stress thought to be placed upon the crayfish by the process (Scudamore, 1947). Whether the gastroliths were absent, small, or so large as to indicate that the animals were in molting condition, there was no evidence that their tolerance had been affected. Furthermore, crayfish were found to molt successfully in the acclimatization baths at 12 and 30°, in the course of tests at temperatures as high as 36°, and at room temperature after the period of exposure to the test temperature. They did not molt at 4°.

Discussion

The literature on temperature relations of fish has made it clear that heat-tolerance is so dependent upon acclimatization temperature that expressions of either lethal temperatures or median tolerance limits are incomplete unless accompanied by information on the animal's thermal history. According to the results of the present tests, as well as those described by Bovbjerg (1952), the same can be said of the crayfish. As a consequence, an attempt to compare the heat-tolerance of the crayfish with those of other aquatic animals is hampered by the fact that in most cases the acclimatization temperatures have been different from those used in this study. Such a comparison is limited further by differences among the criteria used in the measurement of heat-tolerance. Whereas 12- and 24-hour median tolerance limits have been sought here, largely so that the results could be related to the more highly developed literature on the temperature relations of fish, most of the data on aquatic arthropods have been expressed in terms either of the death temperatures of animals subjected to ever-increasing temperatures or of the time required for them to die when transferred to constant test temperatures.

Although one gains the general impression that the crayfish is among the more heat tolerant of the aquatic arthropods that have been investigated, direct comparisons are attempted here with but five species (Table II), these having been selected because their acclimatization temperatures were reasonably close to that of the crayfish from the stock tanks. The median tolerance limit given in Table II for *Orconectes rusticus* is that for the 12-hour test period. The figure for *Hyalella* is the temperature at which about half the specimens survived for nearly 11 hours, so that it approximates the 12-hour median tolerance limit. It seems unlikely that increasing the acclimatization temperature by two or three degrees would have increased the median tolerance limit to that of the crayfish. In fish, at any rate, the acclimatization temperature must be increased by several degrees to bring about

an increase of one degree in the median tolerance limit (Fry, Brett and Clawson, 1942; Brett, 1944; Hart, 1947). A temperature of 35° is almost certainly lower than the 12-hour median heat-tolerance limit of Bovbjerg's (1952) acclimatized crayfish, inasmuch as they lived for days between 34 and 35°. On the other hand, 37° is probably somewhat above that of the sand crab, all specimens tested by Edwards and Irving (1943) having died during or shortly after a period of four hours at this temperature. The figures shown for the lobster larvae are the temperatures at which the hearts stopped beating as the animals were warmed at a rate approximating 0.4° a minute; the 12-hour median tolerance limits must have been considerably lower. Although conclusions can be no more than tentative, the sand crab and the three species of crayfish appear to have similar heat-tolerance limits, these being greater by several degrees than those of the lobster larvae and *Hyalella azteca*.

The methods and criteria being more nearly alike, the temperature relations of the crayfish can be compared with those of fish with more confidence. Evidently the crayfish has a heat-tolerance of the same order as that of the more tolerant of

TABLE II

Estimated heat-tolerance limits of several aquatic arthropods related to the 12-hour median heat-tolerance limit of Orconectes rusticus acclimatized to 22–26°

Animal	Acclimatization temperature, °C.	Heat-tolerance limit, °C.	Author
Orconectes rusticus	22–26	36.4	—
O. propinquus	18–28	more than 35	Bovbjerg, 1952
Cambarus fodiens	18–28	more than 35	Bovbjerg, 1952
Lobster, stage 4	25	less than 34.2	Huntsman, 1924
Lobster, stage 5	20	less than 35.4	Huntsman, 1924
Emerita talpoida	20	less than 37	Edwards and Irving, 1943
Hyalella azteca	20	33	Bovee, 1949

the fishes. Of the several dozen species of fresh water fish for which comparable data are available (Fry, Brett and Clawson, 1942; Brett, 1944; Hart, 1947; Black, 1953), the acclimatization temperatures and criteria of tolerance being essentially the same as those used in this study, the brown bullhead (*Ameiurus nebulosus*) and the goldfish (*Carassius auratus*) are among the most tolerant of high temperatures. That crayfish are at least as tolerant as the bullhead is indicated by the fact that bullheads acclimatized to 26° had a lethal temperature (the equivalent of the 12-hour median heat-tolerance limit used here) of 35.3° (Brett, 1944), whereas the corresponding limit for crayfish of similar acclimatization was 36.4°. Apparently the crayfish does not exceed the bullhead in its potential range of tolerance, however, for the 12-hour median tolerance limit of crayfish acclimatized to 30° was about the same (36.6°) as that indicated for the bullhead by Brett's (1944) data. Like the bullhead, the crayfish appears to be less heat tolerant than the goldfish, specimens of which had a 12-hour median heat-tolerance limit somewhat greater than 37° when acclimatized to 28°, according to figures given by Brett (1946). The rather marked similarities between crayfish and fish with respect to loss and gain of heat-tolerance, as described in a preceding section, suggest that the basic

principles of temperature acclimatization are much alike in the two groups, if not identical.

Summary

1. Crayfish (*Orconectes rusticus*) taken from environmental temperatures between 22 and 26° lived for not more than six hours at 37° and for at least ten days at 35°. Their 12-hour and 24-hour median heat-tolerance limits were 36.4 and 35.6°, respectively.

2. Upon being transferred to about 4° and maintained at this temperature, their heat-tolerance, as measured by the 12-hour median tolerance limit, fell to 35.3° after four days, to 34.8° after eight days, to 34.1° after twelve days, and to 33.5° after sixteen days. Heat-tolerance was lost in a similar manner at about 12°, but more slowly.

3. The heat-tolerance lost after thirteen days of exposure to 4° was regained within one day after they had been returned to the original environmental temperature, the rate of gain of heat-tolerance being much higher than the rate of loss.

4. When tested after one week at 30°, the 24-hour median heat-tolerance limit had risen to 36.6° from the initial value of 35.6°. Two more weeks at 30° did not bring about a further increase in this tolerance limit, however.

5. Apparently the heat-tolerance of these crayfish was not affected by sex, size, or the stage of the molt cycle.

6. The results indicate that crayfish are among the more heat tolerant of the aquatic arthropods and fish for which appropriate data are available.

7. The similarities between the temperature relations of crayfish and fish suggest that the basic principles are alike in the two groups.

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THE MOLTING CYCLE OF THE SPINY LOBSTER, PANULIRUS ARGUS LATREILLE. II. PRE-ECDYSIAL HISTOLOGICAL AND HISTOCHEMICAL CHANGES IN THE HEPATO-PANCREAS AND INTEGUMENTAL TISSUES [1]

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The external manifestations of an approaching molt and of molting itself are the result of more basic physiological phenomena related to growth. Growth in the arthropods is markedly conditioned by the properties of the exoskeleton, which are in turn conditioned by the epidermis and other tissues. In the Crustacea, as in other arthropods, growth is cyclical, periods of comparative rest alternating with periods of activity. These cyclic periods of growth are accompanied by cyclical changes in the epidermis, sub-epidermal tissues and the hepatopancreas. The present paper is a study of the pre-ecdysial histological and histochemical changes in the integumental tissues and the hepatopancreas of *Panulirus argus* Latreille.

Materials and Methods

Animals

Male and female spiny lobsters ranging in carapace length from 80–89 mm. were obtained and handled as previously described (Travis, 1954).

Designation of stages in the molting cycle

In a study of the molting cycle, some method of designating the essential stages is necessary. Two main methods have been resorted to, namely: 1) indication of actual time periods, and 2) indication of morphological characters of skeleton and tissues. The first of these methods offers certain advantages. By this method, it is possible to determine the length of the intermolt periods in each size group and to determine the duration of existing morphological characters of the skeleton at each season of the year. In this manner any animal in the laboratory can, from its previous laboratory record, be placed in the proper stage of the molting cycle either by days following molt or by the morphological characters of the skeleton. The second method consists of breaking down the molting cycle into four major stages, A, B, C, D, and subdividing each of these stages, according to Drach (1939).

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Its major advantage is that it provides a means of grouping animals into the major stages of the molting cycle without reference to the interval of time over which such stages extend. Another advantage of Drach's method is that, regardless of age or size animals of corresponding stages can be compared even though the duration of these stages varies with size and season.

Both methods should be used since they are supplementary and necessary for interspecific comparison. The following time intervals for the molting cycle of *Panulirus* refer to animals of 80–89 mm. carapace length. The length of the entire intermolt period in the summer months is usually 65–70 days (Travis, 1954).

Stage A—Stage immediately following molt. The exoskeleton is of the consistency of a soft membrane. The animals do not feed. The duration is about 24 hours.

Stage B—Preliminary hardening of the skeleton occurs, which attains the consistency of parchment. The carapace is rigid in certain regions while the branchiostegites remain soft. No feeding occurs. The duration is from one day postmolt through the fifth or sixth day.

Stage C—The skeleton is entirely hardened but continues to thicken during a good portion of the period. This is a period of active feeding and is the longest of

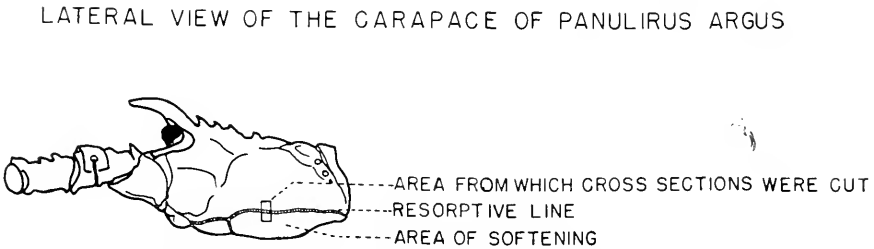


FIGURE 1. Lateral view of the carapace of *Panulirus* to show the area from which sections of the integument were cut.

the cycle, lasting from approximately the seventh to the fifty-first day following molting, or a total of about forty-four days. During late Stage C, the membranous layer and the principal layer are completed. The term "intermolt animal" will be applied to animals with a fully developed skeleton, a condition which falls approximately midway in days between two ecdyses, *i.e.*, 28–35 days following one molt and preceding the next.

Stage D—The new future skeleton is progressively constructed under the old, while the old is gradually broken down by resorption of both mineral and organic constituents. The period lasts ten to fourteen days during which the animals do not feed.

Histological and histochemical methods

For the histological and histochemical studies pieces of skeleton were removed from the carapace of *Panulirus* (Fig. 1) approximately five, three, and one days before a molt. Also, pieces of integument were removed on each of eight consecutive days following molt (a study of which will be reported in a subsequent paper) and from intermolt animals (late Stage C). By removal of pieces of skele-

ton during the pre-ecdysial period (late Stage D) the extent of the resorption in the endocuticle could be detected.

The right posterior lobe of the hepatopancreas was removed at approximately three days and one day before molt, on each of seven consecutive days following molt, and from animals in late Stage C.

All integumental tissues were embedded in celloidin and cut at 10 μ. The posterior lobe of the hepatopancreas was embedded in paraffin and cut at 8 μ. One to three animals were used to represent each of the days mentioned above. Portions

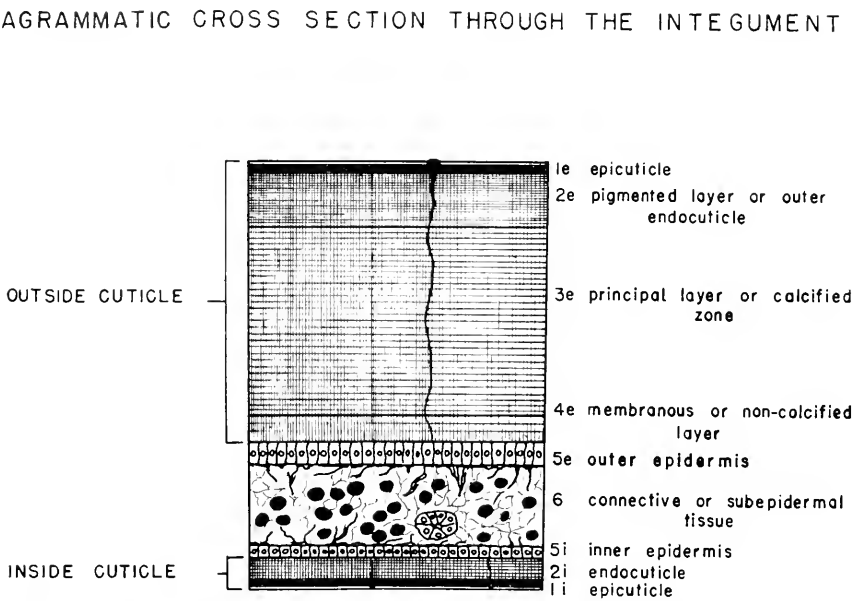


FIGURE 2. Diagram of a section through the integument of the branchial region (late Stage C). Note outside cuticle consisting of four distinct layers with its epidermis and inside cuticle consisting of two distinct layers with its epidermis. The inside cuticle borders the outer periphery of the gill chamber. The spongy sub-epidermal connective tissue is sandwiched between the outer and inner epidermis. The large black oval cells represent reserve cells.

of skeleton and hepatopancreas fixed in Helly's and alcoholic Bouin's fluid were stained by the following methods:

1. Mallory's triple stain.
2. Periodic acid-Schiff (PAS) of McManus, as described by Lillie (1948).
3. Bensley and Bensley's method (1938), demonstrating mucins by means of toluidine blue. The thiazine dyes have three absorption bands: alpha, beta, and gamma. The alpha form is orthochromatic (blue), whereas beta metachromasia (violet) can be caused by highly polymerized carbohydrates or by phosphate-containing compounds. Substances which give gamma metachromasia (red or pink) are usually acid mucopolysaccharides (Pearse, 1953). Since chitin is considered to be a neutral mucopolysaccharide (Meyer, 1938) and is closely associated with protein in the arthropod cuticle, since there is no real histochemical distinction

possible between neutral mucopolysaccharides and mucoprotein (Pearse, 1953), and since in early stages of skeletal deposition gamma metachromasia is exhibited, the pink color produced by the dye probably indicates the presence of muco- or glycoproteins.

For the detection of calcium deposits, portions of the skeleton were fixed in nine parts of 95% alcohol and one part of 40% formaldehyde and were stained with the following:

1. Mallory's triple stain.
2. Schmorl's purpurin (Lillie, 1948).
3. Alizarin red S (Manigault, 1939).
4. Von Kossa's method (Lillie, 1948). Before following this procedure, tissues were washed in 5% aqueous KNO_3 for five minutes or more to remove some of the chloride present.
5. Microincineration (Scott, 1933) was used to confirm the presence of calcium deposits detected by the stain mentioned above.

The hepatopancreas was also fixed in formol-alcohol and was stained with Mallory's triple and Alizarin red S, the latter stain being used to detect calcium.

Portions of the skeleton and hepatopancreas were fixed in cold 80% alcohol, and alkaline phosphatase was determined by the method of Gomori (1941). Control sections (Fig. 32) were made, using distilled water without added substrate during incubation.

Portions of the hepatopancreas which were fixed in 10% neutral formalin were embedded in carbowax (method of Blank and McCarthy, 1950), cut at 10 and 15 μ and stained for lipids with Sudan black B.

In sections of exoskeleton from premolt animals where the resorptive line becomes apparent, the folding, and in some cases the loss of the old cuticle made detection of this line impossible even in celloidin sections. Therefore, sections made by hand or whole mounts were used to study resorption in this area.

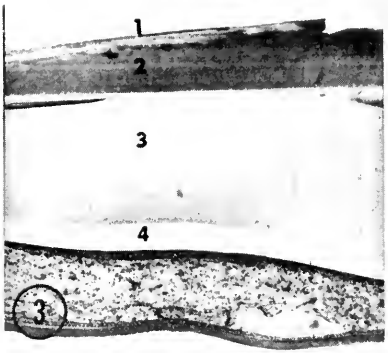
Observations

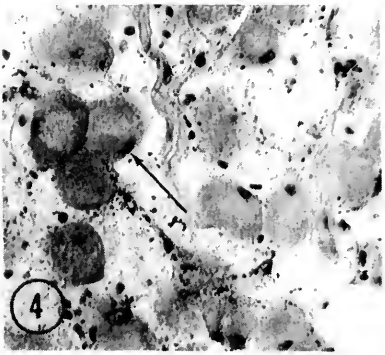
A. The intermolt animal (late stage C)

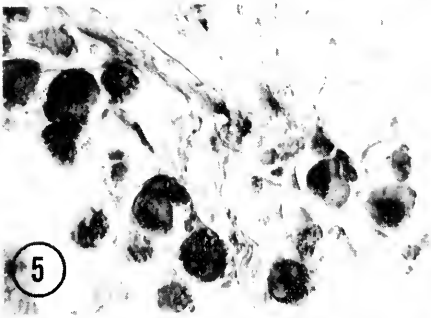
1. *The integument and integumental tissues*

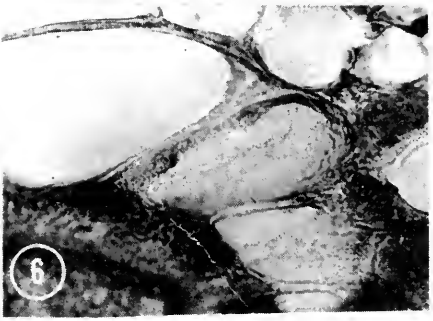
a. *Tissues*

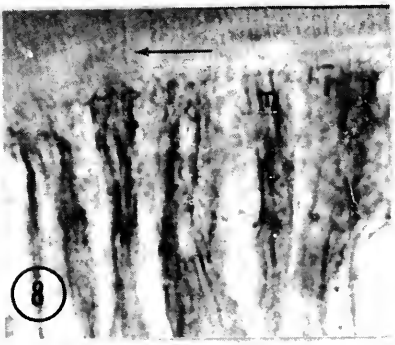
In pieces of exoskeleton from the lateral portion of the carapace the attached tissues are bordered by two integuments or cuticles as indicated in Figures 2 and 3. The inner integument, which forms a boundary for the gill chamber, is approximately 1/20 the thickness of the outer one (Fig. 3). Underlying each of these integuments is a layer of columnar epidermal cells with centrally located nuclei, and between these two layers is sub-epidermal connective tissue of a loose spongy type (Figs. 2, 7). Large oval reserve cells (Figs. 4, 5, 9), which Cuénot (1893) called "protein reserve cells" in *Astacus fluviatilis* and which resemble Type 1 Leydig cells (Kükenthal, 1926–1927) and Langer's vesicles of Mollusca (Bronn, 1896–1897), constitute by far the most predominant type of cells in this tissue. They appear as vesicular cells with a capsule-like envelope of cytoplasm containing a peripheral nucleus. These reserve cells vary in appearance during the molting

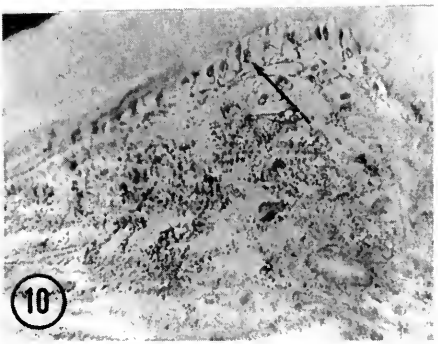












FIGURES 3–10.

cycle, binding or storing large amounts of polysaccharide material and calcium (Travis, 1951a). In the intermolt state the cells apparently store only polysaccharide (Fig. 4), whereas following molt they store calcium as small or large spheres and take on a "mulberry" appearance (Fig. 5). They may store fat as well. Although no material was fixed for the determination of the presence of lipid in the integumental tissues, similar cells in the connective tissue of the hepatopancreas contain much lipid. Cuénot (1893) described cells in the connective tissue of *Palinurus vulgaris* which stored large quantities of fat. His drawings indicate that these "fat cells" are similar in every respect to the reserve cells.

On first examination of the sub-epidermal tissues, it would appear that the reserve cells vary in number during various stages of the molting cycle, but this is not the case. When binding or storing reserves they are greatly increased in size, and their boundaries and nuclei are easily observed, especially with Helly's fixative and the PAS method. During periods when reserves are depleted, for example in late Stage C, many cells decrease in size and become clear and vesicular. Then cell boundaries are difficult to determine, but PAS and toluidine blue bring them out successfully.

In addition to the reserve cells (Type 1) there are: very small spindle cells with branching processes, *i.e.* Leydig cells, Type 2; rectangular cells (Fig. 6) which surround the walls of arteries, *i.e.*, Leydig cells, Type 3 (Kükenthal, 1926–1927); and amoebocytes in the blood spaces and channels of the connective tissue.

Tegumental glands were occasionally observed in the area from which these sections were cut. Collagen-like fibers are found throughout the connective tissue. At intervals within this tissue, the basal ends of some of the epidermal cells from each integument become long and attenuated and extend from one cuticle to the other (Fig. 7). The tonofibrillae (Fig. 7) which traverse the length of the epidermal cells are in close proximity to parallel collagen-like fibers which probably strengthen these supporting epithelial colonnades (Fig. 7) described by Vitzou (1882) in *Astacus fluviatilis* and *Homarus vulgaris*.

FIGURE 3. Photomicrograph of the integument of an intermolt animal (late State C). The four layers of the outer cuticle are represented by numbers. 1, epicuticle; 2, pigmented zone; 3, principal or calcified zone; 4, membranous layer. Note that outer integument is about 20 times thicker than inner cuticle. 90 ×.

FIGURE 4. Large, oval reserve cells (Leydig cells, Type 1) of the connective tissue with peripheral nuclei, one of which is indicated by arrow. Above arrow, collagen-like fibers found throughout the connective tissue. The small granules are glycogen (late Stage C). 450 ×.

FIGURE 5. The reserve cells take on an irregular "mulberry" appearance and their contents become divided up into small spheres as they store calcium following molt. 430 ×.

FIGURE 6. Large rectangular cells which surround the walls of arteries (Leydig cells, Type 3). The numeral (6) is within the lumen of an artery. 760 ×.

FIGURE 7. Colonnades of support, as indicated by arrow, are found at intervals throughout the connective tissue. 80 ×.

FIGURE 8. Tonofibrillae (T) which traverse the length of the epidermal cells. Arrow points to a pore canal, a protoplasmic extension of one of the epidermal cells. 1000 ×.

FIGURE 9. Photomicrograph showing very small amount of glycogen in the integumental tissues of an intermolt animal (late Stage C). Note small number of granules (a few are indicated at the tip of the arrow) localized between the reserve cells of the connective tissue. 450 ×.

FIGURE 10. Nuclei and cytoplasm of the integumental tissues are almost devoid of alkaline phosphatase during late Stage C. Nuclei indicated at tip of arrow show a positive reaction because of the presence of calcium. 80 ×.

b. *The Integument and its composition*

The outer integument, about 750 μ thick, consists of four distinct layers (Figs. 2, 3):

1. The most external layer, a thin epicuticle, is divided into two portions and is about 11 μ thick. It is composed of a tanned lipoprotein, and is impregnated with calcium salts.

2. A thicker layer, approximately 200 μ, is hardened by quinones as well as by calcium salts. This is called the pigmented layer of the outer endocuticle.

3. The third layer, about 460 μ in total thickness, is heavily calcified but non-pigmented and is called the principal layer or the calcified zone.

4. The fourth layer, approximately 90 μ in thickness, is non-calcified, non-pigmented, and is in direct contact with the underlying epidermis. This is called the membranous layer (Drach, 1939) or the non-calcified layer.

The thin inside cuticle next to the gill chamber is composed of two layers, a very thin epicuticle, and a uniformly staining endocuticle (Fig. 2).

Both the epicuticle and pigmented zone are formed before the molt. Drach (1939), therefore, prefers to call them pre-exuvial layers. He calls the principal layer and the membranous layer post-exuvial layers because of their formation after molt.

The basic components of the crustacean cuticle are, as in insects, chitin and protein, the properties of which may be altered by quinones, impregnation with lipids or calcium salts. Results obtained with toluidine blue would indicate that the principal and membranous layers of *Pannlirus* contain a muco- or glycoprotein complex. These give gamma metachromasia (pink) while the pigmented layer shows beta metachromasia (violet). This difference is probably due to the fact that the properties of the protein and closely associated chitin units of the pigmented zone have been changed by quinones. Richards (1952) has similarly found that application of Schiff's reagent for histochemical demonstration of polysaccharides and glyco- or mucoproteins does not always give a positive reaction even though carbohydrate is present. He points out that the carbohydrate in the insect skeleton may be masked wherever sclerotization has occurred.

The horizontal laminations observed in all layers of the cuticle (Fig. 2) with the exception of the epicuticle, have been attributed by Drach (1939) to the rhythmical discharge of secretory material from the epidermal cells. Richards and Anderson (1942), however, suggest that these laminations may be the result of chemical changes in the constituents after secretion.

The vertical cross striae observed in sections of the cuticle are pore canals (Fig. 8), originally associated with protoplasmic extensions of the epidermis. The function of these is not entirely clear. Wigglesworth (1933, 1948) believes that they enable the epidermal cells to act at a distance upon the superficial layers of the cuticle and that hardening of the insect cuticle by quinones is effected through these canals. In *Pannlirus argus,* hardening of the pre-exuvial layers by calcification would appear to be effected through these canals following molt (Travis, 1951a, 1951c).

c. *Localization of glycogen, phosphatase and calcium*

During late Stage C, there is little glycogen, phosphatase, and calcium evident in the integumental tissues. Glycogen when observed is sparsely localized between

the reserve cells or the connective tissues (Figs. 4, 9). Alkaline phosphatase appears to be completely lacking in the cytoplasm and nuclei of the integumental tissues. Some calcium, though sparse, is present in all nuclei (Fig. 10), occasionally present in distal ends of epidermal cells, and is infrequently bound in some polysaccharide complex in the few apparent reserve cells. All of the integument with the exception of the membranous layer is fully calcified.

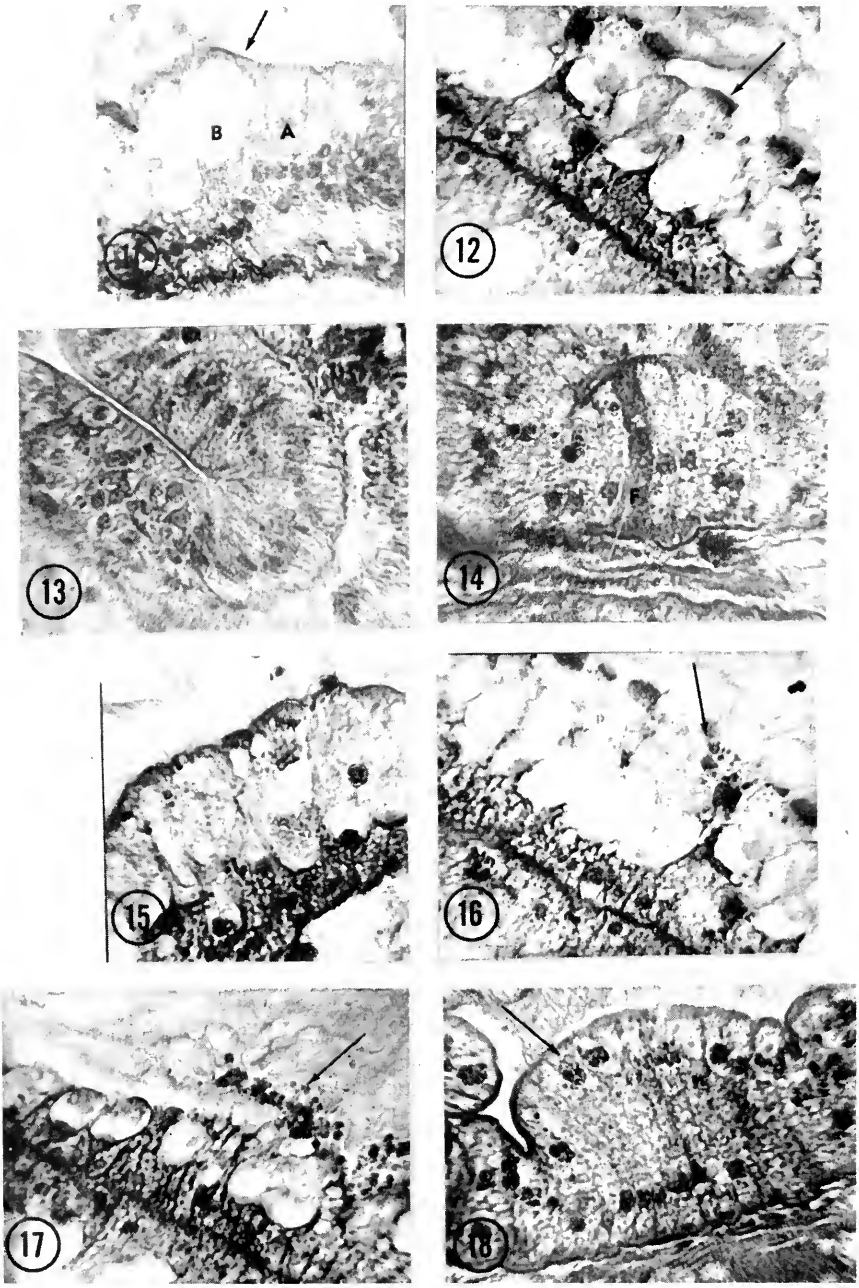
2. The hepatopancreas

a. *Tissues*

The hepatopancreas, a bilateral evagination of the midgut, functions in secreting digestive enzymes and absorbing and transforming food. It is also a major storage depot of organic and mineral reserves in Stage D and is, consequently, the major organ from which these reserves are mobilized when needed by other tissues. The gland itself is composed of innumerable tubules separated from each other by a loose connective tissue, which encloses large blood sinuses and branches of the hepatic arteries. There are collagen-like fibers intermingled between the large oval and vesicular reserve cells (as described in the discussion of the integumental tissues) which constitute the most numerous type of cells in the connective tissue. Amoebocytes are also present.

The tubular tissue proper is composed of columnar epithelium, delimited at its base by a basement membrane and characterized at its distal end (next to the lumen) by a striated border. The nuclei of these epithelial cells are basally located.

In an intermolt animal (late Stage C), a period during which active feeding occurs, the most conspicuous cells observed in the epithelial tissue of the hepatopancreas are large, mature, secretory cells (vesicular or B_2 cells of Hirsch and Jacobs, 1928, 1930). These are swollen, enclosing large vacuoles (Fig. 11), some of which contain stainable material. Their contents plus adjacent cytoplasm are discharged into the lumen, leaving only the basal region and nucleus of the cell intact (Figs. 12, 16, 17). Hirsch and Jacobs (1928, 1930) observed a complete breakdown of the secretory cells in *Astacus leptodactylus, i.e.*, holocrine secretion. Secretion in *Panulirus,* however, occurs in an apocrine rather than a holocrine fashion. The restitution of secretory cells in *Panulirus* is probably not nearly as complex as that observed in Crustacea where holocrine secretion takes place, and it is probable that these cells in *Panulirus* are reconstructed from the remaining basal end. Embryonic cells (E-cells, Fig. 13) predominate at the blind ends of the tubules. Hirsch and Jacobs (1928) pointed out in *Astacus leptodactylus* that these E-cells are followed proximally by B-cells and still more proximally by R-cells (Absorption cells). In *Panulirus* such a distinction is not so clear cut. Secretory cells and what would appear to be absorbing cells (Fig. 11) are frequently observed side by side. Fibrillar cells (Fig. 14) are not numerous, and these plus either absorbing or young secretory cells are scattered throughout the tubular tissue. Mature absorption cells (described in Kükenthal, 1926–1927) are tall columnar cells (Figs. 11, 14, 18, 19) with numerous small vacuoles and with either a basal or central nucleus, depending upon the stage of development. These would appear to be the same as the so-called B_1 cells of Hirsch and Jacobs. Whether the so-called absorption cells (R-cells) are really young secretory cells which have not developed large vacuoles or are special cells limited to absorption alone is not clearly evident.



FIGURES 11–18.

Mature B_2 or secretory cells as well as the tall columnar absorption-type cells contain both fat and glycogen. During Stage D, only the absorption cells contain large numbers of calcospherites. It is not impossible that in their life cycle the same cells perform both functions; absorption in their early stages and secretion in later stages. Certainly the epidermis of the integument performs both functions without the presence of any specialized types within it. In *Panulirus* the predominant hepatopancreatic epithelial cell types from specimens 1–5 days following molt are the long tall columnar cells without large vacuoles, the animal undergoing inanition during this period. Generally, feeding begins on the 6th or 7th day following molting. Because of the difficulty of distinguishing between absorption cells and young secretory cells (B_1-cells) described by Hirsch and Jacobs, and because of the possibility that these cells may perform absorption in their early stage of development and secretion in a later stage of development, the term secretory cell will be applied to those swollen cells containing very large vacuoles at their distal end and undergoing breakdown at the site of the large vacuole (Figs. 11, 12, 15, 16, 17). The name absorption cell will be applied to the tall cylindrical cells which do not contain large vacuoles at their distal end, but which may or may not have small vacuoles, and which may have either a central or basal nucleus (Figs. 11, 14, 18, 19). The presence of a central or basal nucleus may indicate a stage in the maturation of the cell. It will be observed that in Figures 11, 14 and 19 the nuclei of the absorption cells are centrally located, whereas those in Figure 18 are basally located. In the former cases, these cells may be younger than in the latter. In the latter case (Fig. 18), small vacuoles have developed around the calcospherites at the distal ends of the cells, and the nuclei have moved basally. These absorption cells may represent the oldest type before these same cells begin to perform secretion.

FIGURE 11. Portion of the tubular epithelium of the hepatopancreas showing (A) absorption type cells with central nuclei and (B) secretory cells containing large vacuoles. Nuclei in the latter are basally located. Note the striated borders, next to the lumen of the tubule, indicated by arrow. 430 ×.

FIGURE 12. Apocrine breakdown of secretory cells in the hepatopancreas showing ruptured vacuoles. Arrow points to the remnants of a striated border of a secretory cell. Note that the nuclei and basal ends of the secretory cells remain intact. 430 ×.

FIGURE 13. Section through blind end of a tubule of the hepatopancreas showing the numerous young embryonic cells (E-cells). 430 ×.

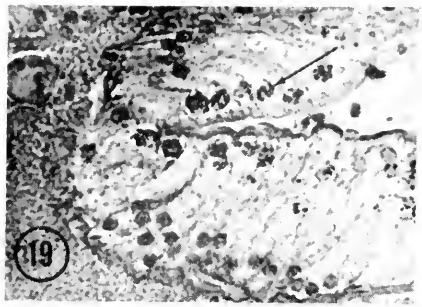
FIGURE 14. The fibrillar cells (F-cells) of Hirsch and Jacobs (1928, 1930). These are rare in the tubular tissue of the hepatopancreas of Panulirus. 430 ×.

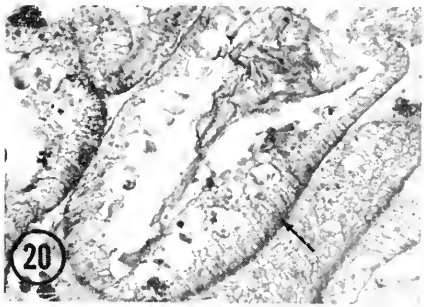
FIGURE 15. Mature secretory cells of the hepatopancreas showing striated borders and basal nuclei. Note the secretory material within the vacuoles.

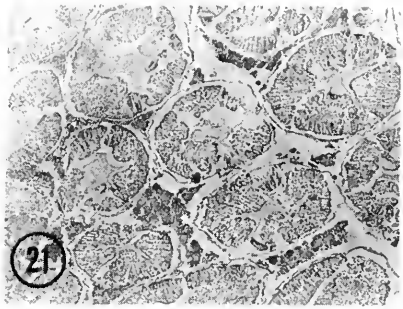
FIGURE 16. Secretory cells of the hepatopancreas which have undergone apocrine breakdown. Note that the basal ends of the cells containing the nuclei remain intact. The secretory granules (at end of arrow) and other contents of the vacuoles are discharged into the lumen of the tubule. Note remnants of striated borders of the secretory cells (to the left of the arrow). 430 ×.

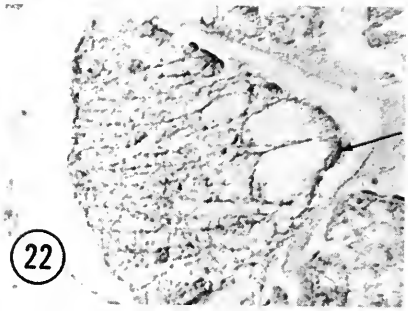
FIGURE 17. Apocrine breakdown in secretory cells of the hepatopancreas. Note discrete secretory granules (at the end of the arrow) extruded into the lumen of the tubule. Basal ends of cells containing nuclei are preserved during secretory breakdown. 430 ×.

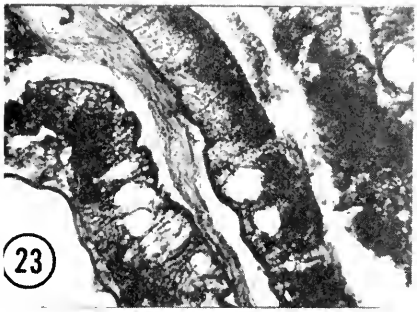
FIGURE 18. Old absorption cells of hepatopancreas with basal nuclei and numerous small vacuoles. The arrow points to calcospherites localized at the distal ends of these cells. Section from a premolt animal, fixed with Helly's and stained with Mallory's triple. 430 ×.

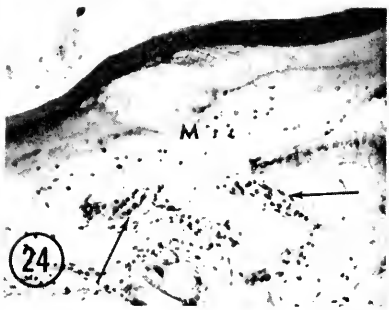


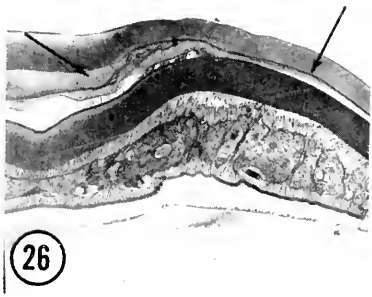












FIGURES 19–26.

b. *Localization of glycogen, phosphatase, calcium and lipid*

During late Stage C, the situation in the hepatopancreas is much the same as that observed in the integumental tissues. The few glycogen granules evident in secretory and absorbing cells (Fig. 20) are found at the bases of these cells and around the periphery of reserve cells in the connective tissue. Renaud (1949) demonstrated quantitatively in *Cancer pagurus* that little free glycogen could be detected in the hepatopancreas during most of Stage C because most appears to be utilized in the construction and growth of other tissues.

Alkaline phosphatase, present in small amounts, is localized at the striated borders of the epithelium and around the periphery of small and large secretory vacuoles (Figs. 21, 22). It would appear that the enzyme is strategically localized to participate in resorptive and secretory processes. Though the cell and nuclear membranes, and blood sinuses in the connective tissue give a positive reaction, control sections incubated without substrate indicate that most of this is due to the presence of calcium salts.

Calcium, though very sparse, is observed in all nuclei, cell membranes and in blood sinuses of the connective tissue. A very small amount is bound in complexes of the reserve cells.

Lipid is very abundant in the hepatopancreas during late Stage C. Large and small droplets are present throughout the epithelial tissues (both absorption and secretory cells, Fig. 23), and reserve cells; and they are frequently observed in the lumina of the tubules. Renaud (1949) has similarly observed numerous droplets of fat in the epithelial tissue of *Cancer pagurus* during this stage of the molting cycle.

FIGURE 19. Younger absorption cells, with basal nuclei, of a premolt hepatopancreas showing calcospherites at distal ends of cells. 430 ×.

FIGURE 20. Cross section of a tubule in the hepatopancreas of an intermolt animal (late Stage C) showing small amount of glycogen. Note that glycogen granules, although few in number, are distributed basally in both absorption and secretory cells (arrow). 100 ×.

FIGURE 21. Alkaline phosphatase distribution in the tubules of the hepatopancreas. 80 ×.

FIGURE 22. Portion of a single tubule of the hepatopancreas (late Stage C) at higher magnification to show that alkaline phosphatase is localized in the striated borders (at end of arrow) of the epithelium and around the periphery of small and large secretory vacuoles. 430 ×.

FIGURE 23. Section through hepatopancreas (late Stage C) to show the abundant distribution of lipid in both absorption and secretory cells of a single tubule. 80 ×.

FIGURE 24. A three-day premolt condition of the inner integument and epidermis (late Stage D). The epidermal cells have increased in number by undergoing extensive folding (not shown in this photograph). Some cells have disintegrated (arrows). Amoeboid cells appear in the molting fluid (M). 400 ×.

FIGURE 25. Outer integument and epidermis at three days preceding molt. Note that these outer epidermal cells have resumed an orderly alignment, have become much elongated and, in contrast to the situation observed in the inner integument and epidermis, have already secreted a portion of the new integument. 760 ×.

FIGURE 26. The condition of old and new integuments at about one or two days preceding molt. Note that the pre-exuvial layers of the new skeleton are almost fully formed, while resorption in the old skeleton is almost complete. Arrow to the left shows that about half of the old principal layer has been resorbed and arrow to the right indicates that all of the old skeleton, with the exception of the pigmented layer and epicuticle has been broken down by the molting fluid. These two latter layers are never attacked by the molting fluid. 90 ×.

B. THE PREMOLT ANIMAL (LATE STAGE D)

1. *External signs of the approaching molt*

The first distinct external evidence that *Panulirus* is approaching a molt is the appearance of a resorptive or ecdysial line (Travis, 1951a, 1954) along the branchiostegites. As Richards (1951) points out, along these lines sclerotization, indicated by lack of color, fails to occur. The ecdysial line in *Panulirus* appears as a result of marked resorption of organic and mineral constituents, thus leaving only a thin, translucent membrane. Histochemical tests have indicated that only the epicuticle and a thin mucilagenous layer remain, the latter being either the transformed membranous layer (Drach, 1939) or a transformed portion of the calcified zone. When resorption is complete in the endocuticle and when most of the calcium is resorbed from the epicuticle, some of these lines become weak and break, while others may act as hinges allowing flexibility and expansion of the soft body beneath the area in question (Herrick, 1895; Drach, 1939; Travis, 1951a, 1954).

In the entire area ventral to the resorptive or ecdysial line, less resorption occurs (20% resorption; Travis, 1951a) than in the line. The author calls this "the area of softening." In the summer months premolt animals can be detected as early as 5 days preceding ecdysis by feeling them along the area of softening. The resorptive line itself, however, is not clearly evident before 3–4 days preceding ecdysis.

2. *The integument and integumental tissues*

a. *Tissues and integument*

One of the most marked changes in the integumental tissues of a premolt animal is that observed in the epidermis. Approximately 10–14 days preceding molting in the summer months, detachment of the epidermis from the integument occurs, with a consequent development of a space between the two. This premolting stage was detected inadvertently on a number of occasions when secretory organs were removed from the eyestalks. Ten to fourteen days following epidermal retraction from the skeleton, the animals molted. Shortly following this retraction, growth of this tissue occurs. The mode by which growth is accomplished in the outer epidermis of the branchial region, whether by cell enlargement or by increase in cell number or both, has yet to be determined. The author has been unsuccessful so far in obtaining, for histological purposes, the precise stage at which the outer epidermis retracts and grows. This is difficult to obtain because by the time external signs of an approaching molt are obvious in the branchial region, the outside epidermis is past the retraction and growth stage and has begun to deposit the pre-exuvial layers. The epidermal cells of the inner cuticle increase in number causing a folding of the tissue. Some epidermal cells appear to disintegrate (Fig. 24). This is evident three days preceding molting. It would appear that processes leading to the growth of the epidermis, resorption from the old skeleton, and development of the new inside cuticle, all lag behind the growth of the epidermis, resorption from the old skeleton, and development of the new outside cuticle. This becomes evident from the following. At three days preceding ecdysis, nuclei, possibly of disintegrating cells of the inner epidermis and others of amoeboid cells, become trapped in what appears to be secreted molting fluid (Fig. 24). Yonge (1936) believes that these cells in *Homarus* attack and break down the chitin. At

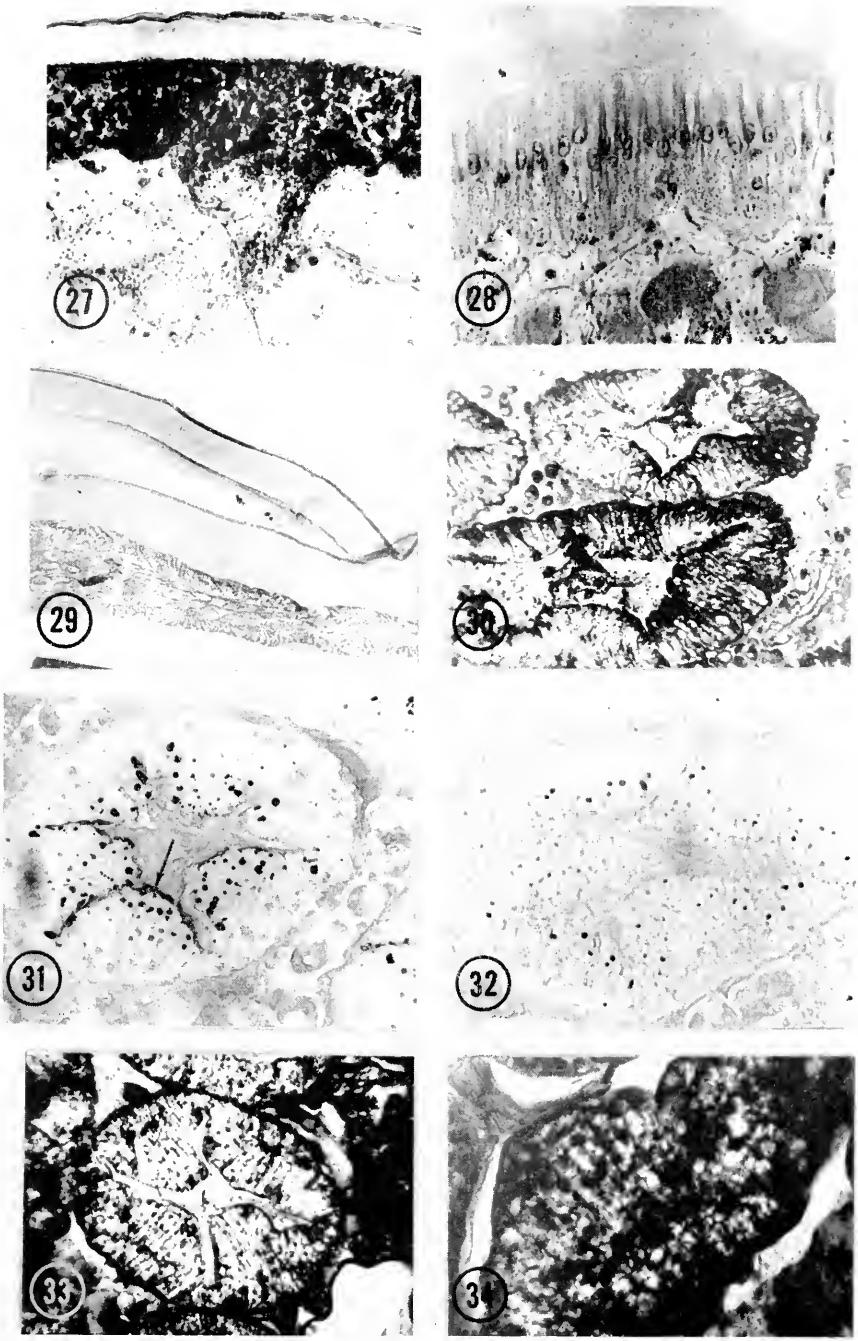
this same time, the epidermal cells of the outside cuticle have resumed an orderly alignment, have become very much elongated, fibrillar in nature, and have already secreted a portion of the new integument (Fig. 25). The new epicuticle of *Carcinus maenas,* shortly after its formation, is hardened by quinones (Krishnan, 1950, 1951). This also appears to be true of *Panulirus.* The thicker of the two pre-exuvial layers, however, becomes hardened by quinones following molt and is then called the pigmented layer. At one to two days preceding molt the old skeleton has thinned considerably (Fig. 26). It will be observed to the left of Figure 26 that almost half of the old principal layer has been resorbed, and to the right of this same figure all of the old skeleton, with the exception of the pigmented layer and epicuticle, has been broken down by the molting fluid. The molting fluid never attacks the latter layers. The immunity of the new epicuticle and pigmented layer to attack by the molting fluid is possibly due to the quinone tanning of the former. The quinone tanning of the epicuticle renders it highly stable and resistant to the enzymes of the molting fluid, but as Richards (1951) has pointed out, there is no adequate explanation of the failure of the newly forming procuticle to be digested by enzymes that must pass through it to reach the molting space.

The almost complete resorption, both mineral and organic, in some of the skeletal areas and partial resorption in others is absolutely necessary for complete separation of the new skeleton from the old and for allowing further thickening of the pre-exuvial layers before ecdysis. Due to resorption from the ecdysial line along the branchiostegites and resorption from an articulating condyle connecting the branchial chamber to the posterior edge of the branchiostegites, lateral expansion of the soft body beneath, and lifting of the old exuvia at molt is allowed (Travis, 1954). As resorption from the old skeleton and building of the new (pre-exuvial layers) progresses in the late premolt period, large numbers of reserve cells become apparent in the connective tissue. These are filled with polysaccharide complexes. This material probably represents not only breakdown products from the old skeleton but reserve substances for the new.

b. *Localization of glycogen, phosphatase and calcium*

During late Stage D, enormous amounts of glycogen are concentrated at the base and throughout the epidermal cells of the inner cuticle (Fig. 27), whereas very little is present in either the outer epidermis (Fig. 28) or the connective tissue. This condition persists until the second day following molt; glycogen then disappears from the inner epidermis for a seven-day period following molt. Little thickening occurs in the inside cuticle after one or two days postmolt, suggesting strongly that this cuticle is formed and completed early. The large amounts of glycogen observed in the inner epidermal cells during late Stage D and Stage A and its disappearance after this integument is completed would suggest that glycogen is a necessary precursor in chitin formation.

Alkaline phosphatase is observed in abundance at the distal ends of the epidermal cells which border on the cuticle (Fig. 29). Krugler and Birkner (1948) have similarly observed a heavy concentration at the distal ends of the epidermal cells of the integument of *Cambarus virilis.* The enzyme appears to be in both reserve cells and cells of Leydig (Type 3). Although these two latter sites are positive in the control sections, they are much lighter in appearance, indicating that the positive



FIGURES 27–34.

reaction of the reserve cells and cells of Leydig (Type 3) is probably due to the presence of calcium and not of the enzyme phosphatase. The enzyme doubtlessly participates in resorptive and secretory processes which are most pronounced at this time.

Although calcium is likewise localized at the distal ends of the outer epidermal cells and in blood sinuses beneath and between these cells, very little is bound in any of the reserve cells during Stage D. While calcium remains in portions of the old integument, *i.e.*, the epicuticle and pigmented layer, no calcification of the new skeleton, in the region of the branchiostegites, occurs until the second day following ecdysis.

3. *The hepatopancreas*

a. *Tissues*

During late Stage D, represented by animals approximately one to three days preceding molt, a marked difference is noted in the tubular epithelium of the hepatopancreas when compared with that of an animal in late Stage C. Likewise numerous reserve cells, filled with lipid and polysaccharide complexes, some of which contain bound calcium, are apparent in the connective tissue. The epithelium lining the tubules becomes high columnar and is composed predominantly of absorption-type cells. One striking difference between the epithelium of a premolt animal and that of an intermolt animal is the appearance of innumerable calcospherites in the distal ends (near the striated border) of the absorbing cells, while none is observed in the fibrillar, and very few are observed in the secretory cells. These calcospherites, spherules of calcium phosphate (previously observed in *Carcinus maenas* by Robertson, 1937), represent calcium salts withdrawn from the old skeleton, some of which are stored in the hepatopancreas during this period prior to molting. These stored calcium salts appear to be used in the hardening of the new skeleton, since calcification proceeds simultaneously with depletion of these stored reserves in the hepatopancreas (to be discussed in a subsequent paper). It is inter-

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FIGURE 27. One- to two-day premolt distribution of glycogen in the inner integument. Note the enormous amounts present in the epidermis while very little is evident in the connective tissue. 760 ×.

FIGURE 28. A one- to two-day premolt distribution of glycogen in the epidermis of the outer integument. Note that only very few granules are present. 450 ×.

FIGURE 29. A one- to two-day premolt distribution of alkaline phosphatase in the integumental tissues. The enzyme is heavily concentrated in the distal ends of the epidermal cells which border the integument. 90 ×.

FIGURE 30. The late Stage D distribution of glycogen in the tubular epithelium of the hepatopancreas. Note great concentrations of glycogen in the epithelial tissue and in the lumina of the tubules. 80 ×.

FIGURE 31. The late Stage D distribution of alkaline phosphatase in the hepatopancreas. A single tubule of this tissue is represented. Heavy localization, as indicated by arrow, in the striated borders of the absorbing cells and around the calcospherites. 100 ×.

FIGURE 32. A late Stage D control section of the hepatopancreas. Within a single tubule represented, only the calcium phosphate spherules remain visible following incubation without substrate. 100 ×.

FIGURE 33. The late Stage D distribution of lipid in the tubules of the hepatopancreas. 80 ×.

FIGURE 34. Portion of the epithelium of a single tubule of the hepatopancreas to show the heavy concentration of lipid droplets in the epithelium. 430 ×.

esting to point out that rather large amounts of calcium in the hepatopancreas have been detected by chemical analysis in *Carcinus maenas* by von Schönborn (1912) and Robertson (1937); in *Cancer pagurus* by Paul and Sharpe (1916); in *Maia squinado* by Drach (1939); and in *Hemigrapsus nudus* by Kincaid and Scheer (1952). All of these previous observations have been made on Brachyura. The observations on *Panulirus* indicate that calcospherites are also present in the hepatopancreas of Macrura.

b. *Localization of glycogen, phosphatase, calcium and lipid*

In late Stage D, glycogen, phosphatase, calcium, and lipid are markedly apparent in the hepatopancreas. Glycogen in abundance is observed in the epithelial tissue and even in the lumina of the tubules (Fig. 30). The connective tissue is, however, virtually devoid of it; glycogen is present in blood sinuses and around the periphery of reserve cells. It has previously been observed that during the premolt stage glycogen accumulates in the hepatopancreas of *Astacus fluviatilis* (Bernard, 1879; Vitzou, 1882; Kirch, 1886), *Homarus vulgaris* (Vitzou, 1882), and *Carcinus maenas* (von Schönborn, 1910). In addition to the increase of glycogen in the hepatopancreas before molt, it also appears in the integumental tissues of *Carcinus maenas, Cancer pagurus, Panulirus argus* and *Panulirus japonicus* (Verne, 1924, 1926; Renaud, 1949; Travis, 1951a; and Schwabe *et al.*, 1952). It will be recalled that the integumental tissues of *Panulirus argus* during this same period begin to accumulate large amounts. These stores, initially accumulated by the hepatopancreas, are mobilized and transported to the integumental tissues for the synthesis of the skeleton.

Alkaline phosphatase is heavily concentrated in the striated borders of absorbing cells (Fig. 31). The most striking localization is that observed around innumerable calcospherites, which are sites of calcium phosphate deposition in the distal ends of the absorbing cells (Fig. 31). This very strongly suggests that alkaline phosphatase is directly participating in the deposition of calcium phosphate calcospherites. The secretory cells have few calcospherites and show very little evidence of phosphatase in their striated borders. Thus the enzyme and calcospherites appear to be predominantly localized in absorption-type cells. Though the reserve cells show darkening, control sections indicate that this darkening is due primarily to calcium which is bound in lipid and polysaccharide complexes and not to the enzyme phosphatase. Calcium is also present in blood sinuses within the connective tissue and is localized in cell and nuclear membranes of all cells.

Droplets of fat are found throughout the epithelial tissue (Figs. 33, 34). There is little change over that observed during late Stage C. Where secretory cells are observed, droplets of fat are found throughout these cells, and much of the material in the large vacuoles is lipid. Fat is also abundant in the reserve cells of the connective tissue. Renaud (1949) noted that lipids of the hepatopancreas in *Cancer pagurus* disappear little by little during the fasting period but are still abundant in late Stage D through Stage B, then undergo a marked decrease. The hepatopancreas serves, among other functions, as an important storage depot of fat. Cuénot (1893) was one of the first to indicate this in *Astacus fluviatilis*. In *Cancer pagurus, Lithodes*, and *Homarus* (Paul and Sharpe, 1916) and in *Cancer pagurus* (Renaud, 1949) there is an accumulation of lipids in the hepatopancreas

preceding molt and a gradual disappearance following molt. Likewise Damboviceanu (1932) showed that blood fatty acids increase before molt in *Astacus fluviatilis* and slowly fall following molt and reach normal levels when the skeleton fully hardens.

Thus during Stage D the hepatopancreas becomes an important storage organ for fat, glycogen and calcium reserves. These, as will be pointed out in a subsequent paper, disappear progressively as growth of the integument and other tissues occurs.

Discussion

External signs of an approaching molt in *Panulirus* reflect profound physiological transformations which occur in the tissues of the organism. These changes manifest themselves in the epidermis and sub-epidermal tissues of the integument as well as the epithelial and connective tissues of the hepatopancreas. All of these changes during the pre-ecdysial period are associated with the growth of the organism. The structure restricting the growth in size to certain periods throughout the year is the rigid exoskeleton. A study, therefore, of changes which occur in the integumental tissues, and changes which effect breakdown of the old skeleton and growth of the new, cannot be considered apart from the more general aspects of growth and molting in the arthropods.

During the intermolt period (late Stage C) the skeleton of the branchial region in *Panulirus* is fully hardened and consists of four distinct layers, the epicuticle, the pigmented layer, the principal layer, and the membranous layer. The first three layers are calcified, whereas the fourth is non-calcified. In the Crustacea, the epicuticle and pigmented layer, in addition to being calcified, are hardened by quinones (Dennell, 1947; Kirshnan, 1950, 1951, 1954). In crustaceans as in insects the basic components of the skeleton are chitin and protein which are firmly associated with one another. Trim (1941), Stacy (1943) and Haworth (1946) regard the arthropod cuticle as a mucopolysaccharide because of the firm combination of the carbohydrate containing amino sugars (chitin) with the proteins. Glycogen, phosphatase and calcium are three necessary constituents, therefore, to consider during the breakdown of the old and the growth of the new skeleton.

During late Stage C, no marked changes occur in the hepatopancreas or in the integumental tissues. Few reserve cells are apparent either in the connective tissue of the liver or in subepidermal tissues of the integument. In both areas, such cells contain little carbohydrate, lipid, and calcium. Even though the animals actively feed throughout this period, glycogen is low in both the hepatopancreas and integumental tissues. This is probably the result of its utilization in the growth of other tissue. Renaud (1949), and Schwabe *et al.* (1952), noted similar situations in *Cancer pagurus* and *Panulirus japonicus,* respectively. There is also little evidence of the enzyme phosphatase in the integumental tissues. It is present, however, where active absorption and secretion occur in the hepatopancreas. Calcium is found in the usual locations, *i.e.,* in all nuclei and cell membranes of the integumental and hepatopancreatic tissues and in blood sinuses in the connective tissues. At this period large amounts of fat are stored in the hepatopancreas.

As the pre-ecdysial period (Stage D) is approached, marked changes are observed in the integumental tissues and the skeleton. Most profound of the internal morphological changes are those in the epidermis. In the summer months it is

caused to retract from the old skeleton 10–14 days preceding the molt, so that a molting space develops between the skeleton and retracted tissue. Following this retraction, growth of the epidermis occurs. The mode by which growth occurs has not been observed in the outer epidermis of the branchial region; however, the epidermal cells of the inner integument appear to increase in number causing a folding of the tissue. Such a condition appears three days before molt, indicating that processes which lead to growth of the epidermis, breakdown of the old skeleton, and growth of the new skeleton bordering the gill chamber, lag behind those processes which occur in the outer integument. While growth of the epidermis and breakdown of the old skeleton of the inside integument occur, the epidermal cells of the outer integument have already resumed an orderly alignment, have become very much elongated, and have secreted a portion of the new skeleton (epicuticle and pigmented layer). At one to two days preceding molt, almost all resorption in the old outside skeleton of the branchial region is complete. Mineral and organic resorption from the skeleton—complete in some areas, partial in others—is necessary to free the new skeleton from the old and to allow expansion of the soft body and further thickening of the pre-exuvial layers before ecdysis (Travis, 1954). During this premolt period, when resorption and growth of the new skeleton are occurring simultaneously, large numbers of reserve cells stand out because they are filled with a polysaccharide complex, which may represent either breakdown products from the old skeleton or reserve materials for the new. At this stage, very little calcium is bound to the polysaccharide complex. During the period, large amounts of glycogen are observed in the hepatopancreas. These glycogen granules are localized at the basal and distal ends of the absorbing cells and are observed frequently within the lumina of the tubules. Integumental glycogen comes largely from the hepatopancreas. Since the animals do not feed during this period, much of the glycogen may come from the conversion of fats stored in the hepatopancreas. Large amounts of glycogen are also concentrated throughout the epidermal cells of the inner integument, while very little is evident in either the connective tissue or the outer epidermis. This condition persists until the second day following molt, at which time the glycogen disappears from the epidermis of the inner integument. As the principal layer of the outer integument is deposited following molt, glycogen is observed to accumulate in the outer epidermis. There is a periodic shift of glycogen from the connective tissue to the outer epidermis (Travis, 1951a, 1951c) which probably indicates that this tissue goes through rhythmical periods of accumulation and utilization as the post-exuvial layers are deposited. This evidence suggests strongly that glycogen is a necessary precursor for chitin formation and is needed not only for this synthesis but for growth of other tissues as well. The fact that glycogen disappears from the inner epidermis on the second day following molt and that little thickening of the inner integument occurs after this period, suggests that the inner integument is completed during a period of three days preceding molt and two days following molt. Large amounts of glycogen, therefore, appear to be utilized at this time by the inner epidermal cells for the synthesis of chitin. That glycogen is a necessary precursor in the synthesis of chitin has also been suggested by Verne (1924, 1926), Renaud (1949), Travis (1951a), Schwabe *et al.* (1952). Renaud (1949) has further proposed a general mechanism by which glycogen may be utilized as a precursor in chitin formation. She suggests that glycogen is first hydrolyzed to glucose, and that this is aminated,

yielding glucosamine which, after acetylation to N-acetyl-glucosamine, undergoes polymerization to yield chitin. During the pre-ecdysial period, hydrolysis of glycogen would contribute greatly to the high blood glucose noted in *Callinectes sapidus* by Baumberger and Dill (1928), in *Astacus fluviatilis* by Damboviceanu (1932), and in *Maia squinado* by Drilhon (1933, 1935). Scheer and Scheer (1951) found that glucose apparently does not serve as a substrate for oxidative metabolism in *Panulirus japonicus* and that it does not stimulate the consumption of O_2 in isolated tissues, since little labeled C^{14} (in injected glucose) was recovered in respiratory CO_2. They further support the idea that the glycogen, present in the hepatopancreas and other tissues, is converted to glucose and that its principal role in the Crustacea is in the formation of chitin. It is interesting that high blood glucose may also be accompanied by increases in organic acids and other intermediates of glycolytic metabolism, thus contributing to the increase in osmotic pressure and facilitating water intake during and following molt. A further, possibly important, function of glycogen during the pre-ecdysial period is an intimate involvement in the deposition of calcospherites in the hepatopancreas. Moog (1946) has suggested the importance of glycogen in calcification of bone. With the abundance of glycogen in the hepatopancreas during Stage D and its possible hydrolysis and subsequent phosphorylation, glycogen could serve indirectly as added substrate (phosphoric esters) for phosphatase action, which, as will be pointed out subsequently, appears to be intimately concerned with the deposition of calcium phosphate calcospherites at the periphery of absorbing cells in the hepatopancreas.

During late Stage D, phosphatase appears in the epidermis and connective tissue. It is localized at the distal ends of the epidermal cells bordering the integument, is present in the reserve cells, and in blood sinuses of the connective tissue. Phosphatase present in these strategic sites of active transfer of materials throughout the integumental tissues, probably participates in those reactions involving hydrolysis and dephosphorylation of glucose phosphate to glucose, which Renaud (1949) suggested to be a probable starting point for chitin formation. Hydrolysis of phosphoric esters would produce molecules which would be able to enter or leave these tissues more readily. The enzyme does not participate in calcification of the branchial integument during the premolt period, since no calcification occurs at this time, although calcium is localized at the distal ends of the epidermal cells of the outer integument and in blood sinuses beneath and among the epidermal cells. In the hepatopancreas alkaline phosphatase is heavily concentrated at the striated borders of absorbing cells. The enzyme is most concentrated around calcium phosphate deposition sites (calcospherites) which are predominantly localized in the distal ends of the absorbing cells. Phosphatase in the hepatopancreas appears not only to be involved in important transfer and dephosphorylation reactions which occur at the surface of the absorbing cells but also to be intimately concerned with the deposition of calcium phosphate calcospherites at the periphery of these same cells. Here the enzyme possibly acts by catalyzing local liberation of phosphate ions from the organic phosphates present in the blood or from those transferred across the cell membranes. Calcium ions which are provided by the blood and which are transferred across the cell membranes then unite with these freed phosphate ions to form deposited calcospherites. It may be pointed out that during this period blood calcium rises from an average intermolt value of 22 mEq/L to a peak premolting average of 41 mEq/L and that total blood phosphorus rises from

3.6 to 5.3 mEq/L. However, little change is observed in blood inorganic phosphate, which remains within the normal intermolt range of 0.28 mEq/L (Travis, 1951a, 1951b). Glycogen, it will be recalled, is abundant in these same tissues and provides a source of phosphoric esters which act as substrate for phosphatase activity. Since the animals do not feed for two weeks preceding and one week following molt, and since the primary source of phosphate is from food (Travis, 1951a, 1951b), all available phosphate must be conserved. The calcospherites represent a means of storing or conserving resorbed phosphate from the old skeleton. Although this will be discussed in a subsequent paper, it may be said that following ecdysis the calcospherites disappear from the hepatopancreas as growth and mineral deposition occurs in the new skeleton.

In addition to the great quantities of glycogen, calcospherites, and heavy localization of phosphatase, the hepatopancreas stores large amounts of lipid during Stages D, A, and B. Other lipid deposits occur around the supra- and sub-esophageal ganglia. It will be recalled that during Stages D, A, and B of the molting cycle, the animals do not feed. It is possible that during such periods of inanition the animals utilize their fat reserves as a major source of energy. This is evident from the work of Renaud (1949) who showed that, although a fraction of protein nitrogen of the hepatopancreas is utilized as an energy reserve during the period of starvation, the reserves that permit *Cancer pagurus* to subsist during this period are constituted mostly from fatty acids and glycerides. Bliss (1952, 1953) has shown that fat and protein appear to be the principal foods oxidized even in normal intermolt *Gecarcinus,* as indicated by the mean respiratory quotient values of 0.77. It would seem, therefore, that during periods of fasting the stores of fat in the hepatopancreas serve as a major source of energy and that they play a principal role in oxidative metabolism of the normal intermolt animal.

The large amounts of fat stored in the hepatopancreas during the pre-ecdysial period may contribute indirectly, through their metabolism, to the rise in blood osmotic pressure preceding molt, which facilitates water intake during and immediately following molt. That fats (fatty acids and glycerols) are present in large quantities within the hepatopancreas and blood preceding molt and that they slowly decline until feeding begins following molt (Stage C) is shown by a number of investigations. Paul and Sharpe (1916) found that prior to molt in *Cancer pagurus, Lithodes,* and *Homarus* there was increased fat storage (fatty acids and glycerides) in the hepatopancreas. Following molt, fatty acids and glycerides decreased in the hepatopancreas and increased in the blood. Damboviceanu (1932) demonstrated a rise in blood fatty acids of *Astacus* before molt, followed by a slow decline during the postmolt period. Drilhon (1935) showed an augmentation before molt of total lipids in the blood, hepatopancreas, and in genital organs of *Maia squinado.* This rise was followed by a fall during and following molt. Renaud (1949) found that total lipids, fatty acids, phosphatides, cholesterol and total unsaponifiable fat increase to a peak in the hepatopancreas preceding molt, slowly fall following molt, and reach their lowest point as the skeleton is hardened (Stage C). Bliss (1952, 1953) reported that eyestalkless *Gecarcinus* approaching molt have an R.Q. greater than one, which to her suggested metabolism of organic acids. It is highly likely that such a situation may occur in normal animals approaching molt. This is very interesting because it would seem from the evidence available that the increase in blood fatty acids, phosphatides, cholesterol and glycerides, as well as organic acids which ac-

cumulate during glycolysis, might very well contribute, as Baumberger and Olmsted (1928) pointed out, to the rise in blood osmotic pressure preceding molt which would facilitate water absorption shortly preceding and following molt.

The fatty acids and cholesterol conveyed to the integumental tissues preceding molt are without doubt used in the formation of the epicuticle. Renaud (1949) showed that in *Cancer pagurus* immediately before formation of the epicuticle, the amount of lipids (fatty acids, cholesterol and unsaponifiable fatty acids) in the epidermis rises from 19% in late Stage C to 33% in Stage D when the epicuticle is being formed. After the epicuticle is formed, the hypodermis is emptied of 1/3 of its lipids.

It is pertinent to suggest the importance of many of these organic acids, products of oxidative and glycolytic metabolism, as carriers of calcium. Such carriers play a part in conveying calcium to the hepatopancreas for storage preceding molt and likewise in conveying it to the integumental tissues as the skeleton progressively calcifies following molt.

It is evident from the foregoing discussion that the marked histological and histochemical changes which occur in the hepatopancreas and integumental tissues during the pre-ecdysial period are associated with the growth of the organism. The hepatopancreas participates intimately in the important processes related to growth of the epidermis. Because it is a major storage depot for glycogen, fat and mineral reserves (calcium phosphate calcospherites) during the pre-ecdysial period, the hepatopancreas is the major organ from which these reserves are mobilized when needed in growth of the integument and other tissues. The possible functions of these reserves in such processes have been suggested.

Summary

1. The integumental tissues from the branchial region of an intermolt spiny lobster (late Stage C) are bordered by an outer and an inner integument. The outer integument consists of four layers: a thin epicuticle, the pigmented layer, the principal layer, and the membranous layer. Both the epicuticle and pigmented layer are formed before molt. The inner integument, about 1/20 the thickness of the outer integument, is composed of two layers, a very thin epicuticle and a uniformly staining endocuticle.

2. The integumental tissues are composed of columnar epidermal cells and a sub-epidermal connective tissue of a loose spongy type. Large oval reserve cells constitute by far the most predominant type of cells in this tissue. These cells store abundant amounts of carbohydrate, lipid and calcium at various stages throughout the molting cycle; they are also found in the hepatopancreas.

3. Within the tubular epithelium of the hepatopancreas are two major types of cells, secretory and absorptive. The former are swollen cells containing very large vacuoles at their distal ends. Secretion is of the apocrine type, leaving only the basal region and nucleus of the cell intact. Reconstitution of secretory cells probably occurs by regeneration from the remaining basal ends. The latter type of cells (absorptive) are tall and cylindrical with a basal or central nucleus.

4. During the intermolt period (late Stage C), a period of active feeding, there is little glycogen, phosphatase, and calcium evident in either the hepatopancreas or the integumental tissues. Lipids, however, are abundant in the epithelial tissues of the hepatopancreas.

5. External signs of an approaching molt (Stage D) become evident by the appearance of a resorptive or ecdysial line along the branchiostegites 3–4 days preceding molting in the summer.

6. The external signs of an approaching molt in *Panulirus* reflect the more basic internal changes which occur in the integumental tissues and hepatopancreas. Growth of the outer epidermis occurs shortly after its retraction from the outer integument, 10–14 days preceding molt. Growth of the epidermis of the inside integument is accomplished through an increase in cell number at 3 days preceding molt. Resorption from the old and completion of the new inner integument occurs in a period of five days, three days preceding and two days following molt. At three days preceding molt the epidermal cells of the outer integument have already resumed an orderly alignment, are greatly elongated, and have secreted the epicuticle and pigmented layer of the new skeleton. By this time most of the principal layer of the old outer integument has been broken down by the molting fluid. The almost complete mineral and organic resorption in some areas, partial in others, allows for complete freeing of the new skeleton from the old and for further thickening of the pre-exuvial layers before ecdysis.

7. During the pre-ecdysial period (late Stage D) large numbers of reserve cells of the integumental tissues become greatly swollen, and bind or store large amounts of polysaccharide material. Glycogen is abundant in the epithelial tissues of the hepatopancreas and simultaneously accumulates in the epidermal cells of the inner integument as the latter is being formed. Available evidence (see discussion) would suggest strongly that glycogen is a necessary precursor for chitin formation. Furthermore, glycogen is perhaps intimately involved in the deposition of calcospherites in the hepatopancreas.

8. During late Stage D, phosphatase appears in the distal ends of the epidermal cells bordering the integument and in reserve cells of the connective tissue. It is suggested that phosphatase in these strategic sites of active transfer probably participates in producing molecules which are able to enter or leave the cells more readily, and in those reactions involving hydrolysis and dephosphorylation of glucose phosphate to glucose, a possible starting point for chitin formation. It is not participating in calcification of the branchial integument at this time, because calcification does not begin until the second day following molt. In the hepatopancreas, on the other hand, alkaline phosphatase is heavily concentrated at the striated border of the absorbing cells and around calcium deposition sites (calcospherites). Here the enzyme appears to be involved in important transfer and dephosphorylation reactions which occur at the surface of the absorbing cells and also to be intimately concerned with the deposition of calcium phosphate at the periphery of these same cells (see discussion).

9. Evidence available suggests that lipids, abundant in the hepatopancreas at this time, function as a major source of energy. Furthermore, the accumulation of fatty acids, glycerides, and other intermediates of oxidative as well as glycolytic metabolism during late Stage D and Stage A may contribute to the rise in osmotic pressure preceding molt, facilitating water intake. Further evidence suggests that some of the fatty acids, cholesterol and unsaponifiable fatty acids conveyed to the integumental tissues preceding molt are used in the formation of the epicuticle.

10. It is suggested that many of the organic acids, products of oxidative and glycolytic metabolism, are perhaps important carriers of calcium. Such carriers

participate in conveying calcium to the hepatopancreas for storage preceding molt, and in conveying it to the integumental tissues as the skeleton progressively calcifies following molt.

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ADENOSINETRIPHOSPHATASE ACTIVITY OF SQUID MANTLE MUSCLE (LOLIGO PEALII) [1, 2]

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Studies on the biochemistry of vertebrate striated muscle clearly indicate the presence of two distinct enzymes which catalyze the hydrolysis of the terminal phosphate from adenosinetriphosphate (ATP). One adenosinetriphosphatase (ATPase) is calcium-activated and appears to be identical with myosin (Engelhardt, 1946; Szent-Gyorgyi, 1947). The second ATPase is magnesium-activated and is associated with the particulate matter of the cell (Kielley and Meyerhof, 1948; Perry, 1952; Kitiyakara and Harman, 1953; de Villafranca, 1954). Although it has not been clearly established, most workers assume that myosin-ATPase and the contraction phenomenon must be closely associated. A contribution to the understanding of the enzymes' role, as well as to the general understanding of muscular contraction, could be made by a careful examination of their distribution in muscle tissue from various members of the animal kingdom.

Very few data on muscle ATPase from invertebrates have been published. Humphrey (1949) concluded that there is both a myosin-ATPase and a water-soluble-ATPase in the adductor muscle of the oyster, *Saxostrea commercialis*. More recently Gilmour and Calaby (1952) report two ATPases present in locust muscle (*Locusta migratoria* and *Gastrimargus musicus*); one a calcium-activated, myosin-ATPase and the second, much more concentrated, a magnesium-activated, water-soluble-apyrase. The latter differs from that found in mammalian muscle in not being associated with the particulate matter of the cell.

The large quantity of easily available muscle in the mantle of the squid makes it a highly desirable organism for a comparative study of the biochemistry of muscle. The present paper deals with an attempt to locate and isolate the ATPase activity in the mantle muscle of the squid, *Loligo pealii*. A preliminary account of this work has been published (de Villafranca, 1953).

Materials and Methods

Fresh squid were decapitated and the mantle placed on ice. After removing the fins and pen, the epidermis was stripped from the remainder of the mantle. The mantle was then washed with distilled water and strips, about 5 mm. wide, were cut into 10 volumes of ice cold distilled water and homogenized for one minute in a Waring Blendor run at top speed. The homogenate was centrifuged twice, first at 2300 rpm for 5 minutes and then the supernatant at 4600 rpm (up and

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[2] It is with great pleasure and appreciation that I acknowledge that the work reported here was done while I held a Lalor Foundation Fellowship at the Marine Biological Laboratory, Woods Hole, Mass., during the summer of 1953.

down) thus separating the homogenate into a water-soluble extract (S_1) and into a residue of debris and water-insoluble material. The combined residues of the two centrifugations (2300 rpm and 4600 rpm) were added to 10 volumes of cold 0.5 *M* KCl to extract the myosin and the KCl homogenate was centrifuged at 2300 rpm for 5 minutes. Precipitation of the myosin by diluting the KCl extract with 6 volumes of cold distilled water was performed twice.

Throughout the extraction the material was kept as cold as possible in ice water baths. Centrifugations were carried out at room temperature. The pH was maintained above neutrality, approximately pH 7.4, by the addition of solid sodium bicarbonate when necessary.

ATPase activity of the various fractions was determined by measuring the increase in inorganic phosphorus after ten minutes incubation at 25° C. of duplicate samples each containing, in addition to the tissue, approximately 0.002 *M* ATP, 0.05 *M* histidine buffer at pH 5.9, 0.05 *M* KCl, and 0.01 *M* $CaCl_2$ or some other ion as specified. The total volume of the reaction mixture was one ml. The reaction was started by the addition of substrate and stopped by trichloroacetic acid (TCA) to a final concentration of 5%. The inorganic phosphorus was determined on the TCA supernate in a Bausch and Lomb colorimeter at 660 mμ according to the method of Fiske and Subbarow (1925), and the enzyme activity expressed as Q_P (Bailey, 1942); *e.g.*, microliter equivalents of phosphorus hydrolyzed in an hour by one mg. of tissue protein.

Protein was estimated by multiplying nitrogen values, obtained by semi-micro Kjeldahl distillation and titration, by the factor 6.25. The tissue proteins were first precipitated with TCA and then digested with a selenium, copper, sulfuric acid mixture before the distillation.

ATP was purchased as the sodium salt (chromatographically pure) from the Schwarz Laboratories, New York City. Inosine-triphosphate (ITP) was a gift from Dr. H. M. Kalckar.

Results

The data from a typical experiment, summarized in Table I, indicate the relative lack of water-soluble ATPase in squid mantle muscle. In this particular experiment the water-soluble fraction (S_1), although containing 18.5% of the mantle protein nitrogen, contains only 0.9% of its ATPase activity. Microscopic examination of this fraction reveals a homogeneous suspension of particles which are, presumably, the mitochondria. Numerous attempts were made to obtain a second ATPase (in addition to a myosin-ATPase) by extracting mantle with molar sucrose, or 0.5 *M* KCl, and following this extraction with differential centrifugation at 20,000 g and 100,000 g to spin down the mitochondria and microsomes, but the results were essentially negative (de Villafranca, 1953). Mitochondrial preparations obtained from sucrose extracts did have somewhat higher proportions of ATPase activity, but these preparations exhibited the same ion activation and pH optimum as the myosin-ATPase. Furthermore, the bulk of the activity could be removed from this type of preparation by washing with 0.5 *M* KCl. The ATPase activity of the soluble fraction (including the particulate matter) was thus considered to be due to myosin-ATPase contamination and not due to an inherent particulate ATPase.

In contrast to the S_1 fraction, the 0.5 *M* KCl extract (MS) of the water-insolu-

TABLE I

The ATPase activity of fractions isolated from squid mantle muscle

Fraction	Q_P	Percentage activity	Percentage nitrogen
Whole homogenate	1140	100.0	100.0
Water-sol. ext. (S_1)	54	0.9	18.5
Residue after KCl ext.	1335	7.6	6.5
KCl extract (MS)	1558	92.3	68.1

ble material contains most of the mantle ATPase activity (Table I). It was from the MS fractions that myosin was precipitated by dilution with 6 volumes of distilled water.

In Figure 1 may be seen the effect of magnesium, manganese, or calcium ions on the ATPase activity of twice-precipitated myosin. Only calcium activates the enzyme to any extent, activating optimally at a concentration of 0.01 *M* $CaCl_2$ both at pH 5.9, as is illustrated, or at pH 8.93. Manganese activates slightly while magnesium is without effect. ATPase activity in the presence of 0.02 *M* $CaCl_2$ was found to be optimal at 0.05 *M* KCl but other concentrations of KCl, up to 0.1 *M*, were only slightly less effective.

The curve illustrating the relationship between myosin-ATPase activity and pH is shown in Figure 2. A definite optimum exists in the region of pH 5.9 with

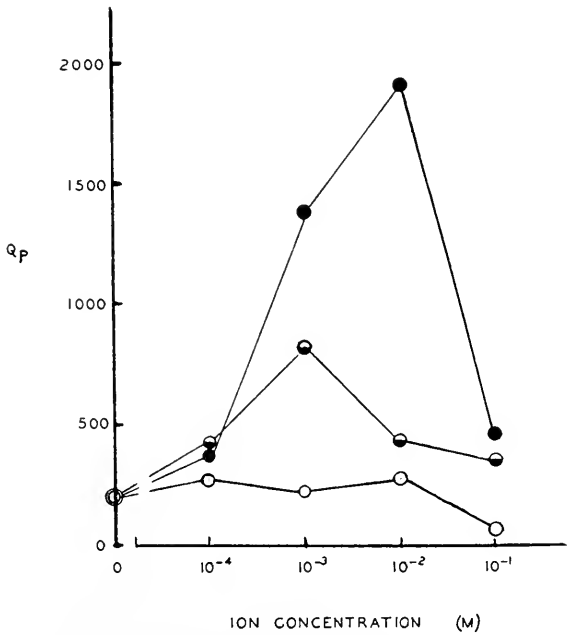

FIGURE 1. The effect of different concentrations of calcium, magnesium, or manganese on the ATPase activity of twice-precipitated squid myosin. Activity was measured in 0.05 *M* histidine buffer (pH 5.9), 0.05 *M* KCl, 0.002 *M* ATP, and the ions as indicated: $CaCl_2$, ●; $MnCl_2$, ◒; and $MgCl_2$, ○.

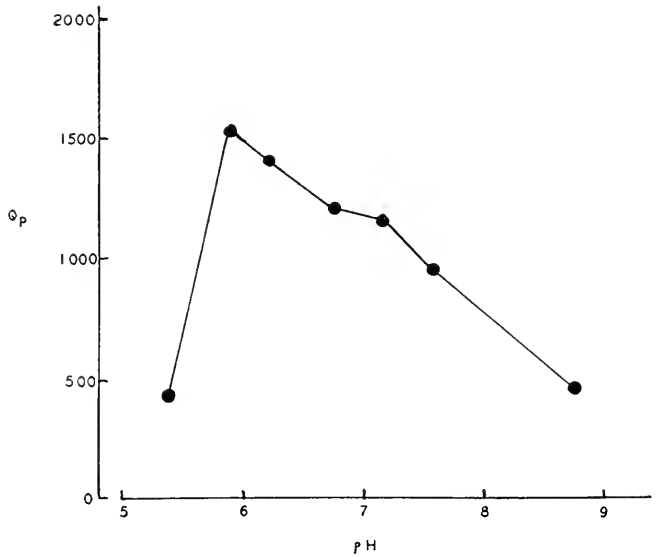

FIGURE 2. ATPase activity of twice-precipitated myosin as a function of pH. Activity measured in 0.05 *M* histidine, 0.02 *M* $CaCl_2$, 0.05 *M* KCl, and 0.002 *M* ATP.

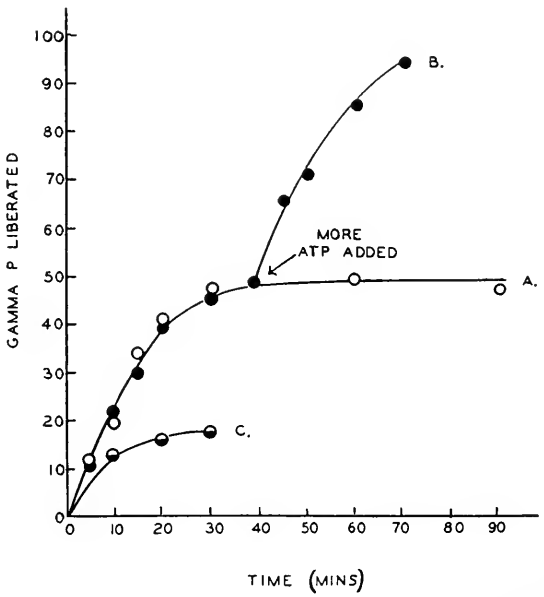

FIGURE 3. Rate of hydrolysis of ATP and ITP by twice-precipitated myosin. In experiment A, 124 gamma of "7 min." P were present; experiment B, 147 gamma and an additional 147 gamma of "7 min." P, aliquot of ATP was added as indicated. Experiment C had ITP, 123 gamma of "7 min." P, as substrate. Activity in all the experiments was measured in 0.05 *M* histidine buffer (pH 5.9), 0.01 *M* $CaCl_2$, and 0.05 *M* KCl.

much greater activity than at pH 8.8 or higher. The most alkaline pH at which the activity was measured was pH 9.4. In this case the activity was proportionally lower than that shown at pH 8.8 (Fig. 2).

That the myosin is probably a true ATPase, splitting only the terminal phosphate from ATP, is indicated in the progress curves (Fig. 3). At the end of 60 minutes approximately 40% of the available "7 min." P had been hydrolyzed (Fig. 3A). If at the end of 40 minutes more substrate is added, additional inorganic phosphorus is released (Fig. 3B), indicating that the enzyme is still active. The enzyme, however, is rapidly inactivated when it is incubated, without substrate, at 37° C. for as little as 10 minutes: 97% of its ATPase activity is lost. It was also noted that much of the activity is lost if the squid are not freshly killed prior to extraction.

Inosinetriphosphate was actively hydrolyzed by squid myosin; Q_P of 1005. This value is not a completely accurate estimate of the ITP hydrolysis since the few experiments performed may reflect a lack of substrate saturation of the enzyme possibly due to high inosinediphosphate content: *i.e.*, at the end of 30 minutes the enzyme preparation had hydrolyzed only 14–16% of the total "7 min." P available (Fig. 3C).

On the assumption that the enzyme hydrolyzed all of the terminal phosphate present in the ATP preparations, the results (in Fig. 3A and of other experiments) indicate that approximately 40% of the "7 min." P is due to the terminal labile group of ATP, or that the substrate preparation contains about 80% ATP. The calculations of the Michaelis-Menten constant were, therefore, based on ATP concentrations calculated as 80% of the total "7 min." P. By this method K_m values, ranging from 2.58 to 3.63×10^{-4} with an average for 5 separate determinations of 3.05×10^{-4}, were obtained.

Discussion

Apparently squid mantle muscle lacks a magnesium-activated, water-soluble or particle-bound, ATPase comparable to that found in vertebrate striated muscle (rat, Kielley and Meyerhof, 1948; de Villafranca, 1954: rabbit, Perry, 1952: or pigeon, Kitiyakara and Harman, 1953) and in the muscle of many invertebrates such as the locust (Gilmour and Calaby, 1952), the oyster (Humphrey, 1949), and, very probably, the cockroach and housefly (Sacktor *et al.*, 1953; Sacktor, 1953). A small amount of this enzyme, too small an amount to be detected under the present conditions, might be present in squid mantle muscle, but its contribution to the total ATPase activity, as compared with the contribution made by myosin-ATPase activity, is negligible. This contrasts strongly with the situation Gilmour and Calaby report for locust muscle where the Mg-activated apyrase dominates the ATPase activity of the whole muscle, particularly in the highly active flight muscles.

The major portion of squid mantle ATPase activity exhibits much the same response to ions as vertebrate (Mommaerts and Seraidarian, 1947; de Villafranca, 1954) and other invertebrate myosin-ATPase; strong activation with calcium (approximately 10^{-2} *M*) and no activation to slight activation with magnesium. In contrast, myosins from the molluscs, *Mya arenaria* (Humphrey, 1948) and *Sepia officinales* (Nguyen-van-Thoai and Pin, 1950), both exhibit greater activity with magnesium than with calcium. It should be remembered, however, that mag-

nesium activation is also a characteristic of undissociated rabbit actomyosin (Szent-Gyorgyi, 1947).

Although the pH optimum for *Loligo* myosin-ATPase is considerably more acid than the optima for mammalian myosin (pH 6.2–6.5 and pH 9.2, Mommaerts and Seraidarian, 1947) it is comparable to the optima reported by Nguyen-van-Thoai and Pin (1950) for other molluscs: pH 5.8 for *Aplysia depilans*, *Pecten maximus*, and *Sepia officinales*. More alkaline optima were reported for *Mya arenaria* (pH 8.5, Humphrey, 1948) and for *Saxostrea commercialis* (just below pH 8, Humphrey, 1949). Since the unique effect of different buffers on myosin-ATPase activity is well known (Mommaerts and Seraidarian, 1947; Bailey, 1942), it is possible that the acid optimum (pH 5.9) reported here might be a function of the buffer used. Veronal buffer, however, showed no alkaline optimum. Experiments comparing the activity in histidine buffer, glycine buffer and "Tris" buffer at pH 8.8 to 9.0, revealed activity to decrease in that order. The apparent activating effect of histidine is being studied at this time.

It is interesting to note that the specific activity of squid myosin-ATPase is of the same order of magnitude as reported for mammalian myosin; Q_P-s ranging from 1000–6000 (Bailey, 1942). The values here (1000–2000) may be contrasted with the Q_P values reported by Humphrey (1948; 1949) which ranged from 9 to 60 for other invertebrates. The low values reported by Humphrey might be due to inactivation of the enzyme by the high temperatures, 35–38° C., at which it was tested. The squid enzyme, as stated previously, was quickly inactivated at temperatures of that order and the high Q_P values were obtained at 25° C.

Evidently the metabolism of squid muscle deviates somewhat from that of other animals. It would be of more than passing interest to ascertain whether the oxidative enzymes of the tricarboxylic acid cycle, found bound to the particles of mammalian muscle cells, were similarly bound in squid muscle. It would also be of interest to compare the function and chemistry of this muscle, which is composed of smooth muscle fibers (Dahlgren and Kapner, 1908; and the author's own unpublished observations), with striated muscle in an endeavor to clarify the real significance of striations.

Summary

1. It is highly probable that the mantle muscle of the squid, *Loligo pealii,* has only one ATPase, associated with the myosin-like proteins extracted with 0.5 *M* KCl. It lacks a water-soluble, or particulate-bound, ATPase comparable to that found in vertebrate and certain insect muscle.

2. The extremely labile myosin-ATPase is activated almost 10-fold by 0.01 *M* $CaCl_2$ and almost 4-fold by 0.001 *M* $MnCl_2$. Magnesium is without effect. The pH optimum for this enzyme is approximately pH 5.9. A true ATPase, being specific for the terminal phosphate of ATP, it also hydrolyzes ITP at a somewhat reduced rate. An average Michaelis-Menten constant of 3.05×10^{-4} and a Q_P as high as 2000, comparable to mammalian myosin-ATPase and much higher than other marine invertebrates, have been obtained.

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SOME MINUTE MOVEMENTS IN PROTOPLASM

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In biological literature there are widely scattered records of apparently automatic movements of minute entities within the protoplasm of both unicellular and multicellular animals and plants; motions not referable to cyclosis nor to pedesis; having yet no name, accepted meaning or cause.

In the ciliated protozoa Folliculinidae, I studied these motions with the Zeiss immersion lens, in 1912, 1913 and again in 1945, 1946, and 1950, as well as in the related Stentoridae. Since these observations were scarcely mentioned in my papers (1923, 1945, 1946) I now bring them to attention, in the hope that others will find these organisms favorable for extended investigation with exact measurements and experiments to gain some better comprehension of such phenomena.

In folliculinids and stentors food is cast into the endosarc from the oesophagus to be carried in a food vacuole or "gastriole" slowly and individually through a long course, until finally undigested refuse is thrown out at the cytoproct. The transit of gastrioles is not in a visible tract nor in cyclosis, but each is individually handled as so often seen when yeast cells boiled and stained with Congo Red are taken as food, but not digested. Moreover, individual grains of yeast may be carried through the animal without large enveloping sacs, and the same is true of small linear crystals of the above stain.

The protoplasm of folliculinids and stentors is colored, commonly blue-green, and this color is concentrated chiefly in color bodies, "protrichocysts," scattered through the interior but crowded in lengthwise lines in the ectosarc. In *Stentor coeruleus,* where the interior is grossly vesiculated, these blue granules move about between vesicles as if gliding along films, but in clear homogeneous plasm they have no visible track. They go individually in various directions.

To overcome the difficulty of focusing in the thick mass the attenuated stalk of the fully extended animal is most favorable, as here the clear plasm forms a thin film but eight microns wide. In *Stentor igneus* individual red grains went in such stalks more than 15 microns at an estimated rate of 2¼ microns per second. All the interior was active with such movements resembling autos in a city going in diverse directions, stopping and starting. Some passed close to others going in opposite directions. In *Parafolliculina americana* Hadzi the trajectories of such particles were curved as well as straight. Their size was from ½ micron to 3 microns. In camera lucida sketches the particles magnified 2400 diameters went 10 to 100 times their own lengths in single trips at a rate of 2 to 3 microns per

second. The motions of the blue grains in *Stentor coeruleus* have been seen recently by Weisz (1949) who emphasizes the chemical structure of these bodies and concludes that they are mitochondria. That he saw the color bodies in the ectosarc constantly active in pedesis and going into the interior suggests that coverglass pressure may have liquefied the area observed. I considered the color bodies to be end products of digestion, of no use in metabolism; since in cannibal stentors and in blepharismas the ingested prey is digested except the color bodies which are gathered in a mass and cast out at the cytoproct. Moreover, when stentors stand long in one place color bodies are cast out and accumulate as a green mass about the feet; while in folliculinids lines of ectosarcal color bodies are cast out as part of the secretion that forms the temporary dwellings.

Some records of minute movements similar to these in the Folliculinidae and Stentoridae may be briefly enumerated as follows. Schultz (1863), who emphasized flowing as fundamental in living things, studied in detail motions in the fine anastomosing pseudopodial threads of Foraminifera which serve both for feeding and for locomotion. He saw that many granules acted independently, like people on a crowded sidewalk, swarming about amongst others, at times halting and trembling, but always at length going to an end of the pseudopod. Dead objects, grains of carmine, showed the same phenomena. Schneider (1906) also saw particles moved independent of flow in both Foraminifera and Radiolaria as well as in the stamen hairs of a common plant, the gourd, which proved a very favorable subject when Martin Heidenhain (1897, 1911) studied these cells with Zeiss water immersion and apochromatic oil immersion lenses. He saw individual particles moved along and concluded they were carried by invisible "minimum waves" of the surrounding plasm.

Somewhat as in forams, mycetozoa (myxomycetes) move and feed by means of protoplasmic networks within which currents of protoplasm are seen to carry innumerable colored granules; however, these currents reverse rhythmically, as so well demonstrated by Kamiya (1942). Loewy (1949), from facts discovered by Seifriz, concluded the cause of these flows was within them; every flow would be made up of minute individual acts; apparently erratic individual motions were due to temporary lack of agreement with the motions propelling the majority that made up the flow. This concept was independently arrived at by several investigators of that period.

That mitochondria also exhibit individual motions was demonstrated by the Lewises (1915) in tissue cultures of chick embryos.

Chromatophores, color-bearing cells of some animals capable of rapid color changes, also show both flow and individual motions of colored grains. Degner (1912) studied shrimp chromatophores and likened the movements of grains to those seen by Schultz in forams. The chromatophores of fish were studied by Ballowitz (1913) and in another species by Mathews (1931). In amphibia Haberlandt (1919) studied motions of the minute colored vesicles due to staining with intra-vitam stains. In reptiles chromatophores were studied by Schmidt (1917) in the scales of a lizard.

Additional instances of small motions are: those of nucleoli, seen only in certain stages of mitosis in Salvinia, by Hiraoka (1949); the movements of chondriosomes studied by Buvat (1953), in root cells of chickory; movements of gran-

ules in the erythrocytes of elasmobranchs, as demonstrated by Parpart (1950) by means of television microscopy; slight, but important, movements of chromosomes; and transport of chloroplasts in common green plants.

While the work that has been done on seeming automatic movements in protoplasm contains no extensive records of sizes and speeds, it seems that size varies much as in the folliculinids, while speed may be much greater. Observers fail to agree upon causes for such movements, but most all seek the causes in the plasm and regard the particles as passively moved by contraction, surface tension, sol-gel changes or a combination of chemical and physical activities.

Folliculinids and stentors, being complex animals of small size, offer advantages for observation and measurement and as various foreign bodies may be got into the protoplasm by the normal feeding mechanisms it should be possible to make comparisons between rates and courses of different inert particles, to aid in comprehending the forces involved in their propulsion. Are they propelled by forces acting only at the beginning of their trajectories or acting all along the lines of their courses? Experiments upon folliculinids might be used to test, or to confirm, some of the very interesting explanations of Professor William Seifriz (1953) arrived at in his recent work in Paris. He holds that the movements of particles, individually or in flow, are caused by action of the adjacent plasm, acting not only by contraction as in muscles, but also instigated by nerve-like foci in the plasm. Underlying these contractions, as well as contractions of muscles and activity of nerves, are inferred energy supplies from molecules having electric fields.

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MONOGENETIC TREMATODES OF GULF OF MEXICO FISHES. PART I. THE SUPERFAMILY GYRODACTYLOIDEA [1]

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The series of papers of which this is the first installment deals with the monogenetic trematodes which were recovered from 313 individual fishes representing 25 families. The hosts were collected during the period from September, 1951 to January, 1954. In all a total of 637 fishes of 98 species were examined throughout the study, but only 49 of these species carried the parasites to be reported in this series. When finally completed, a total of 75 species of Monogenea belonging to over 46 different genera will have been considered. The present paper presents data on several species belonging to the subfamilies Gyrodactylinae and Tetraonchinae.

While this study began essentially as a faunal survey and as such constitutes an entirely new locality record for all species, several aspects of perhaps greater importance have developed as it progressed. Some of these are: (1) an improvement of the taxonomy of the order, (2) the redescription of many previously described species, (3) an increased knowledge of monogenetic host-specificity, and (4) the discovery of several unusual structures, *e.g.* a giant cell in one species and what is thought to be a binucleated cell in another. These features will be treated in this and future installments.

The purpose of this work is to add to existing knowledge of the taxonomy and distribution of this very interesting and successful group of parasites. The Gulf of Mexico was chosen as the collection site because it represented an almost unexplored source of material.

Although the early workers, particularly European, have contributed much to our knowledge of the Monogenea by their pioneering research, the most useful and natural taxonomic schemes have been developed only recently. Most valuable of the recent studies have been those of Johnston and Tiegs (1922), Price's comprehensive series (1936 to 1943b) and Sproston's synopsis (1946). Price (1936 to 1943b), Sproston (1946) and Mizelle (1938) have treated the literature prior to 1946 so thoroughly that further comment is unnecessary. Since publication of Sproston's synopsis most of the 25 or 30 papers which have been published are species descriptions, locality records or morphological studies, but several of

[1] Contribution from the Oceanographic Institute and the Department of Zoology, Florida State University and the Biological Laboratories of the Citadel.

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The entire work is dedicated to Dr. Perry C. Holt and Mrs. Dolores M. Hargis.

broader scope have appeared. The most important of these are those of Brinkmann (1952), Palombi (1949) and Yamaguti (1953).

The taxonomic scheme employed in the present series is essentially that of Sproston (1946), who drew heavily on Price's works (1936 to 1943b). However, some emendations and additions have been made in the light of evidence which has been accumulated since 1946. Much of this evidence is derived from the recent contributions of Yamaguti (1942 and 1953) and studies of the present collection. An attempt has been made in the pages that follow to indicate by position or discussion the phylogenetic position of the families or subfamilies, but little has been done with the lower taxa because of present limitations of our knowledge. In dealing with the monogeneids it must be borne in mind that many of the existing systematic ideas are undoubtedly somewhat temporary. This is due to the dearth of information, embryological, faunal and morphological, concerning the order.

Materials and Methods

Collections were made at several points on the north and east coasts of the Gulf of Mexico. The sites are given with the discussion of each species. Hosts captured in waters adjacent to the Alligator Harbor Marine Laboratory of Florida State University were identified by the writer and Mr. E. B. Joseph. Those from distant sites were identified by the collectors.

Worms were prepared by the relaxation-fixation method of Hargis (1953). This technique has been found to yield good results as far as morphological detail, significant measurements and accurate ecological data are concerned and is especially suited for mass collections. Whole mounts were used exclusively, and where possible a large number of individuals were studied for comparison.

All measurements were made using an ocular micrometer and are cited in millimeters. In the case of curved structures measurements are of lines subtending the greatest arcs of those structures. In the descriptions given below the mean is given first, followed by the minima and maxima in parentheses. The number of measurements used to derive the mean is actually the same as the number of individuals measured; otherwise the actual number employed appears in parentheses before the measurements. All drawings were made with the aid of the camera lucida.

Taxonomic particulars, *i.e.* common names and authors, of the hosts are given with the description or discussion and are not repeated elsewhere.

Results

Order Monogenea Carus, 1863
Suborder Monopisthocotylea Odhner, 1912

Palombi (1949) is one recent author who apparently agrees with Fuhrmann's (1928) separation of this suborder into two suborders with different names, Monopisthodiscinea Fuhrmann, 1928 and Monopisthocotylinea (Ohdner, 1912), Fuhrmann, 1928, but until a more thorough evaluation of these groupings can be made, the writer prefers to follow the classification of Price (1937a), Sproston (1946) and Brinkmann (1952) and retain Odhner's groupings.

Superfamily Gyrodactyloidea Johnston and Tiegs, 1922
Family Gyrodactylidae Cobbold, 1864
Subfamily Gyrodactylinae Monticelli, 1892

It has been assumed by most workers that the members of the subfamily Gyrodactylinae, and perhaps also the Isancistrinae Fuhrmann, 1929, are more primitive than those of the other subfamilies of the superfamily Gyrodactyloidea. The writer would like to suggest that they may actually be more advanced. The reasons underlying this suggestion are: (1) both subfamilies are viviparous, usually considered an advancement, (2) both lack eyespots, usually suggestive of a long history of parasitic existence, (3) if elements of the haptor are really homologous as is presently suspected, it is just as possible that the dorsal anchors have been lost and their bars retained as it is that the evolution of these dorsal elements is just beginning with the bar appearing first, (4) the marginal hooks of *Gyrodactylus* are strikingly different from those of other gyrodactyloidids, apparently being more advanced. These possibilities should be investigated further. However, no change will be made in the relative position of Gyrodactylinae until additional evidence is available.

Genus *Gyrodactylus* Nordmann, 1832

Four species of this interesting genus were encountered on euryhaline fishes of the family Cyprinodontidae.

One of the difficulties encountered in morphological studies of members of the genus *Gyrodactylus* is their diminutive size. Another is the displacement and distortion of the internal organs caused by the uterine embryos. Both of these factors have combined to cause some uncertainty as to the precise identity of certain differentially staining structures and the proper terminology to be applied to others. For instance, some workers have applied the term "ootype" to a structure which is located at the posterior end of the uterus and often contains an ovumlike object. Because the ootype is usually considered to be that part of the oviduct where egg formation takes place it is possible that the name ootype is not applicable in this case since the worms are viviparous and may not form egg shells.

Gyrodactylus funduli n. sp.
(Figs. 1–3)

Host: *Fundulus similis* (Baird and Girard), Longnose Killifish, a euryhaline bentho-littoral cyprinodontid.

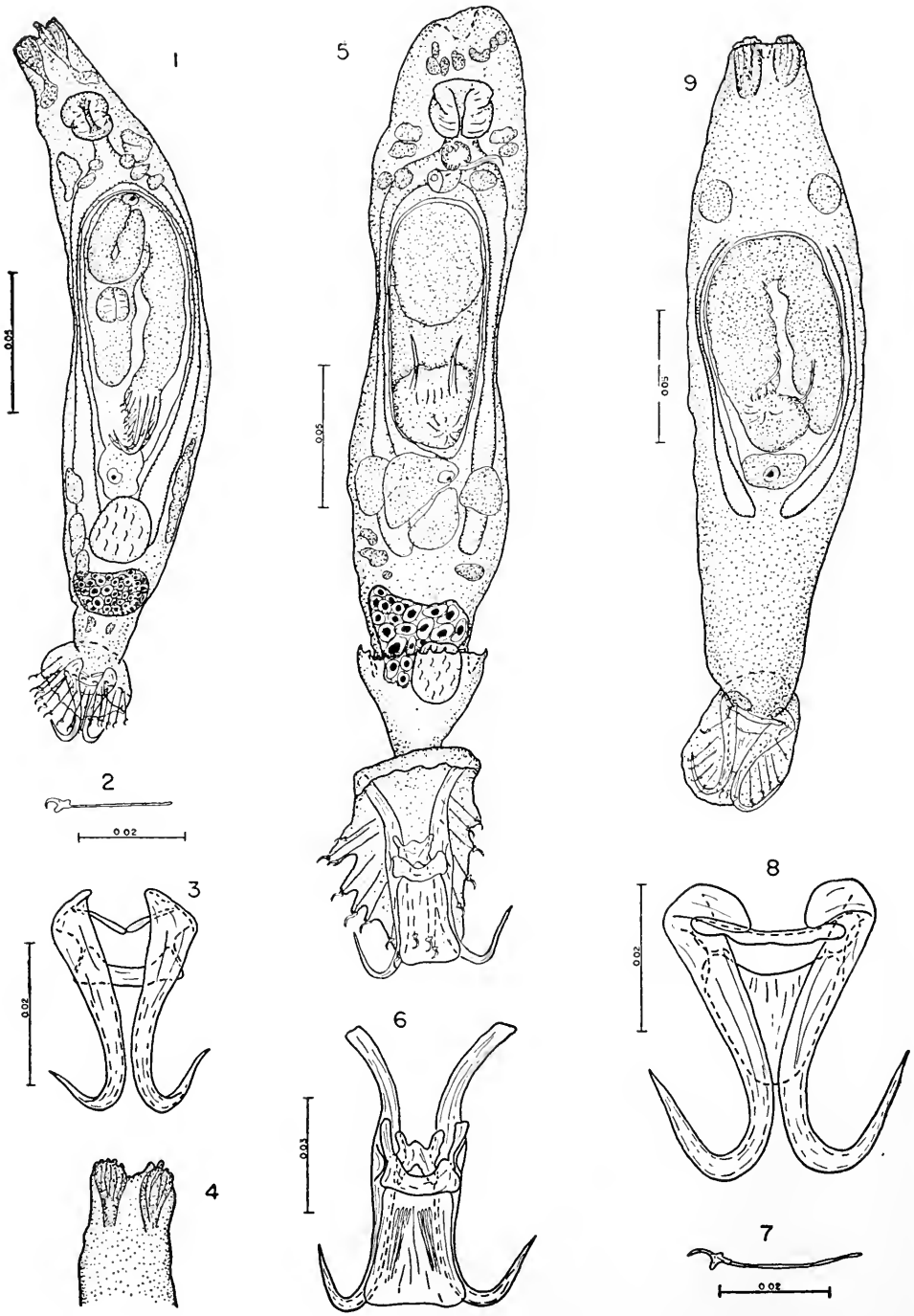
Location: Gills. Probably also on skin.

Locality: Alligator Harbor, Franklin Co., Florida.

Number studied and measured: 3.

Holotype: USNM Helm. Coll. No. 49330.

Description: Body weakly fusiform, 0.261 (0.255–0.268) long by (2) 0.051 wide. Cuticle thin and smooth. Prohaptor of a pair of antero-lateral, papillate head organs connected to posterior cephalic glands. Opisthaptor a concavo-convex disk, 0.049 (0.044–0.051) long by 0.035 (0.032–0.038) wide, opening ventro-posteriorly; armed with 2 anchors, 2 bars and 16 marginal hooks. Anchors stout, 0.032 (0.031–0.034) long by 0.008 wide, with short, angular, truncated



FIGURES 1–9.

roots, ventro-mesial knobs and strongly recurved tips. Ventral bar thick, (2) 0.012 (0.011–0.013) long by (2) 0.003 (0.002–0.004) wide, with anteriorly directed, truncate ends that articulate with the ventro-mesial knobs of the anchors. Dorsal bar delicate (2) 0.010 (0.008–0.012) long by (2) 0.001 wide, somewhat variable in shape, often appearing bipartite. Haptoral hooks (2) 0.021 (0.020–0.022) long, with long thin shafts and small, sickle-shaped tips. Pharynx bilobed, 0.019 (0.016–0.024) long by 0.014 (0.012–0.018) wide, strongly muscular; esophagus short. Gut bifurcate, crura unramified and not confluent posteriorly. Structure interpreted as testis roughly spherical, about (1) 0.024 in diameter, situated dorsally between posterior ends of intestinal crura. Structure interpreted as cirrus, globular, armed with a large curved spine and possibly smaller ones. Several darkly staining bodies laterally placed near the cirrus are not identifiable. Ovary elongate, somewhat bean-shaped, post-testicular. Structure interpreted as the "ootype" located immediately anterior to the testis. Vitellaria two irregular groups of darkly staining bodies lateral to the posterior ends of the intestinal crura. Large embryo *in utero,* (1) 0.090 long, and advanced enough to contain a second embryo.

Discussion: Though the closest relatives of *Gyrodactylus funduli* n. sp. are not apparent, it differs from all other known species of the genus in the following respects: (1) anchor roots obliquely truncate, (2) shape of ventral bar, (3) host.

Gyrodactylus prolongis n. sp.
(Figs. 4–7)

Synonym: *Gyrodactylus* sp. of Linton, 1940.

Host: *Fundulus grandis* Baird and Girard, Gulf Killifish, a euryhaline bentholittoral marine cyprinodontid.

Location: Gills. Probably also on skin.

Locality: Alligator Harbor, Franklin Co., Florida.

Previously reported host and locality: *Fundulus heteroclitus,* skin, at Woods Hole, Mass.

Number studied and measured: 3.

Holotype: USNM Helm. Coll. No. 49331.

Description: Body somewhat cylindrical, 0.348 (0.312–0.376) long by 0.076 (0.057–0.089) wide. Peduncle unusual, surrounded by an anteriorly directed skirt whose edge is armed by several cuticularized points; rest of cuticle thin and

Gyrodactylus funduli n. sp.

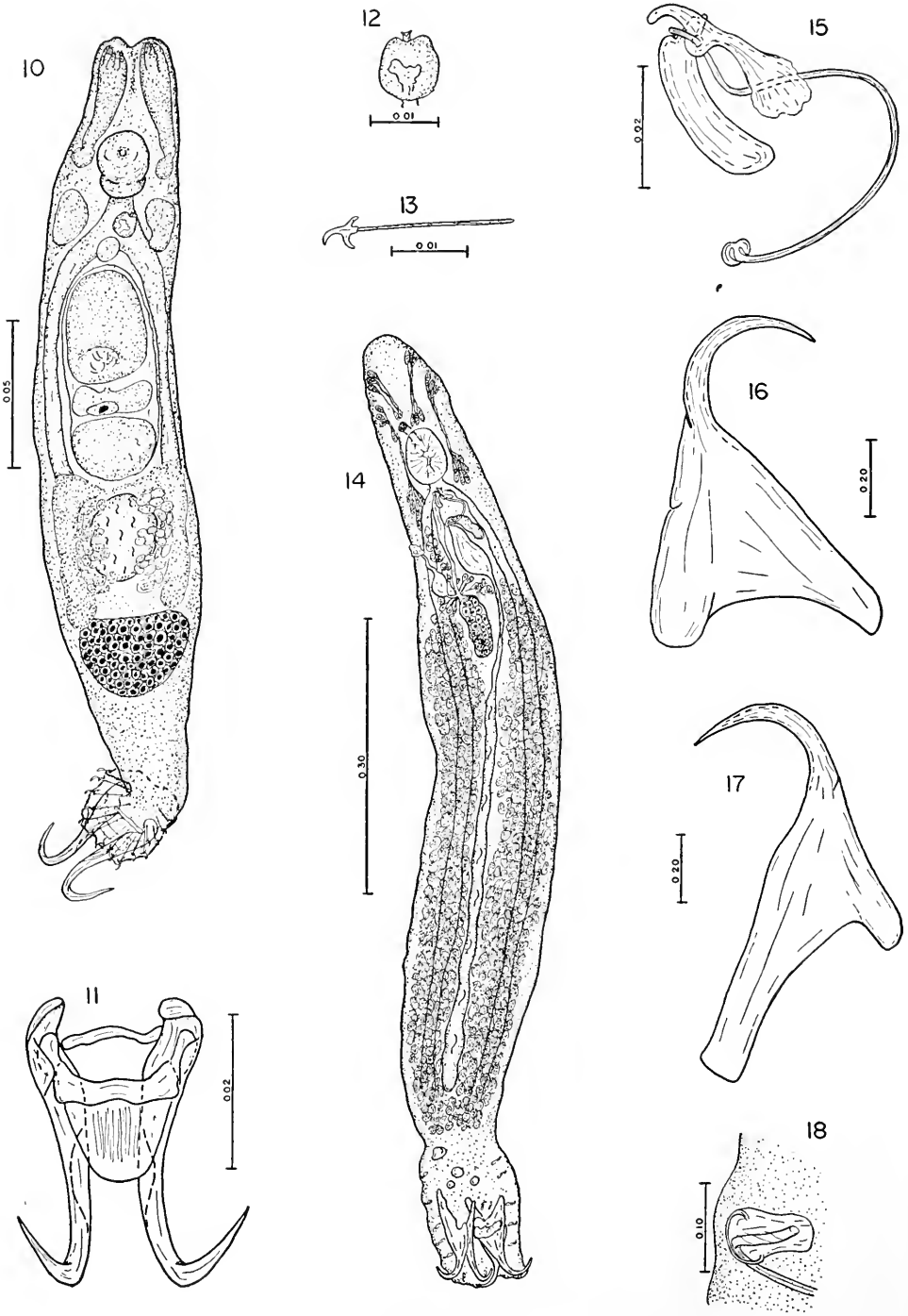
FIGURE 1. Whole mount, dorsal view.
FIGURE 2. Haptoral hook.
FIGURE 3. Anchor complex, dorsal view.

Gyrodactylus prolongis n. sp.

FIGURE 4. Enlargement of anterior end, showing head organs.
FIGURE 5. Whole mount, ventral view.
FIGURE 6. Anchor complex, ventral view.
FIGURE 7. Haptoral hook.

Gyrodactylus sp.

FIGURE 8. Anchor complex, dorsal view.
FIGURE 9. Whole mount, dorsal view.



FIGURES 10–18.

smooth. Prohaptor a pair of anterolateral papillate head organs, no cephalic glands observed. Opisthaptor irregularly oval to rectangular in shape, 0.081 (0.076–0.083) long by 0.049 (0.044–0.051) wide, armed with 2 anchors, 2 bars and 16 marginal hooks. Anchors relatively long, 0.075 (0.074–0.076) long by 0.010 wide, with exceptionally long roots, ventro-mesial knobs and strongly recurved tips. Ventral bar stout, 0.019 (0.018–0.023) long by 0.006 (0.005–0.007) wide, with long anteriorly projecting ends. Ventral shield rectangular, projecting posteriorly from ventral bar. Dorsal bar stout, butterfly shaped, 0.011 (0.009–0.014) long by 0.007 (0.005–0.008) wide. Haptoral hooks radially arranged, with long, thin shafts and sickle-shaped tips, (2) 0.041 long. Pharynx somewhat bilobed, strongly muscular, (1) 0.022 long by (1) 0.027 wide; esophagus short. Gut bifurcate, crura unramified and not confluent posteriorly. Testis ovoid, (1) 0.016 long by (1) 0.014, in posterior half of body; indication of vas deferens observed proceeding sinistrally from cirrus, suggesting vas deferens may course anteriorly on the left side of body. Cirrus muscular, spherical, 0.010 (0.009–0.012) in diameter, apparently armed with the usual hooks or spines. Unidentifiable darkly-staining bodies located near level of cirrus. Ovary irregular, partially dorsal to testis. "Ootype" subtriangular, appearing to contain an ovum, lying to right of midline between intestinal crura. Vitellaria consists of several irregular bodies near level of ootype. Embryo *in utero,* (1) 0.040 long by (1) 0.016 wide.

Discussion: This species was first reported by Linton (1940) but, apparently because of insufficient material, was not formally described. It differs from all other known species in the following respects: (1) unusual, skirt-like accessory holdfast on peduncle, (2) unusually long anchor roots, (3) rectangular shape of ventral shield.

The occurrence of this fluke on both *Fundulus heteroclitus,* Atlantic Ocean, and *F. grandis,* Gulf of Mexico, is probably a reflection of the close relationship of the hosts. This possibility is strengthened by the occurrence of *Gyrodactylus stephanus* Mueller, 1937 on both fishes.

Gyrodactylus sp.
(Figs. 8–9)

Host: *Cyprinodon variegatus* Lacépède, Variegated Minnow, a euryhaline bentho-littoral cyprinodontid.

Location: Gills. Probably also on skin.

Locality: Alligator Harbor, Franklin Co., Florida.

Gyrodactylus stephanus Mueller, 1937

FIGURE 10. Whole mount, dorsal view.
FIGURE 11. Anchor complex, ventral view.
FIGURE 12. Cirrus.
FIGURE 13. Haptoral hook.

Amphibdelloides narcine n. sp.

FIGURE 14. Whole mount, ventral view.
FIGURE 15. Cirrus complex.
FIGURE 16. Ventral anchor.
FIGURE 17. Dorsal anchor.
FIGURE 18. Vagina.

Number studied and measured: 1.

Slide in personal collection.

Description: Body weakly fusiform, 0.287 long by 0.064 wide. Prohaptor a pair of antero-lateral, papillate head organs; cephalic glands not seen. Cuticle thin and smooth. Opisthaptor oval, 0.044 long by 0.038 wide, concavo-convex, opening ventrally, armed by 2 anchors, 2 bars and 16 marginal hooks. Anchors stout, 0.040 long by 0.007 wide, with anteriorly recurving tips and characteristic long, mesially folded roots. Ventral bar larger than dorsal, 0.024 long by 0.005 wide, ends articulating with each anchor. Ventral shield subtriangular, projecting posteriorly from ventral bar. Dorsal bar slight, 0.023 long by 0.001 wide, somewhat irregular in outline and situated between the anterior ends of the anchors distally. Haptoral hooks 0.024 long with long, thin shafts and sickle-shaped tips. Pharynx not observed. Gut apparently bifurcate, crura unramified and not confluent posteriorly. Embryo *in utero* 0.094 long by 0.049 wide. Intensely staining body posterior to embryo may be a part of "ootype" because it appears to contain an ovum.

Discussion: Although this trematode is probably new to science, the present material is insufficient for formal description and naming. It differs from other *Gyrodactylus* spp. in the following respects: (1) length and abrupt medial curvature of anchor roots, (2) shape of ventral shield, (3) host.

Gyrodactylus stephanus Mueller, 1937
(Figs. 10–13)

Host: *Fundulus grandis* Baird and Girard, Gulf Killifish, a euryhaline, benthollittoral cyprinodontid.

Location: Gills. Probably also on skin.

Locality: Alligator Harbor, Florida.

Previously reported host and locality: *Fundulus heteroclitus* from marine habitats in the vicinity of Baltimore, Md.

Number studied: 6.

Number measured: 2

Homotype: USNM Helm. Coll. No. 49332.

Redescription: Body weakly fusiform, almost cylindrical, 0.328 (0.299–0.357) long by 0.044 (0.038–0.051) wide. Cuticle apparently thin and smooth. Prohaptor a pair of antero-lateral papillate head organs connected posteriorly to small cephalic glands. Opisthaptor oval, 0.048 (0.044–0.051) long by 0.038 (0.032–0.044) wide, concavo-convex, opening ventrally; armed with 2 anchors, 2 bars and 16 marginal hooks. Anchors stout, 0.042 (0.039–0.044) long by 0.007 wide, with ventro-mesial knobs and dorsally-directed wing-like expansions. Ventral bar stout, 0.020 (0.019–0.020) long by 0.004 wide, ends curved anteriorly and articulated with the ventral knobs of the anchors. Delicate ventral shield directed posteriorly from ventral bar. Dorsal bar somewhat irregular in outline, slight, 0.016 long by 0.001 wide. Pharynx bilobed, strongly muscular, 0.019 (0.018–0.020) long by 0.016 (0.012–0.019) wide; esophagus short. Gut bifurcate, crura unramified and apparently not confluent posteriorly. Testis ovoid, slightly postequatorial, 0.031 (0.028–0.034) long by 0.030 (0.028–0.031) wide, located between the posterior ends of the intestinal crura. Cirrus spherical,

muscular, 0.008 in diameter, armed with a clearly discernible spine, located dorsal to gut fork and slightly dextral to midline. Unidentified darkly-staining bodies placed lateral and posterior to the cirrus. Ovary somewhat irregular, post-testicular. Vitellaria apparently in confluent masses, probably also follicular, located antero-lateral to testis around intestinal crura. Ovoid structure immediately anterior to the testis appears to be a seminal receptacle because of presence of sperm. "Ootype" immediately anterior to receptacle. Embryo *in utero,* 0.040 long by 0.033 (0.032–0.034) wide.

Discussion: *G. stephanus* has been redescribed and figured because of the lack of detail in the original description.

As mentioned above, both *G. prolongis* n. sp. and *G. stephanus* occur on *Fundulus heteroclitus* and *F. grandis* and probably reflect the close relationship of these hosts. The latter species of fish is considered the Gulf counterpart of the former by some ichthyologists. Further study of the monogenetic trematodes infesting these euryhaline fish which have fresh-water relatives may prove interesting from ecological and zoogeographical standpoints.

Family Dactylogyridae Bychowsky, 1933
Subfamily Tetranochinae Monticelli, 1903

Tetraonchids are so small that, despite their usual large numbers, comparatively few workers have collected them. Well over a dozen species were taken during this study although only nine will be reported in the present series. The rest will be treated at some future date.

The systematics of this subfamily are in such an unsatisfactory state that a careful and thorough revision is indicated. Because of inadequacies of material and time this revision must be delayed; therefore, the species reported in this series are placed in available genera according to contemporary criteria in the hope that such action will facilitate the future sorting of species.

The chief reasons underlying these taxonomic difficulties appear to be the following. The characters used to separate many genera are not of sufficient importance to support such action. Also, the number of known species and genera has increased so rapidly that it has not been possible for systematists to keep abreast. Many of the recent workers who have made revisions in the subfamily have drawn their samples from either fresh-water or marine habitats, rarely both, and consequently have presented a somewhat distorted view; this is largely due to the current usage of different taxonomic criteria in the two ecological groups. The lack of a clear terminology also aids in obscuring details of importance, *e.g.* the term "accessory piece" is used by some to include both ornamentations of the cirrus itself and extra sclerotized structures located near the cirrus, others restrict this term to the latter, as it should be.

With the addition of the new genera of Yamaguti (1942 and 1953) and the recent reinstatement of *Haplocleidus* Mueller, 1937 by Hargis (1952) (Palombi, 1949, also recognized this genus) the number of tetraonchid genera stands at 24. This appears to be too many. In the light of new evidence it now appears that the taxonomic action by Hargis (1952) with regard to the genus *Haplocleidus* is not very significant because it is improbable that length and slight shape differences between the dorsal and ventral anchor pairs are of generic value. Later

work may show these groups to be subgeneric or infrageneric but they must be re-evaluated.

Some of the other characters which must also be reviewed are: (1) confluency or separation of the posterior ends of the intestinal crura; in actual practice this character is often difficult to detect because the gut is obscured by the vitellaria; (2) the side on which the vaginal pore opens; Brinkmann (1952) and others have contended that even complete absence of the vagina in monogenetic worms is not of much significance; (3) similarity or dissimilarity of bars; Yin and Sproston (1948) have remarked that bar dissimilarity may vary according to the opinion of the worker and is, therefore, not dependable as a taxonomic gauge. There are other difficulties but those listed will suffice to indicate the unsatisfactory state of affairs. The above statements may be easily verified by attempting to use the keys of Price (1937a) and Sproston (1946) and comparing their key generic characters with those of Yamaguti (1942 and 1953).

In the opinion of the present writer the taxonomy of Tetraonchinae will be considerably improved if, in addition to the evaluation recommended above, future workers make it a practice to give detailed figures of the entire worm being described and not just the taxonomic "hard parts." Too often characters of significance in the soft body parts and the general body shape are thus obscured. Also data concerning the hosts and their systematic or ecological relationships with each other may provide valuable systematic clues.

Genus *Amphibdelloides* Price, 1937

This genus is distinguished from its close relative *Amphibdella* Chatin, 1874, by the lack of lobes on the opisthaptor and the presence of a haptoral bar. The present writer suspects that these characters are not of generic importance and that later study will confirm this suspicion. Perhaps they are subgeneric in stature. However, Price's genus is retained pending further evidence.

Amphibdelloides narcine n. sp.
(Figs. 14–18)

Host: *Narcine brasiliensis* (Olfers), Torpedo Ray, a bentho-littoral marine torpedinid.

Location: Gills.

Locality: Alligator Harbor, Franklin Co., Florida.

Number studied: 274.

Number measured: 5.

Holotype: USNM Helm. Coll. No. 49333.

Paratype: USNM Helm. Coll. No. 49334.

Description: Body elongate, weakly fusiform, 0.903 (0.790–1.873) long by 0.161 (0.147–0.191) wide, rounded anteriorly, narrowed posteriorly to a wedge-shaped haptor. Cuticle thin and smooth. Prohaptor 2 paired clusters of about 3 small head organs each, connected posteriorly to cephalic glands. Opisthaptor wedge-shaped, rectangular in frontal view, 0.124 (0.108–0.146) long by 0.149 (0.102–0.248) wide; armed with 2 pairs of centrally placed anchors, 1 bar, 16 marginal hooks, and usually with several circular, cuticularized pieces variously

located. Anchors slightly dissimilar in shape but similar in size, shafts and tips curved; ventral anchors, 0.093 (0.085–0.099) long, with wide base, roots fused to form a triangle, differing from dorsal anchors in the completeness of root fusion and shorter superficial roots; dorsal anchors, 0.100 (0.093–0.108) long, base wide, roots more completely separated, superficial roots longer, situated lateral to the ventral anchors. Haptoral bar stout, 0.035 (0.026–0.051) long by 0.013 (0.009–0.018) wide, somewhat variable in shape, usually terminally expanded, apparently homologous to the ventral bar of other tetraonchids. Six pairs of haptoral hooks marginally located, (4) 0.008 (0.007–0.009) long, with bulbous shaft terminations and small sickle-shaped tips, one pair between roots of ventral anchors. Mouth mid-ventral, immediately anterior to pharynx; buccal tube short. Gut bifurcated, crura unramified, apparently not confluent posteriorly. Testis usually very long and narrow, (4) 0.396 (0.338–0.456) long by (4) 0.025 (0.019–0.030) wide, extending from near posterior ends of vitelline fields to a level just posterior to ovary; vas deferens sinistral to ovary, between intestinal crura, expanding anteriorly to form a seminal vesicle before narrowing to join cirrus. Cirrus a long, narrow, curved cuticular tube, 0.065 (0.054–0.070) long by 0.001 wide, with a flange-like proximal base; two accessory pieces, piece I, to right, a stout, slightly curved bar with parallel sides and variously sculptured anteriorly, (4) 0.027 (0.023–0.031) long by (4) 0.005 (0.004–0.005) wide; accessory piece II to left, (4) 0.019 (0.018–0.022) long by (1) 0.003 wide, widened posteriorly, constricted medially, anterior end in the shape of a hook with a hole in its proximal protuberance through which the cirrus passes. Prostate reservoir saccate, usually posterior to cirrus. Genital pore common, slightly dextral to midline near gut bifurcation. Ovary saccate, pretesticular at about one-third level of body; oviduct short. Ootype short; uterus a thin-walled tube to genital pore. Vagina dextromarginal, with an exterior cuticularized plate surrounding the vaginal pore, vaginal tube cuticularized, expanding mesially to join the seminal receptacle which narrows to join oviduct anterior to ovary. Mehlis' gland around oviduct immediately anterior to ovary. Vitellaria follicular, near intestinal crura, mostly posterior to ovary, terminating near posterior ends of crura, a few anterior follicles dorsal to right crus; transverse vitelloducts probably joining oviduct near level of Mehlis' gland. Eggs not observed. No eyespots.

Discussion: *Amphibdelloides narcine* n. sp. is very closely related to *A. maccallumi* (Johnston and Tiegs, 1922) Price, 1937 which was found to be parasitic on *Tetranarce occidentalis* (Storer), probably the natural host, and *Squalus acanthias* Linn., probably an "unnatural" host, at Woods Hole, Mass. (Recently Alexander, 1954, reported a form that he identified as *A. maccallumi* from the gills of *Torpedo californica* Ayers taken from Cortez Bank off the southern California coast.) A study of USNM Helm. Coll. slide No. 35701 (listed by Price 1937b, p. 153 as 25701) shows that *A. narcine* differs from *A. maccallumi* in the following respects: (1) body smaller, (2) shape of vaginal pore sclerite, (3) accessory pieces, although arranged in the same fashion, are differently sculptured and shaped, accessory piece I more simply sculptured, (4) anchor roots more deeply cleft, (5) host. Both worms are very similar morphologically, indicating that they are closely related and have, perhaps, differentiated only recently. In addition, this close relationship of the parasites probably reflects a corresponding

relationship between their hosts, *Narcine brasiliensis, Tetranarce occidentalis* and *Torpedo californica.*

The occurrence of *A. maccallumi* on *Squalus acanthias* Linn. is probably accidental or abnormal although nothing is known of the conditions under which the hosts were captured or held prior to autopsy by the original author, MacCallum (1916), who reported it as another species. It is strongly suspected that many of MacCallum's host records are not "natural" due to the sources of host material, *e.g.* the New York Aquarium and the New York fish market.

Summary and Conclusions

The present paper is at once the introduction to and the first installment of a projected series of papers which will include the data gathered during a recent study of the monogenetic trematodes recovered from 49 species of Gulf of Mexico fishes. The introduction given herein includes a discussion of the scope and important aspects of the entire work and a short discussion of the literature of the order. In addition, the present state of the taxonomy of the order Monogenea has been briefly considered and the conclusion reached that monogenetic systematics is relatively young and plastic and that much research remains to be done before it can be stabilized.

Only the first portion of the data concerning the superfamily Gyrodactyloidea has been presented herein. Two new, one previously described and one unknown species of the genus *Gyrodactylus* have been described and discussed. These are *G. funduli* n. sp., *G. prolongis* n. sp., *G. stephanus* Mueller, 1937 and *G.* sp., respectively. It has been suggested that members of this interesting genus may actually be phylogenetically more advanced and not more primitive than other gyrodactylids as has heretofore been commonly assumed.

The systematics of the subfamily Tetraonchinae has been considered and the conclusion reached that the entire subfamily and its taxonomic characters should be carefully reviewed in an effort to establish a more natural scheme. In addition, *Amphibdelloides narcine* n. sp., the first of several new tetraonchids discovered, has been described and its relationship with *A. macallumi* considered.

Part II will continue presentation of data on the subfamily Tetraonchinae of the superfamily Gyrodactyloidea.

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ON THE DEVELOPMENT OF ENUCLEATED TRITON EGGS WITH AN INJECTED BLASTULA NUCLEUS

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The experimental method of nuclear transplantation in amphibian eggs reported by Briggs and King (1952, 1953) and King and Briggs (1954a, 1954b) provides a promising new tool for the study of nuclear differentiation during development. They have shown that it is possible to activate and enucleate eggs of the grass frog, *Rana pipiens,* by pricking, and then to inject nuclei from late blastula, gastrula, or older cells into them. Following this treatment, development in the best cases was almost normal to the tadpole stage. Their results indicate that, in Rana at least, nuclei from undetermined blastula cells and chorda and medullary gastrula cells are able to replace the zygote nucleus in development. However, their results also indicate (King and Briggs, 1954b) that as the differentiation of endoderm cells proceeds through neurula and early tailbud stages, the nuclei of these cells become less and less capable of participating in total development. Nevertheless, the nuclei of determined gastrula cells are themselves undetermined and totipotent in developmental capacity since (p. 77, King and Briggs, 1954a), ". . . nuclei from the presumptive medullary plate are *not* limited to participitation in neural differentiation but may participate as well in mesodermal and endodermal differentiation. The same situation exists with respect to the chorda-mesoderm nuclei, which also participate in all types of differentiation."

Briggs and King's method of activating unfertilized eggs of *R. pipiens* was by pricking with a clean glass needle. This, although activating the eggs, was not sufficient to cause them to undergo normal parthenogenetic development beyond the early cleavage stage. This is in agreement with the initial observation by Bataillon (1910) and many later investigators. In the hands of Briggs and King, the enucleation of activated *R. pipiens* eggs following the method of Porter (1936) was almost 100% successful. Three separate checks were used in determining success in enucleation; they were: a) pricked and enucleated eggs failed to cleave or to form cleavage furrows of any kind, b) enucleated eggs fertilized by normal sperm developed without exception into androgenetic haploids as determined by nucleolar number and epidermal nuclear size measurements at tailbud stage 18, and c) Feulgen-stained sections of morula to gastrula stage embryos with injected blastula nuclei showed Feulgen-positive material in the exovate resulting from

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enucleation which was interpreted as the remains of egg nucleus chromatin. These latter cases also possessed diploid-size nuclei in the embryonic cells which the authors concluded were derived from the injected blastula nucleus.

Waddington and Pantelouris (1953) have attempted nuclear injection experiments on egg fragments of the newt, *Triturus* (*Triton*) *palmatus*. They obtained cleavage and partial blastulae; however, no cases advanced to gastrula or later developmental stages. They suggest that technical difficulties in handling the material might be responsible for their failure to obtain development beyond the blastula stage.

It seemed appropriate to attempt to repeat the experiments of Briggs and King, and Waddington and Pantelouris on whole eggs of *Triton palmatus* and *Triton alpestris*, and to include chromosome analyses of experimental eggs. These species are particularly suited for cytological study owing to the low number ($N = 12$) and large size of the chromosomes. Application of the smear technique given in the following description of methods enables one to make chromosome counts on Triton eggs at any time during development. Thus, one is not dependent upon nucleolar number and size measurements of interphase nuclei in older embryos for this critical criterion for determining success in enucleation and nuclear transplantation.

It should be pointed out at this time that although none of the cases in the following experiments developed beyond the early gastrula stage, the results in general outline support the observations reported by Briggs and King, and Waddington and Pantelouris. In early developmental stages at least, an injected blastula nucleus of Triton can replace the zygote nucleus during cleavage and blastulation; however, the totipotency of post-blastula nuclei remains in question.

Material and Methods

1. Induction of ovulation

Gravid females collected in nature were injected on two successive days with 50 International Units Choriongonadotropic Hormone (Ciba, No. 542) dissolved in 0.1 cc. 2X Niu-Twitty solution (Flickinger, 1949; Niu and Twitty, 1953). Three or four days following the first injection, the females generally began to deposit eggs. They were then opened and the unfertilized eggs in the oviducts were removed to dry Petri dishes; there they were kept moist by evaporation from a small piece of wet filter paper cemented to the inside of the cover. Usually from 30 to 70 eggs were obtained from a single female.

2. Activation of the egg by irradiated sperm

The eggs of Triton, unlike those of Rana, cannot be activated by pricking with a clean glass needle, nor has it been possible in 64 attempts to activate the eggs by the injection of blastula nuclei alone. Since the experiments required chromosome-free eggs as hosts for injected blastula nuclei, an activation technique other than that used by Briggs and King had to be used in these experiments. The most successful method involved x-irradiation of sperm at 50,000 roentgens (7.5 minutes irradiation at 31 KV through a beryllium window and a 0.1-mm.

aluminum filter at 7 cm.), which is the dosage recommended as optimal for inactivation of sperm chromatin by Rugh (1939). The sperm ducts were removed from freshly killed males, placed on filter paper moistened with Niu-Twitty solution, and transferred to cellophane-covered Columbia watch dishes. The sperm were irradiated while still in the sperm ducts. Fertilization was carried out in the usual manner by tearing open the sperm duct and applying a small drop of undiluted sperm to the animal surface of the "dry" eggs. After 15 minutes the eggs were flooded with sterile tap water and were allowed to stand for 5 minutes before being removed by watch-maker's forceps from the rapidly swelling jelly capsules. In experimental and control series, 60% to 90% fertilization was generally obtained. Although polyspermy normally occurs in Triton and very frequently multiple sperm entry points can be seen as dark spots on the surface of the egg, Fankhauser (1925, 1932) has shown that only one sperm nucleus participates in normal development; the remainder degenerate. When several sperm penetrate an enucleated egg fragment, cleavage is abnormal and such germs never advance beyond the blastula stage (Fankhauser, 1934a). In the present experiments, haploid chromosome counts were consistently obtained from blastulae resulting from the fertilization of normal eggs with irradiated sperm; thus, there was conclusive evidence that the irradiation treatment was adequate for the inactivation of sperm chromosomes without seriously reducing the fertilizing capacity of the sperm.

3. Enucleation of activated eggs

It was not possible to eliminate the egg chromosomes by any x-ray dosage which did not in addition greatly lower the viscosity of the egg cytoplasm. It was decided therefore that more reliable results with less damage to the eggs could be obtained by using the enucleation method for Triton described by Curry (1931, 1936). Eggs were fertilized by irradiated sperm and removed from the jelly capsules in the manner described above. The eggs were then placed in numbered depressions on a wax-coated Petri dish filled with sterile Niu-Twitty solution. Each egg within its membrane was then pricked by a fine glass needle in the center of the lightly colored "egg spot" which marks the location of the second maturation spindle of the egg nucleus (see Fig. 1A). By means of a flat-tipped mouth- or hand-pipette the cytoplasm and maturation spindle in the region of the "egg spot" were then sucked out through the puncture in the egg membrane (Fig. 1B). Enucleations were carried out in groups of eight to twelve eggs immediately after they had been removed from the jelly capsules, that is, within thirty minutes following fertilization. The effectiveness of this technique decreases rapidly thirty to forty minutes after fertilization, since, by this time the second maturation division is complete and the egg pronucleus migrates from a superficial position into deeper levels of the egg as has been described for the axolotl by Stauffer (1945, Fig. 1). In the present experiments, the enucleation technique of Curry was approximately fifty per cent successful as determined by chromosome counts made on blastulae resulting from eggs fertilized by normal sperm. About one-half of such eggs proved to be haploid and half were diploid; the latter group represent failures in the enucleation technique.

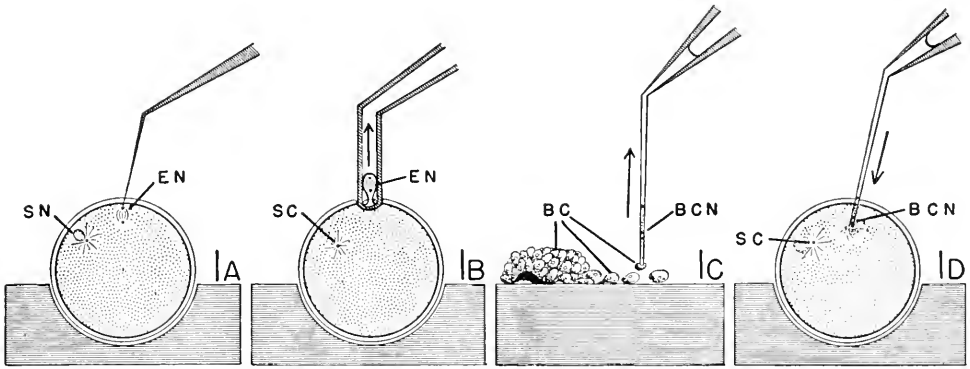


FIGURE 1. Diagram of transplantation technique. (A) Diagrammatic optical section of an egg fertilized with irradiated sperm (SN); the second maturation spindle (EN) of the egg is near the animal pole; a needle is shown pricking the "egg spot"; nuclei are shown greatly enlarged and are not visible in the living egg. (B) The same egg figured in 1A while the maturation spindle (EN) is being sucked out through the puncture in the egg membrane. The egg will then contain no active chromatin but the sperm centrosome (SC) may remain functional. (C) Donor blastula cells (BC) and the method of drawing one cell into the injection pipette are shown; note that the cell membrane of the blastula cell is not ruptured at the inner end and that the blastula cell nucleus (BCN) is visible as a small sphere during the injection procedure. (D) Injection of a blastula cell into an enucleated egg which then contains a diploid blastula cell nucleus (BCN) and a functional sperm aster (SC).

4. Injection of blastula nuclei

Eggs used in the injection experiments were fertilized with irradiated sperm and enucleated in the manner described above. The donor embryo consisted of a young normal diploid blastula (stage 7, Gläsner, 1925) which had been removed from its jelly capsule and egg membrane. It was placed in the operating dish along with the eggs to be enucleated. A small piece of the donor blastula was then removed by means of a hair or platinum loop (Baltzer, 1941). Always some of the cells along the edge of the excised piece detached from the fragment and these isolated cells were used for injection. Injections were performed immediately after enucleating a set of host eggs. Entire donor cells were used and no attempt was made to eliminate the cytoplasm. All injections were carried out free-hand by mouth-pipette. The tip of the injection pipette was a thin-walled capillary with an inside diameter of approximately one-third the diameter of the cell to be injected. Correctly constructed pipettes permitted a cell to be drawn into the lumen of the tube without breaking the cell membrane until almost all of the cell contents were contained within the tube. Very frequently the nucleus of the blastula cell could be clearly seen as a transparent sphere in the column of yolky cytoplasm drawn into the capillary tip (see Fig. 1C). The method of handling cells for injection involved first filling the pipette to the level of capillary equilibrium; then, a small amount of fluid was blown out just before a single blastula cell was picked up. The return flow of fluid into the pipette was used to draw the cell slowly into the capillary tip. The pressure exerted was gentle and constant and when the correct amount of fluid was first expelled, the cell contents remained as an undiluted mass at the tip of the pipette. This procedure had

the great advantage of eliminating the necessity of controlling the position of the cell in the capillary by oral pressure while puncturing the host cell; it also permitted the blastula cell to remain undiluted and prevented the injection of operating medium. Injection was accomplished by inserting the pipette into the animal pole of the egg and the blastula cell was then forced into the egg by gentle pressure on the mouth pipette. The cell membrane, if not already broken, was ruptured at this time. At the same time the pipette was withdrawn slowly so that the upper level of the cytoplasm in the pipette was constantly in view (Fig. 1D). As the last of the cytoplasm was expelled, the pipette tip was withdrawn from the egg and the surface coat of the host cell closed the point of puncture almost immediately. When eggs of *T. palmatus* were used, expulsion of the injected cell and cytolysis resulting from enucleation and injection only rarely occurred, owing to the exceptional elasticity and rapid healing property of the surface coat. Eggs of *T. alpestris* were not as satisfactory in this respect.

5. Cytological preparations

Chromosome analyses in the cases to be reported were made from semipermanent smear preparations of late cleavage and blastula stage embryos. The technique was a modification of that given by Darlington and La Cour (1947) for aceto-orcein smears of Drosophila salivary gland chromosomes. The stain consisted of 2.0 gm. orcein refluxed for four hours in 100 cc. 70% acetic acid; when cool, the stain was filtered and used full strength. Blastulae were divided along the animal-vegetal axis into two halves so that two smears could be obtained from a single embryo. A half-blastula in a drop of Niu-Twitty solution was placed on a clean dry cover glass and oriented so that the non-coated (inner) surface of the embryo was in contact with the glass. All excess fluid was removed and a large drop of alcohol-acetic fixative (three parts 100% ethyl alcohol and one part glacial acetic acid) was added directly to the tissue. After 10 to 15 seconds a second clean cover glass was placed on the fixed cells to flatten them and encourage their adhesion to the lower cover glass surface. After one minute, the preparation was flooded with alcohol-acetic fixative which freed the upper cover glass and permitted it to be slipped off leaving the flattened smear firmly attached as a single sheet to the lower cover glass. The preparation was then transferred to a Columbia cover glass staining jar containing additional fixative and was allowed to harden for two hours. For staining, the cover glass smears were placed flat in covered Petri dishes since aceto-orcein softens the tissue and the smears will detach and slip from the cover glass if held vertically. Smear preparations were stained in full strength aceto-orcein for 30 minutes; differentiated in alcohol-acetic fixative until the smear had bleached to a light pink (approximately 5 minutes); dehydrated in 100% ethyl alcohol, and mounted directly in euparal, or cleared in xylol and mounted in Canada balsam.

Following the above procedure, the individual cell membranes, although broken, remained sufficiently intact to permit the identification of single cell contents. Successful smear preparations have been made from two-, four-, and eight-cell stage eggs; however, in early stages such as these it was found advisable to wait for 30 to 60 seconds before adding the second cover glass to flatten the fixed cells in order that a degree of hardening could take place before the cell mem-

branes were ruptured. In this way it was possible to retain the identity of individual cells even at the earliest cleavage stages.

6. Controls

In each experiment, eggs from one female were used and they were divided among the experimental and two control groups. One control consisted of normal eggs fertilized by normal sperm. These cases provided the basis for determining per cent fertilization and normal tempo of cleavage in a given egg sample. Usually from 60% to 90% fertilization was obtained and at room temperature the eggs began first cleavage within five and one-half to six hours; successive divisions began at approximately one and one-half hour intervals. Experimental eggs usually lagged from one-half to two or three hours behind the normal eggs.

The second control consisted of normal eggs fertilized by irradiated sperm. These were used to determine the effectiveness of the radiation treatment in inactivating the sperm chromatin. The eggs were smeared and stained in the manner described above at the mid-blastula stage. Successful irradiations at 50,000 r consistently gave haploid embryos with all chromosomes of maternal origin. In these cases, minute chromatic particles were frequently observed in the smears and they are believed to represent fragments of sperm chromatin (see Fig. 2D). In series which were considered successfully inactivated by irradiation, none of these "sperm" fragments was of sufficient size to be confused with normal chromosomes. In general the larger "sperm" fragments were uneven in thickness and often appeared as club-shaped masses as shown in Figure 2D.

Experimental Results

A total of 14 experimental series was carried out in which inactivation of sperm by irradiation was considered successful. There were 278 experimental cases in all and, of these, 70 whole eggs were fixed in Zenker solution at various developmental periods (see Column I, Table I) and stored in 70% alcohol for later cytological examination. The general results from the experiments are summarized in Table I and, in addition, Table II gives in greater detail the observations obtained from Series 62 which was the best single experiment.

1. Types of cleavage

The 252 experimental cases which were not preserved before the onset of cleavage can be divided into the three groups given in Column II, Table I.

a. Eighty-nine failed to cleave as a result of failure in fertilization or from operative damage.

b. One hundred and eight underwent abnormal cleavage that resembled in variety and kind the cleavages reported by Fankhauser (1925, 1934b) for andromerogonic eggs. To this group belong Cases 12, 15, 18, 24, and 32 figured in Table II. As in the case of andromerogonic embryos, many such cases, although showing initial irregularities in cleavage, can develop into blastulae which externally appear to be normal (see Cases 12, 15, and 24, Table II). The study of sectioned embryos of this kind has not been done but one might expect that these irregularities may stem from accessory sperm asters which produced cells with-

TABLE I

Summary of results from injecting blastula nuclei into enucleated Triton eggs

Experimental series	Total cases	I Whole embryos fixed			II Cleavage			III External appearance of blastula			IV Chromosome counts on young blastula			V Chromosome counts on old blastula		
		Before cleavage	During cleavage	As blastulae	None	Clearly abnormal	Normal or nearly normal	Cytolyzed	Clearly abnormal	Normal or nearly normal	Haploid	Diploid or hyperdiploid	None	Haploid	Diploid or hyperdiploid	None
26	10	—	—	1	6	—	4	—	—	4	—	—	—	—	—	3
29	35	—	—	3	6	15	14	6	8	15	1	3	3	6	2?	5
30	15	—	—	—	10	4	1	—	3	2	1	1	3	—	—	—
35	14	—	—	5	2	9	3	2	5	5	—	—	—	1	—	4
36	10	—	—	—	3	6	1	1	3	3	2	—	3	—	—	1
37	12	—	—	—	6	3	3	1	3	2	1	1	3	—	—	—
38	12	—	—	—	5	5	2	—	3	4	—	—	—	2	—	5
43	31	—	—	—	8	15	8	—	9	14	11	3	9	—	—	—
44	10	—	—	—	3	6	1	—	3	4	—	—	—	—	—	7
48	30	18	9	—	3	5	4	—	—	—	—	—	—	—	—	—
55	9	—	—	2	1	6	2	—	3	5	1	1	4	—	—	—
56	9	—	3	—	4	4	1	—	—	2	2	—	—	—	—	—
62	32	—	3	—	18	12	2	1	4	6	3	7	—	—	—	—
64	49	8	11	7	14	18	9	—	6	10	4?	1?	4?	—	—	—
Total	278	26	26	18	89	108	55	11	50	76	22+4?	16+1?	25+4?	9	2?	25
Column Totals		70			163			126			72			36		

out chromatin (as described in andromerogons by Fankhauser, 1934b), or with non-functional irradiated sperm chromatin (as found by Briggs, Green and King, 1951).

c. Fifty-five, or approximately 30% of the experimental eggs which cleaved, developed normally. That is, they passed through normal or nearly normal two- and four-cell stages and gave rise to blastulae which were superficially normal or nearly normal in appearance (see Cases 6, 9, and 31, Table II). Aside from the previously mentioned delay in the beginning of cleavage, these cases maintained a nearly normal division rate.

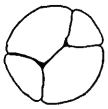
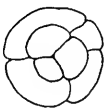
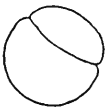
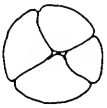
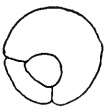
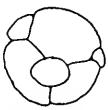
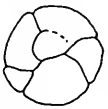
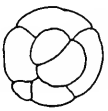
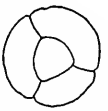
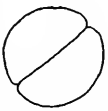
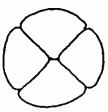
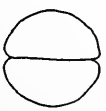
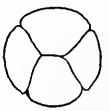
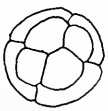
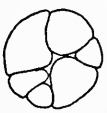
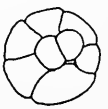
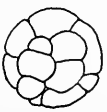
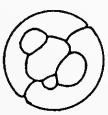
2. Types of blastulae

Of the 126 cases which developed into partial or complete blastulae (Column III, Table I), 18 were preserved entire as is indicated in Column I, Table I. The remaining 108 cases were used in the preparation of chromosome smears.

The 36 cases listed in Column V, Table I, were permitted to develop until the normally fertilized control eggs had advanced to the late gastrula stage. None of the experimental eggs developed beyond the earliest crescent blastopore stage and most cases gave no indication of gastrulation at all. When these cases were opened, masses of loose cells were found filling the interior of the germ and it

TABLE II

Summary of results from experimental series 62

CASE NO.	CLEAVAGE FIRST (5½–7½ HRS)	CLEAVAGE SECOND (7–9 HRS)	CLEAVAGE THIRD (9–16 HRS.)	APPEARANCE OF BLASTULA (16–18 HOURS) EXTERNAL	APPEARANCE OF BLASTULA (16–18 HOURS) BLASTOCOELE	CHROMOSOME ANALYSIS FIGURES COUNTED	CHROMOSOME ANALYSIS PLOIDY
6				NORMAL	NORMAL	7	HAPLOID
9			DELAYED	NORMAL	?	1	HAPLOID
24			DELAYED	NORMAL	NONE	2	HAPLOID
12				NORMAL	NONE	8	DIPLOID
15			DELAYED	NORMAL	NONE	8	DIPLOID
17			DELAYED	UNEQUAL CLEAVAGE	NONE	2	DIPLOID
7				HALF UNCLEAVED	NONE	11	HYPER-DIPLOID
18				UNEQUAL CLEAVAGE	NONE	9	HYPER-DIPLOID
31		DELAYED	DELAYED	NORMAL	REDUCED CAVITY	9	HYPER-DIPLOID
32		DELAYED		SOLID MORULA	NONE	2	HYPER-DIPLOID

See explanation in text.

was evident that developmental advance had stopped during blastulation as Waddington and Pantelouris (1953) also have reported.

In the present experiments ectodermal donor cells were usually used for injection; however, in 47 cases endodermal cells were used. No appreciable difference in results was noted when the two types of donor cells were employed and germs with transplanted endodermal nuclei developed as well as those with injected ectodermal nuclei. Unfortunately no positive data have been obtained which throw light on the question of nuclear determination in blastula cells, since none of the cases have developed far enough to show morphological or histological differentiation.

The 72 cases given in Column IV, Table I, were sacrificed for smear preparations between late cleavage and mid-blastula stage 7 while the individual cells still appeared to be quite healthy. However, when these embryos were opened, even those cases which externally appeared to be most nearly normal showed internal anomalies. The internal malformations ranged from a thickened animal hemisphere and reduced blastocoele to solid blastulae such as those given in Cases 7, 17, and 18, Table II in which the internal yolk mass was only partially cleaved or exhibited unequal rates of cleavage in different parts of the germ.

3. Chromosome analysis

Thirty-six cases were permitted to continue development in the hope that advanced embryonic stages could be obtained but none gastrulated. When chromosome smears were made from these embryos, very few chromosome counts could be obtained. Most of the nuclei were pycnotic and apparently mitotic activity for the most part had stopped during the latter half of the blastula period. In only nine cases could valid chromosome counts be obtained (Column V, Table I) and these were all haploid and undoubtedly represent cases in which the techniques of enucleation and injection had failed. Two questionable cases in Series 29 were found which had tangled masses of chromosomes which could not be counted but seemed to represent appreciably more than the haploid number; these are recorded in Column V, Table I, as questionable diploids.

The most instructive chromosome analyses were obtained from 72 cases sacrificed during late cleavage and early blastula stages. At this time division is still largely synchronous and many cases were presumably at interphase when smeared and presented no countable mitoses for study (see the 29 cases in Column IV, Table I). In the remaining 43 cases, mitoses were found and counts were made on all anaphase figures which were spread sufficiently to be interpreted. A very considerable aid in making the chromosome diagnoses was provided by the excellently detailed study of Triton chromosome morphology by Fankhauser (1934c). He has demonstrated that most of the chromosomes can be individually recognized by structural characters. The most readily identifiable of the twelve chromosomes of Triton is "Number 8" which is characterized by a near-terminal achromatic region on one arm which is attached to a small "satellite" chromatic region. Special attempts were made to identify the homologues of chromosome No. 8 as a supplement to the total chromosome count in determining ploidy in each germ. The results of the best experimental series, No. 62, are presented in some detail in Table II and examples of chromosome configurations obtained from smears of Cases 6, 18, and 7 are shown in Figures 2A, 2B, and 2C, respec-

tively. In these figures, chromosome No. 8 has been drawn in heavy lines to make it more readily distinguishable from the others. Column IV, Table I, gives a total summary of results on the 43 cases in which valid counts could be made. With this tabular material, it is unnecessary to give a detailed discussion of individual cases; however, it should be noted that the embryos fall into two major groups, namely:

Euhaploid (22 + 4? cases) which represent failures in technique.

Eudiploid or hyperdiploid mosaics (16 + 1? cases) which probably represent complete or partial success of the experimental method used.

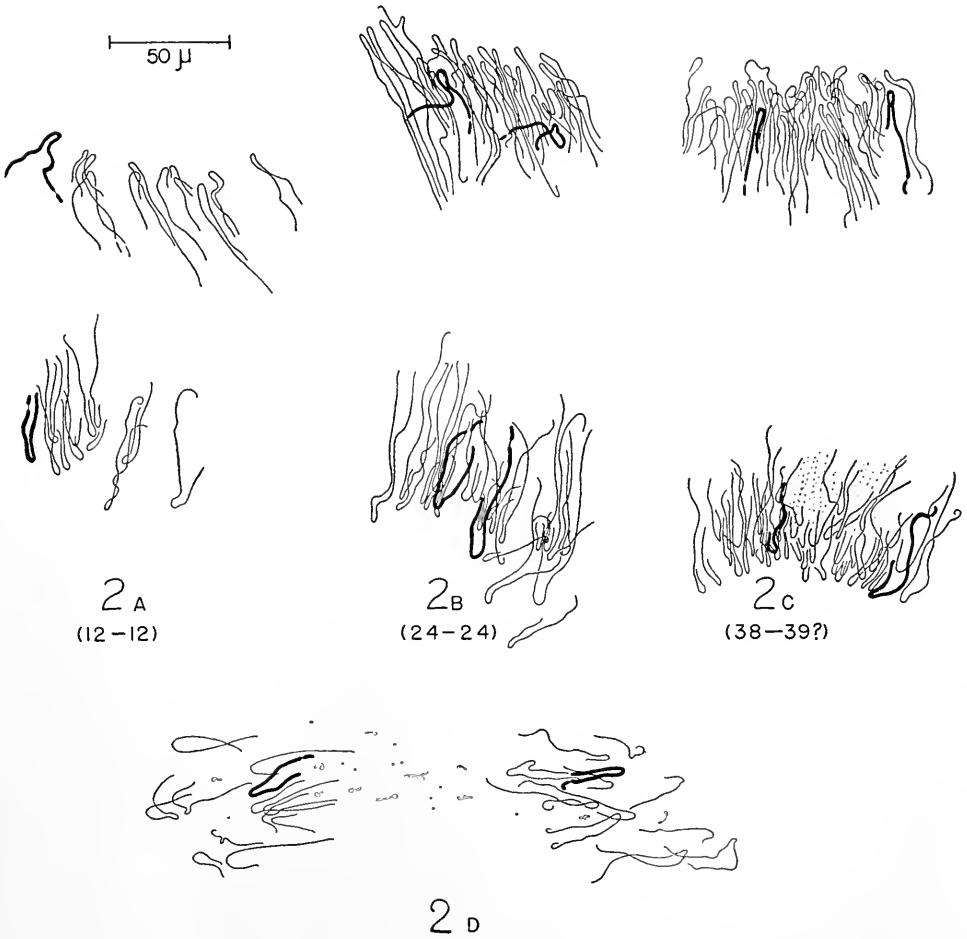


FIGURE 2. Camera lucida tracings of chromosome configurations obtained from aceto-orcein smears of Triton blastulae. (A) Euhaploid anaphase figure from Case 9, Series 62; Chromosome No. 8 (Fankhauser, 1934c) drawn in heavy lines. (B) Eudiploid anaphase figure from Case 18, Series 62; this case was a diploid-hyperdiploid mosaic. (C) Hyperdiploid anaphase figure from Case 7, Series 62; dotted lines in the lower half of the figure represent areas of crowding in which chromosome details are uncertain. (D) Euhaploid anaphase figure from a normal egg fertilized with irradiated sperm and showing an exceptionally large number of "sperm" fragments which are shown in outline; maternal chromosomes are shown in solid lines; See discussion in text.

Conclusions and Summary

The limited amount of data now available on nuclear injections in Triton does not permit one to draw definite conclusions concerning the developmental capacities and determination of injected blastula nuclei. However, several noteworthy facts can be pointed out which are derived from the data presented here.

1. Triton eggs can be enucleated, fertilized with sperm inactivated by irradiation at 50,000 r, and can develop under the influence of a blastula nucleus injected into the uncleaved egg cytoplasm. Some of these eggs will cleave and in almost all cases the beginning of cleavage and the time of successive divisions approximates that found in normally fertilized controls. It is possible that the division apparatus of the irradiated sperm exerts some influence on the time of first cleavage in these eggs.

2. Cleavage is highly variable and strictly normal cleavage patterns are rare. One frequently encounters unequal cleavages which in many ways are comparable to those found in haploid embryos. The blastulae which result from these abnormally dividing embryos externally may appear to be normal or nearly normal; however, internally they are generally atypical and at best possess a reduced blastocoele. No cases were obtained which developed beyond the earliest crescent blastopore stage. Similar types of stopped "solid" blastulae have been reported from hybridization experiments (Lüthi, 1938; Baltzer *et al.* 1939; Baltzer and Schömann, 1951; Baltzer, 1952) and it is of particular interest that cells of the blastula wall in such embryos are indefinitely viable when transplanted to healthy diploid hosts. The application of this transplantation technique to the current type of experiment is the most promising approach available at present for assaying the developmental capacities of tissues from embryos developing with transplanted nuclei from determined and undetermined embryonic tissues of Triton.

3. In a relatively high percentage of cases, chromosome analyses can be carried out by the aceto-orcein smear technique described herein when applied to cleavage and blastula cells. The following chromosomal constitutions have been obtained in experimental cases involving attempts at enucleation of the egg followed by injection of a diploid blastula nucleus:

a. Euhaploid. The simplest explanation of cases such as these (see Cases 6, 9, and 24, Table II) is that there was a failure of the enucleation technique and these cells developed strictly under the influence of the egg nucleus. In these cases the injected cell nucleus is assumed to have degenerated, or at least to have failed to participate in development.

b. Eudiploid. Here it is assumed that the injected diploid blastula nucleus had taken over the function of the zygote nucleus (see Cases 12, 15, and 17, Table II). In such cases it is also assumed that there was success both in enucleation of the egg and in injection of the blastula cell. It is possible, although unlikely, that eudiploids could result from a combination of failure in enucleation of the egg followed by suppression of the second maturation division with the result that all chromosomes would be of maternal origin. There is no way at present to determine with certainty that these diploid cases do indeed represent successful nuclear transplantations.

c. Hyperdiploid mosaics. These embryos possessed individual cells which contained different chromosome numbers that ranged from 24 to as many as 38

or more (see Cases 7, 18, 31, and 32, Table II). Mosaicism and heteroploidy cannot be adequately explained at present; however, it is possible that the maturation spindle of the egg was not removed or that some egg chromosomes remained in the egg following the attempt at enucleation of the host egg. If this had occurred and these chromosomes of maternal origin later became incorporated with those of the injected blastula cell, this could account for hyperdiploid mosaics. King and Briggs (1954a) have reported only one case of mosaicism in their experiments and it was haploid-diploid; however, they did encounter a rather high incidence of polyploidy and state (p. 77, 1954a) that "7 out of 17 embryos resulting from transfers of early gastrula nuclei were polyploid, while among the embryos derived from transfers of late gastrula nuclei, 13 out of 16 were polyploid." In their studies, ploidy was determined by nuclear size of epidermal cells and nuclear number and this method cannot yield precise information on heteroploidy; their results cannot, therefore, be compared directly with the results obtained with Triton. Nevertheless, their explanation of polyploidy in Rana might also apply to Triton. They suggest (Briggs and King, p. 458, 1952) that "The blastula nuclei are undoubtedly in various stages of mitosis when transplanted and there should be ample opportunity for doubling of chromosomes to occur before the nucleus has been transplanted and before the egg cytoplasm is ready to cleave." In order to test this possibility, smear preparations of individual Triton blastula cells were made. The cells were first sucked into an injection pipette in the manner described for the transplantation technique. Stained single cells revealed nuclei at all interphase and division stages and it seems quite probable that in different injected germs, partial or complete doubling of chromosome number could have occurred before the egg cytoplasm was prepared for division. It is therefore possible that the cases showing hyperdiploid counts may possess chromosomes that are all of donor origin. It is hoped that the study of preserved material fixed before cleavage may throw additional light on the behavior of injected blastula nuclei before the first cleavage of the host cell.

4. Under the conditions of these experiments and the techniques employed, there is no direct evidence to indicate that enucleated Triton eggs injected with a blastula cell can develop into normal post-gastrula embryos. Whether this is due to the lack of totipotency on the part of the injected nucleus or due to operative technique is not clear at present.

5. The present study, as well as that of Waddington and Pantelouris (1953), indicates that the eggs of Triton are a relatively unfavorable material as compared with Rana for nuclear injection experiments. The necessity of using irradiated sperm, the low percentage of successful egg enucleations, the irregular behavior of injected cell nuclei, and the cessation of development at the end of blastulation all tend to reduce the probability of obtaining clear-cut solutions to the problems attacked by nuclear transplant experiments.

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GROWTH OF OYSTERS WITH DAMAGED SHELL-EDGES

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During the past few years several papers have appeared dealing with the structure and other characteristics of oyster shells. Medcof (1944) discussed the structure, deposition and quality of the shell of the American oyster, *Crassostrea virginica*, while Korringa (1951a, 1951b) described at length the structure of the shell of the European oyster, *Ostrea edulis,* and also considered it as a habitat harboring numerous species of the epifauna of oysters. Korringa also incorporated in his papers an extensive review of the literature on shells of other oysters and related mollusks. Owen (1953) commented on the shells of lamellibranchs but confined himself almost exclusively to the matter of shell form.

No systematic observations, however, had been made to determine whether damage to oyster shells, such as breaking off their edges, would result in a more rapid growth, which would partly or wholly compensate for the loss caused by the breakage. The present studies were undertaken because we thought that information on this subject would be of both biological and practical significance.

A review of the literature shows that only the articles of Glaser (1903, 1905a, 1905b) and Nelson (1921) discussed a subject related to our problem. Glaser found that mis-shaped oysters, *Ostrea virginica* (now *Crassostrea virginica*), which had grown under unfavorable, overcrowded conditions, would eventually attain normal shape if replanted with ample room for unobstructed shell-growth. He also found that oysters, the growth of which had been inhibited by physical obstruction, would show rapid growth of shell in the affected areas after the obstruction was removed. Nelson (1921) later came to the same conclusions.

Breaking of shell-edges is common when heavy dredges are used for gathering oysters, as is the practice in Long Island Sound and in many other areas where the oyster beds are located at a considerable depth. The damage is especially severe if the oysters are dredged during periods when the new shell-growth is still thin and brittle. In Long Island Sound the most rapid increase in the length of oysters takes place during May, June and July, representing, respectively, 22.57, 19.03 and 22.12 per cent of the total annual increment (Loosanoff and Nomejko, 1949). The oysters dredged during that period usually have a considerable part of the new shell-growth broken off. Since in some areas the oysters may be handled several times annually, and because each time many of them may have the edges broken off, the accumulated loss of shell may be quite significant.

Our first experiment was begun July 18, 1946. Fifty oysters were divided at random into two equal groups. The oysters of the first group, designated as the control, were measured to determine their length, representing the greatest anterior-posterior dimension (Table I). The oysters of the second group were measured in a similar way, but after the initial measurements had been made the edges of their shells were filed off.

TABLE I

Original mean length and increase in mean length of shells of normal oysters and of oysters the shell-edges of which were filed off. Milford Harbor, 1946, 1947 and 1949. Measurements in millimeters

Group	Original length	Length after filing	Decrement due to filing	Length at end of season	Increase over		Per cent increase over	
					Original length	Filed length	Original length	Filed length
1946								
Control	69.5	—	—	91.7	22.2	—	31.9	—
Damaged	68.7	61.7	7.0	89.6	20.9	27.9	30.4	45.2
1947								
Control	92.7	—	—	102.9	10.2	—	11.0	—
Damaged	91.4	84.5	6.9	104.4	13.0	19.9	14.2	23.6
1949								
Control	85.4	—	—	112.8	27.4	—	32.1	—
Damaged	83.4	79.7	3.7	111.1	27.7	31.4	33.2	39.4

In filing, the shell material was removed all the way around the "bill" instead of only in one place, near its end (Fig. 1). The filing reduced the mean length of the shells by about 7.0 millimeters (Table I). The filed-off material consisted not only of recently-formed, thin and transparent shell but also of the thicker, old portion, some of which may have been formed the preceding year. After the filing the oysters were re-measured, and both groups were placed in wire trays, which were suspended side by side in Milford Harbor.

Since the filing was done by hand and slowly, the oysters had enough time to contract the edges of the mantle, avoiding injury to it. Because of this the amount of mantle tissue remained the same as before the filing, thus still possessing the original area of secretory surface for the formation of new shell.

For clarity of description, all the oysters, or groups of oysters, the shells of which were filed off in these experiments, will be called the damaged oysters, while the unfiled ones will be referred to as the control. The oysters used in these experiments were adults, three to six years old.

On December 6, 1946, when the water in Milford Harbor was already cold enough to stop shell-growth, the oysters were again examined. The measurements showed that during the period of the experiment the damaged oysters had added more new shell-growth than the controls. In general, since the date of filing, the mean length of the damaged oysters showed an increase of 27.9 mm., while that of the controls was only 22.2 mm.

The experiment was repeated again in the summer of 1947 using 50 individually-marked oysters for each group (Table I). The shells of one group were filed off on August 14, and then both the damaged and the control oysters were suspended together in the same tray in Milford Harbor. After the beginning of the hibernation period the oysters were again examined. It was found that, as indicated by the increase in length, the damaged oysters had not only added more

new shell-growth than the controls, but had actually outgrown them by the end of the season.

In 1947 both groups of oysters grew less than those used in 1946. This can be attributed to several considerations: first, the 1947 experiment was begun approximately one month later than that of 1946. Secondly, the oysters used in 1947 were considerably larger than those used in 1946 and their rate of growth could be expected to be slower than that of the smaller and younger oysters (Loosanoff, 1947). Finally, since the locations of the trays in which the oysters were suspended varied in the different years, the oysters may have been subjected to a somewhat different set of ecological conditions.

The experiments conducted in 1946 and 1947 showed conclusively that the oysters, the shell-edges of which had been filed off, compensated for this loss by forming new shell at a more rapid rate than the uninjured oysters living under identical conditions. However, since the experiments of these two years were not initiated at the beginning but approximately in the middle of the growing period, the experiments were repeated once more, starting in the spring when the oysters come out of hibernation, and continuing until late fall when the water again becomes too cold for oysters to grow.

On April 14, 1949, two groups, each composed of 110 individually-marked oysters, were treated as described above, and suspended in trays. Because the experiment commenced at the beginning of the shell-growing period, which in our waters usually occurs during the second half of April, the parts of the shells that were filed off consisted exclusively of old shell material that had been formed the year before. The restoration of that portion of the shell could be considered, therefore, as a true regenerative activity on the part of the oysters.

Final examination and measurements of the oysters were made on October 27. Three oysters were found dead in the control group but none in the damaged one.

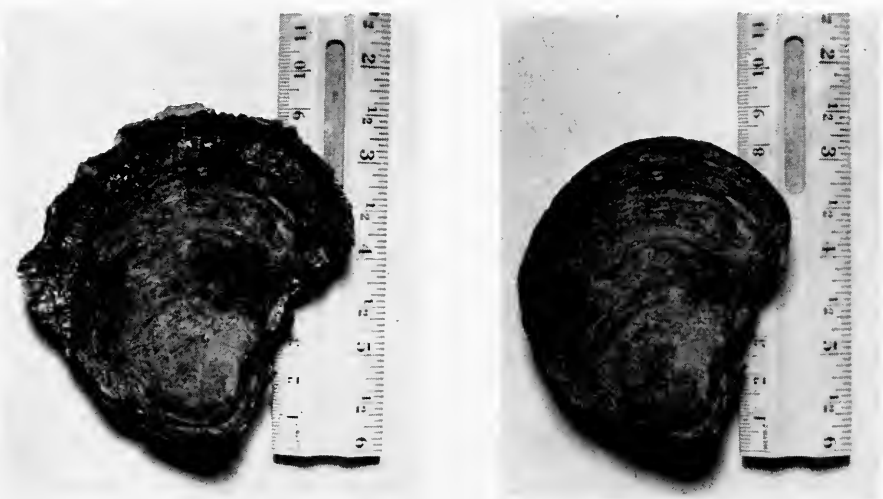

FIGURE 1. Photographs of the same oyster taken before and after the shell-edges were filed off.

The measurements showed that the damaged oysters again increased in length more than those of the control group (Table I).

Since the shell-edges of oysters may be broken off any time during the year, an experiment was devised to determine whether the tendency to compensate for such damages is present throughout the growing season, which extends from April to November (Loosanoff and Nomejko, 1949). The experiment was conducted as follows: On April 8, 1948 oysters that came from the same source were divided at random into five groups each containing 55 individuals, and the mean length of the oysters of each group was determined (Table II). The shells of Group 1, designated as the control, were not filed off during the entire period of observation. The shells of Group 2, however, were filed off on April 8, to provide information on the growth of oysters, the shells of which would be broken early in the spring, at the very beginning of the growing period. After filing, the oysters of Group 2 were again measured. All five groups were then suspended in wire trays in Milford Harbor, presumably under identical conditions.

As the season progressed, the oysters of Groups 3, 4 and 5 had their shells filed off. Each time measurements were taken before and after filing (Table II). Group 3 was filed off on July 13, Group 4 on August 9 and Group 5 on September 7. Thus, the entire season was roughly divided into four periods. Each time a group had its shells filed off and the length recorded, the length of the control group was also measured.

During this experiment 46 oysters were either lost overboard, died, or had their shells broken accidentally, thus becoming ineligible for further observations. The loss was especially serious in our control group when approximately half of it was lost near the end of the experiment. Nevertheless, 229 oysters were still available and our statistical analysis was based on these individuals. The number of oysters remaining in each group was as follows: Group 1—26; Group 2—54; Group 3—54; Group 4—51; and Group 5—44.

TABLE II

Original mean length of five groups of oysters and increase in mean length of normal oysters and of the oysters the shell-edges of which were filed off during different months of the growing period. Milford Harbor, 1948. Measurements in millimeters

Date	Groups				
	1	2	3	4	5
April 8, 1948	88.01	84.26* 81.70**	84.41	82.14	82.42
July 13, 1948	96.95	—	95.56* 90.68**	—	—
August 9, 1948	101.61	—	—	96.01* 91.79**	—
September 7, 1948	106.87	—	—	—	104.19* 99.26**
November 29, 1948	114.61	110.83	113.09	109.16	110.68

* Measurements before filing.
** Measurements after filing.

TABLE III

Decrements and increments of mean length of shells of normal oysters and of the oysters the shell-edges of which were filed off during different months of the growing period. Milford Harbor, 1948. Measurements in millimeters

Aspects studied	Groups				
	1	2	3	4	5
Decrement due to filing	—	2.56	4.88	4.22	4.93
Actual Length Increment (final length less initial length)	26.60	26.57	28.68	27.02	28.26
Per Cent Actual Length Increment	30.22	31.53	33.98	32.90	34.29
Total Length Increment (Actual Length Increment plus decrement due to filing of edges)	26.60	29.13	33.56	31.24	33.19
Per Cent Total Length Increment	30.22	34.57	39.76	38.03	40.27
Increase in length since filing, in millimeters	—	29.13	22.41	17.37	11.42
Corresponding increase of control, in millimeters	—	26.60	17.66	13.00	7.74
Per cent increase in length since filing	—	35.65	24.71	18.92	11.51
Corresponding per cent increase of control	—	30.22	18.22	12.79	7.24

Regardless of the supposedly random sorting of the oysters at the beginning of the experiment into five groups we found later that the control, Group 1, had a somewhat higher mean length than the other four groups (Table II). Analysis of variance showed that the differences in the initial length were significant beyond the .05 level. Therefore, to avoid in the analysis difficulties that would result from a disparity in the initial length, we decided to base the analysis of the amount of growth, shown by the different groups throughout the entire period of the experiment, not on the final length but on the difference between the initial and final lengths. This difference was termed the Actual Length Increment (Table III).

The analysis of variance, to test for homogeneity of the five groups on the basis of their Actual Length Increments, showed no significant difference among the groups. The conclusion was formed, therefore, that by the end of the growing period the damaged oysters showed approximately the same length increment as would have been expected of them if their shells had not been filed off. It should be remembered, however, that the Actual Length Increment does not include the increment needed to make up for the loss in length due to filing of the shells. If these two increments were combined to form the Total Length Increment (Table III), this would clearly show that all the damaged groups of oysters added more new shell and consequently must have increased in length faster during certain intervals than the control.

The stimulation caused by filing of the shells did not induce a significant increase in length beyond that of the oysters in the control group. The observations showed that, regardless of when during the growing period the edges of the shells

were filed off, the oysters grew just enough to compensate for this loss and then added to it the normally expected length increment.

Another measure considered in connection with our data was the Total Length Increment which, as already mentioned, consisted of the Actual Length Increment plus the decrement due to filing of the shells (Table III). (For the control group, obviously, the Actual Length Increment and the Total Length Increment were identical.) The analysis of variance for the four damaged groups on the Total Length Increment indicated that there were significant differences among the groups. This was especially true for Group 2 which showed a smaller Total Length Increment than the other damaged groups. However, this was to be expected because the decrement due to filing varied from group to group. Thus, the oysters of Group 2, which were filed earliest in the growth period, had the smallest decrement and, therefore, had to grow less in total length to achieve the same final length as the oysters of the other groups (Tables II and III).

It was thought that there might be a definite relationship between the amount of shell removed and the final length reached. However, it was established that the amount of damage, within our experimental scope, was not an important factor, that is, regardless of how great or slight the damage was, the individual oysters appeared to be stimulated only to the extent of compensating for the loss in length and then adding whatever additional increment would be expected under normal conditions. In other words, by breaking off more shell along the edges, the oysters could not be induced to reach proportionately greater length by the end of the growing season.

Although the experiments showed that breaking of shell-edges induces oysters to grow faster in length, we had no information concerning the rate at which such an increase proceeded. Because we had made no measurements on the oysters of Groups 2–4 after their shells had been filed, except the final one at the end of the growing period, there were no data for ascertaining the shape of the growth curve after the damage to the shells. It was thought, nevertheless, that since the damaged oysters showed practically the same Actual Length Increment as the control, the stimulating effect of shell-damage did not induce the oysters to grow at an accelerated rate throughout the remainder of the growing period. This was well illustrated by the oysters of Group 2, the shells of which were injured at the beginning of the growth period. This group, subsequently, had approximately eight months in which to grow at the accelerated rate if such rate were a reality. Nevertheless, the Actual Length Increment of these oysters not only did not exceed those of the three other damaged groups, but their Total Length Increment was the smallest (Table III).

Theoretically, there were at least two hypotheses regarding the rate of growth of oysters after damage to their shells. First, it could be assumed that following shell-damage the growth would continue at a somewhat accelerated but, nevertheless, even rate throughout the entire remaining portion of the growing period. The second possibility was that immediately after the damage the growth would proceed at a rapid rate but then, after sufficient growth had been added to the shell-edges to compensate for the original loss, the growth would decrease to the normal rate corresponding to that of the undamaged oysters. We thought the latter hypothesis was more probable because Group 5, which was damaged late

in the growing period, still came up to parity with the other groups in the length increment, thus indicating very rapid growth within a short period. However, to solve this matter definitely we carried on the following experiment:

Two groups of oysters were selected from a common source on May 21, 1954. The average length and width of the oysters of both groups were identical, being 80.6 and 64.5 mm., respectively. After that the shells of one group were filed off, reducing the average length to 77.0 mm. and average width to 60.0 mm. Both groups were then kept under the same conditions. A week later the average length and width of the control oysters were 81.0 and 65.0 mm., respectively, while similar dimensions of the damaged oysters were 79.5 and 62.5 mm. After two weeks the length and width of both groups became identical, being 82.0 and 66.5 mm., respectively, thus indicating that during this short period the oysters with filed shells had caught up with the control oysters. From then on the oysters of the two groups grew at approximately the same rate, showing no significant differences. This experiment has proven, therefore, that the second hypothesis was correct, namely, that in injured oysters the growth proceeds at a rapid rate for a brief period immediately after damage and then decreases.

The rapid growth of the new shell, representing "repair" or regeneration of the portion of the old one removed by filing, is probably due chiefly to the fact that the mantle of the oyster can now protrude farther along the edges of the shell. Because of this the secretory activities of the mantle involved in the formation of the edges of the shell are accelerated. The accelerated growth continues until the shell has grown enough to establish the normal ratio between the size of the oyster body and the length and width of the shell. After that the increase in length continues at approximately the same rate as in the undamaged oysters.

A rapid "repair" of the edges of shells is possible only if the oyster itself, especially its mantle, remains uninjured. If the mantle is injured, the oysters may not be able to show any increase in length until they recover. We have observed a number of such cases under laboratory conditions and also in nature. The best example of the latter was the condition of the Long Island Sound oysters following the extremely severe and prolonged storm of November, 1950. During this storm the oysters were rolled on the bottom by the wave action for many hours and with such force that the shell surfaces acquired almost a polished appearance, while the edges of the shells were rubbed off to such an extent that often the soft parts of the oysters protruded between them. Many oysters died because of injuries, which usually involved the mantle tissue. Those that survived did not resume normal growth but developed into stunted oysters with thick, irregular shells. Oysters of this type are still found in Long Island Sound four years after the storm.

The observations discussed in this paper are concerned almost exclusively with growth of shells as indicated by length measurements. They do not cover other aspects of growth and we know that it is possible that while in some of our experiments the new, thin shell-growth indicated an increase in length, the weight and volume of the oysters may have been smaller than before the shells were filed. Nevertheless, our auxiliary experiments, designed to ascertain the rate of growth immediately after filing, showed that after the edges of the shells had been filed, as shown in Figure 1, the growth in width proceeded at the same rate as the growth in length. This suggests that breakage of shell-edges is repaired by growth of

the same pattern regardless whether it is length or width that is involved in the damage.

Our studies represent only initial steps toward understanding various aspects of the growth of damaged oyster shells. The next experiment, which suggests itself, should consist of studying the growth of oysters, the shell-edges of which were broken several times during the growing period. The growth curve of repeatedly damaged oysters should differ considerably from that of normal oysters, or of oysters the shell-edges of which were broken only once during the season.

Another aspect of these studies could be directed toward ascertaining whether the growth of oysters of different ages and sizes, damaged in the same manner, would differ significantly from that of the oysters on which the present conclusions are based. Because we already know that during the first years the increases in length, width and depth of an oyster proceed at different rates than later in life (Loosanoff, 1947), we may assume such a difference to exist. This assumption would be in agreement with the observations of Glaser (1905b) who found that the recuperative power of overcrowded, mis-shaped oysters varies with age, young individuals recovering their normal shape more rapidly than old ones.

We wish to extend our sincere thanks to Barbara J. Myers for the statistical treatment of the data used in this article and to Miss Rita Riccio for her help in preparing and editing the manuscript. Our thanks are also due to several friends, especially Drs. T. C. Nelson and J. C. Medcof, who offered suggestions and advice.

Summary

1. These experiments, based on length measurements, showed that oysters, the shell-edges of which were broken off, compensate for this loss by forming new shell at a more rapid rate than undamaged oysters living under identical conditions.

2. Damage to shells does not stimulate oysters to grow at an accelerated rate throughout the remainder of the growing period. Immediately after the damage they grow rapidly to compensate for the loss and then continue to grow at the usual rate to add to their length the normally expected annual increment. Thus, no relationship was found between the amount of shell removed and the final length reached.

3. The ability of oysters to repair broken shell-edges and still grow to about the same length as undamaged oysters remains the same regardless of when during the growing season (April 8–September 7) the shells are broken.

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THE DEMONSTRATION OF TWO CHROMATOPHOROTROPICALLY ACTIVE SUBSTANCES IN THE LAND ISOPOD, TRACHELIPUS RATHKEI

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The remarkable ability of decapod Crustacea to undergo adaptive and rhythmic color changes has been under critical study for many years. Early investigators were concerned primarily with the structure of the chromatophores and with the possibility of neural control of pigment migration. Koller (1925), working with *Crago*, gave the first clear cut evidence for humoral control of the chromatophores. Perkins (1928) with *Palaemonetes* and Koller (1928) with *Crago* determined that the substance responsible for these effects was elaborated by some tissue of the eyestalk. Hanstrom (1937) successfully demonstrated that the observed chromatophorotropic activity of the eyestalk was localized in the region of the sinus gland. The extirpation experiments of Brown and Cunningham (1939) and Brown, Ederstrom and Scudamore (1939) presented conclusive evidence of the role of the sinus gland as a source of chromatophorotropins. With the exception of *Crago*, the decapods studied would seem to fall into one of two main categories: (1) those resembling *Palaemonetes* in that sinus gland extracts exert a concentrating influence upon the predominant pigment type and (2) those resembling *Uca* in that extracts of the sinus gland produce a dispersion of the dark pigment.

The presence of chromatophorotropins within the central nervous system was first shown by Brown (1933) for *Palaemonetes*. Subsequent work has established the presence of chromatophore activators within the central nervous system of most decapods. These factors have been found in some cases to have the same action as the hormones of the sinus gland, and in other instances they act antagonistically to them (Knowles, 1939; Brown and Ederstrom, 1940; Brown and Wulff, 1941; Brown, 1950; Sandeen, 1950; Enami, 1951; Brown, Webb and Sandeen, 1952).

The ability of isopod Crustacea to exhibit color responses has long been established and yet the exact nature of the mechanisms controlling these responses has not been clearly demonstrated. The presence of two hormones, one producing a dispersion of the melanin and the other a concentration of the melanin, was indicated in *Ligia oceanica* by the unique experiments of Smith (1938). However, Okay (1945) working with *Idothea baltica* and *Sphaeroma serratum* reported that the responses observed were dependent upon the presence of a single hormone.

Studies on the effects of extracts of isopod heads have yielded apparently conflicting results. In general, extracts prepared from isopod heads when injected into isopods of the same or related species have produced a concentration of the melanin (Kleinholz, 1937; Okay, 1945; Suneson, 1947; Carstam and Suneson, 1949).

Stahl (1938) has shown a dispersion of the red pigment of the decapod

Leander adspersus following the injection of extracts of the entire heads of the isopods *Mesidothea*, *Porcellio* and *Oniscus* and a concentration of the same pigment following the injection of extracts of the heads of *Idothea baltica*. Carstam and Suneson (1949) observed both a dispersing and a concentrating effect upon the red pigment of *Leander* following injection with extracts of the heads of *Idothea neglecta*.

Evidence indicating the presence of structures in *Oniscus asellus* which are apparently homologous to the sinus glands of other Crustacea (Walker, 1935; Stahl, 1938) has suggested the advisability of further investigations of the chromatophorotropins of isopod Crustacea. In addition, Gabe has shown in *Oniscus asellus* (1952a) and in *Sphaeroma serratum* (1952b) histological variations in the sinus glands during the intermolt cycle suggesting the physiological intervention of the gland in this cycle. However, its role in color change was not considered.

The wide variations in color observed in certain species of terrestrial isopods seemed to indicate the possibility that the progressive fixation of pigment, occurring as an adaptation to the terrestrial environment, may not be complete in these forms.

The purpose of this investigation was to determine the degree of chromatic activity occurring within these isopods and, if possible, to cast some light on the nature and distribution of the chromatophorotropins involved in these responses.

The authors would like to acknowledge their gratitude to John R. Cortelyou for his suggestion of the problem and his keen interest and aid throughout the course of this work.

Materials and Methods

Specimens of *Trachelipus rathkei*, a terrestrial isopod, were collected in large numbers from wooded areas in the vicinity of Chicago, Illinois. The animals when collected exhibited wide variations in color ranging from a mottled yellow-brown to an intense black. Males were normally much darker than females. Stock supplies of animals were maintained in covered battery jars containing moistened pieces of decaying wood. These conditions allowed for the successful maintenance of the stock in the laboratory for two to three months. Animals to be light-adapted were placed into clear Petri dishes lined with moistened filter paper. The dishes were then kept on a white background under constant overhead illumination. Animals to be dark-adapted were maintained in a similar manner in Petri dishes painted with black enamel. Attempts were made to establish a relatively constant humidity within the dishes, through the addition of water to the filter paper.

1. Animals

Microscopic examination of the pigmentary system of adult *Trachelipus* reveals that the coloration of the animal is dependent upon the relative distribution of two types of pigment within an apparently syncytial network of chromatophore processes. Although, in many instances, each pigment would appear to be contained within a distinct chromatophore network, the yellow usually lying beneath the black, the presence of extensive dichromatic areas indicates that the syncytium

is complete. Observations of the external surface of the intact dorsal segments showed a cuticle pattern which did not permit clear identification of the chromatophore structure. On the other hand, the internal surface is obscured by a considerable amount of adherent muscle. Histological preparations shed little light on this problem, since the yellow pigment is soluble in acid fixatives and the decalcifying agents used for these studies. At this time, no definite conclusions can be drawn as to the exact structural nature of the pigmentary system of the adult *Trachelipus*.

However, observations of larval *Trachelipus* prior to and immediately following their release from the brood pouch of the female show that in this species there are initially discrete chromatophores (Fig. 1) which rapidly develop into syncytial networks. These appear to be dichromatic and retain this condition during the early stages of fusion. Unfortunately, culture methods were not adequate to maintain the young animals long enough to observe the subsequent development of the chromatophore network.

The nervous system of *Trachelipus* is typically crustacean in nature and appears to be identical to that described for *Oniscus asellus* Linneas (Walker, 1935). The system is relatively unspecialized. The division of the crustacean brain into protocerebrum, deuterocerebrum and tritocerebrum is only weakly apparent in these forms. The protocerebral ganglia are clearly defined and are continuous with the well developed optic tracts. At the distal end of each optic tract in the region of the lamina ganglionaris, there is attached in a slightly ventrolateral position, a small oval structure which is bluish-white when viewed with reflected light. Upon injury this structure is seen to release a bluish viscous fluid, which diffuses through the saline. Both from its morphological appearance and its topographical relationship it resembles the sinus glands of other Crustacea (Fig. 2) and is referred to as such in the subsequent discussion. The deuterocerebrum is greatly reduced and appears as a narrow strip lying behind the protocerebral ganglia. The tritocerebrum, which bears the nerves leading to the antennae, is continuous with the short circumesophageal connective on each side. No tritocerebral commissure was observed although it may have been lost during the dissection. The subesophageal ganglion is not clearly defined and includes the first and second maxillary ganglia and the maxipedal ganglion. The nerve cord is characterized by a uniformity of segmentation of the thoracic ganglia and an anterior fusion of abdominal ganglia. The abdominal nerve cord thus consists merely of the posteriorly directed nerves arising from the fused ganglia.

2. *Preparation of extracts and method of assay*

For the preparation of extracts, the entire nervous system was removed from the animal with the aid of a dissecting microscope ($\times$ 45), and transferred to a syracuse watch glass containing Van Harreveld's solution. Animals from which the nervous systems were taken ranged in length from 9 to 11 mm. Care was taken to remove the entire system in one piece, such that the sinus glands were removed from the animal while retaining their connection to the optic tracts. The surrounding tissue was dissected away in order that extracts would include only the tissue of the sinus glands or of the neural elements.

To determine the relative distribution of the chromatophorotropically active

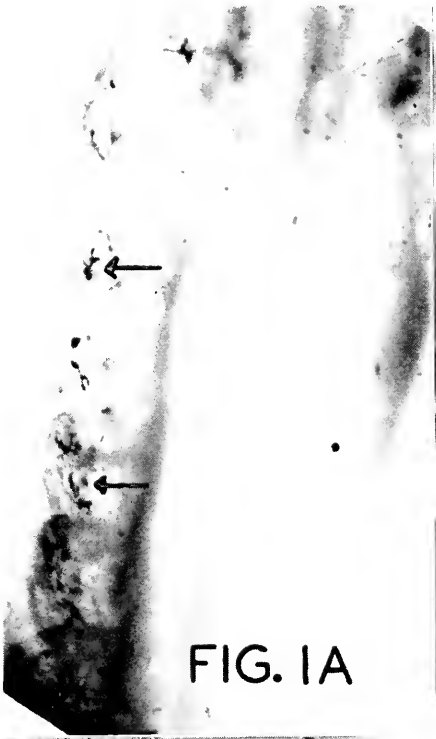

FIG. 1A

FIG. 2

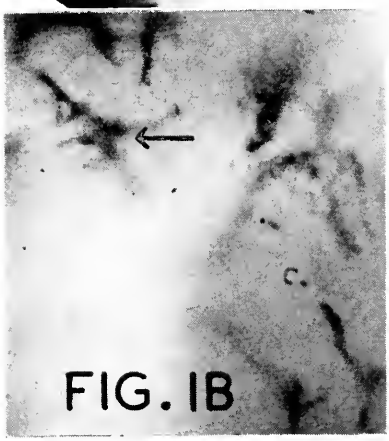

FIG. 1B

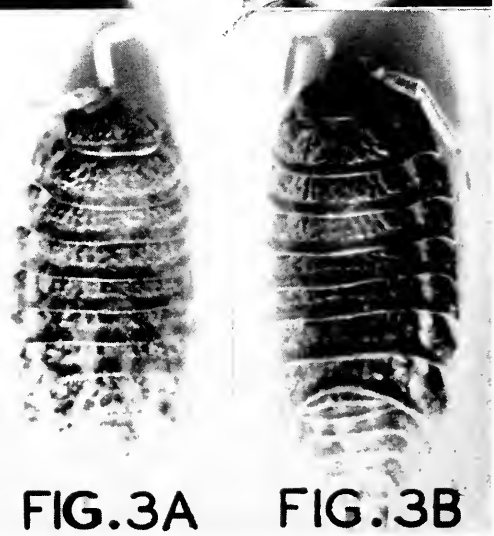

FIG. 3A FIG. 3B

FIGURE 1. Discrete chromatophores of larval *Trachelipus*. (A) × 150; (B) × 440, illustrating early stages of concentration in response to light stimulus.

FIGURE 2. Dissected head of *Trachelipus* demonstrating sinus glands and cerebral region. (× 45)

FIGURE 3. Responses of *Trachelipus* to light. Adult female *Trachelipus* after being maintained in constant (diffuse overhead) illumination while on a light background (A), and after being maintained in total dark (B). (× 13.5)

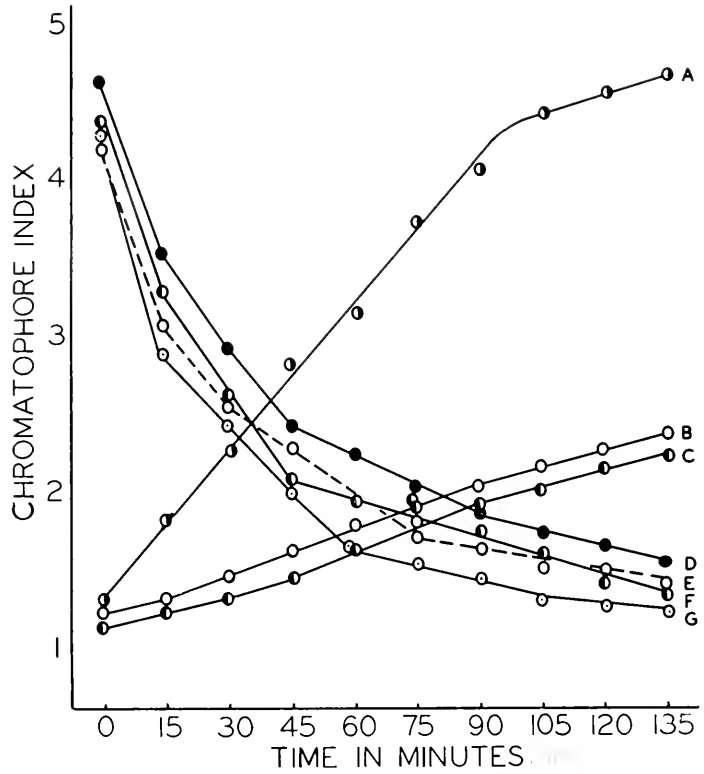

FIGURE 4. Comparative activities of extracts prepared the sinus glands and neural elements of *Trachelipus* when assayed upon the red chromatophores of *Cambarus* (concentration 5). Dispersing activity: sinus glands (A); optic tracts (B); cerebral ganglia (C). Concentrating activity: subesophageal ganglia (D); anterior half of thoracic cord (E); connectives (F); posterior half of thoracic cord (G).

substances, extracts were prepared from nervous systems which had been divided into the following regions: (1) sinus glands; (2) optic tracts; (3) cerebral ganglia (including deuterocerebrum and protocerebrum); (4) circumesophageal connectives (including the tritocerebrum); (5) subesophageal ganglia (including first and second maxillary and the maxipedal ganglia); (6) anterior half of thoracic cord; (7) posterior half of thoracic cord (including the abdominal ganglion). Due to the extreme fragility of the abdominal nerves, no determinations were made of the activity of these elements. The individual fragments were placed into separate mortars. Because the chromatophorotropins undergo a slow inactivation at room temperature, the mortars were placed on ice during the preparation of the extracts. When 10 intact nervous systems had been dissected and divided into the appropriate regions, the fragments were triturated and mixed with one cc. of saline. The extracts were then maintained in small capped vials and refrigerated until further use. Extracts could be kept in this manner for several days without undergoing a loss of activity.

For use in experiments designed to determine the relative effects of concentration, the extracts were prepared in the same manner, from nervous systems divided into two portions, the first of which included the sinus glands, optic tracts and cerebral ganglia; the second included the connectives, subesophageal ganglion and entire thoracic cord. The number of nervous systems used in the preparation of these extracts varied according to the experiment and will be discussed with the appropriate results. In specified instances, extracts were prepared from entire nervous systems, including the sinus glands.

Since boiled extracts showed no significantly marked change in activity, the extracts in most cases were not boiled.

To alleviate the technical difficulties involved in observations of the pigmentary system of *Trachelipus*, assays were made upon isolated portions of the carapace of *Cambarus* sp. The carapace was removed from the animal and divided into pieces approximately 5 × 5 mm. in size. Since the effect upon the chromatophores seemed to proceed from the periphery to the center of the piece, more uniform results were insured by using pieces of approximately the same size. These were then placed into hanging drop slides containing a known concentration of extract. Into each depression were placed pieces of the carapace removed from: (1) a light-adapted animal; (2) an animal whose eyestalks had been removed at least 12 hours before the experiment was begun. In this way, the effectiveness of each extract was determined upon chromatophores which were initially fully dispersed as well as upon those which were completely concentrated. Controls consisting of carapace fragments maintained in Van Harreveld's solution were used in each experiment. The condition of the red chromatophores was then determined at 15-minute intervals using a standard chromatophore index (Slome and Hogben, 1928) where 1 signifies maximum concentration of pigment and 5 maximum dispersion with 2, 3, and 4 indicating the intermediate conditions. Since a difference was noted in the reaction of the chromatophores, average values were determined. Because this difference was more pronounced in older animals, small crayfish having a carapace length of 1.5 to 2.5 cm. were used. Each individual experiment was repeated four to five times.

Extracts of *Cambarus* sinus glands and central nervous systems were used to indicate that the chromatophores of isolated pieces of carapace retain their activity for several hours.

In another series of experiments extracts were injected into young intact *Cambarus*. One type of extract included sinus glands, optic tracts, and cerebral ganglia, while a second consisted of connectives, subesophageal ganglia and thoracic cords. The individuals receiving these extracts were (1) light-adapted for 24 hours and (2) eyestalkless, prepared 24 hours before the time of injection.

Experiments and Results

1. Responses to light

Adult *Trachelipus* exhibit slow and weak chromatic responses to light. Animals maintained upon a light background under the stimulus of diffuse light become slightly but perceptibly lighter than animals maintained in the dark (Fig. 3). More intense illumination, however, results in a darkening of the animals, even

upon a white background. In these cases the additional factor of an increase in temperature was also involved.

The discrete chromatophores of larval *Trachelipus* exhibit rapid and strong responses to light. Under the stimulus of light, the pigment of these cells rapidly concentrates. Although in many instances this results in the typically punctate condition of the chromatophores, in several cases the pigment apparently concentrated within the chromatophore processes, thus presenting a beaded condition. This reaction to light declines rapidly with age, in correlation with the progressive fusion of the chromatophore network.

2. Presence and distribution of chromatophorotropins

To determine the presence and relative distribution of the chromatophorotropic factors, extracts were prepared of sinus glands alone, as well as of isolated pieces of the central nervous system, as described in the preceding section. Since 0.5 cc.

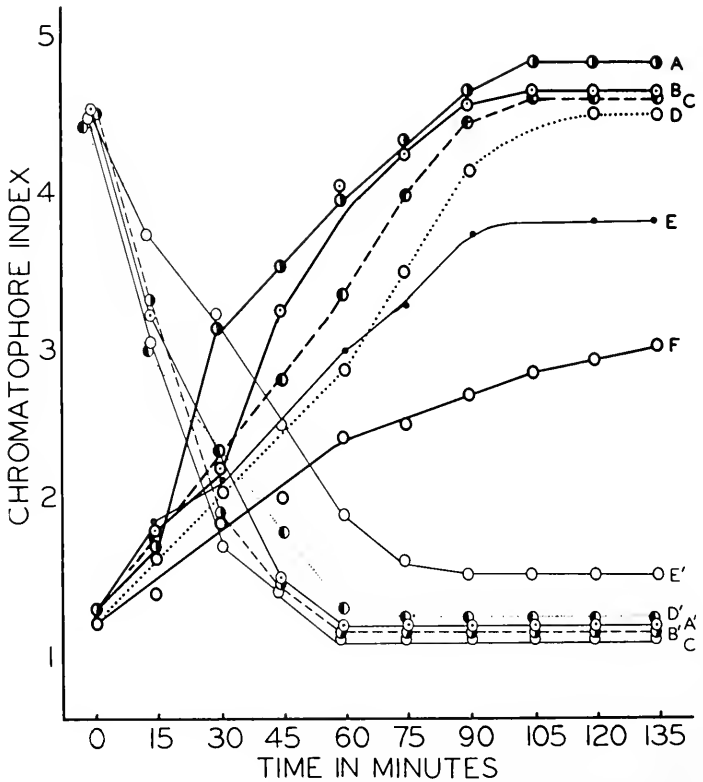

FIGURE 5. Effect of changes in concentration on the rate and magnitude of responses of the red chromatophores of *Cambarus* induced by extracts prepared from the sinus glands, optic tracts and cerebral ganglia of *Trachelipus*. (A—conc. 15; B—conc. 10; C—conc. 5; D—conc. 2.5; E—conc. 0.5; F—conc. 0.25.) Similar effects induced by extracts prepared from the connectives and thoracic cords of *Trachelipus*. (A'—conc. 15; B'—conc. 10; C'—conc. 5; D'—conc. 2.5; E'—conc. 0.5.)

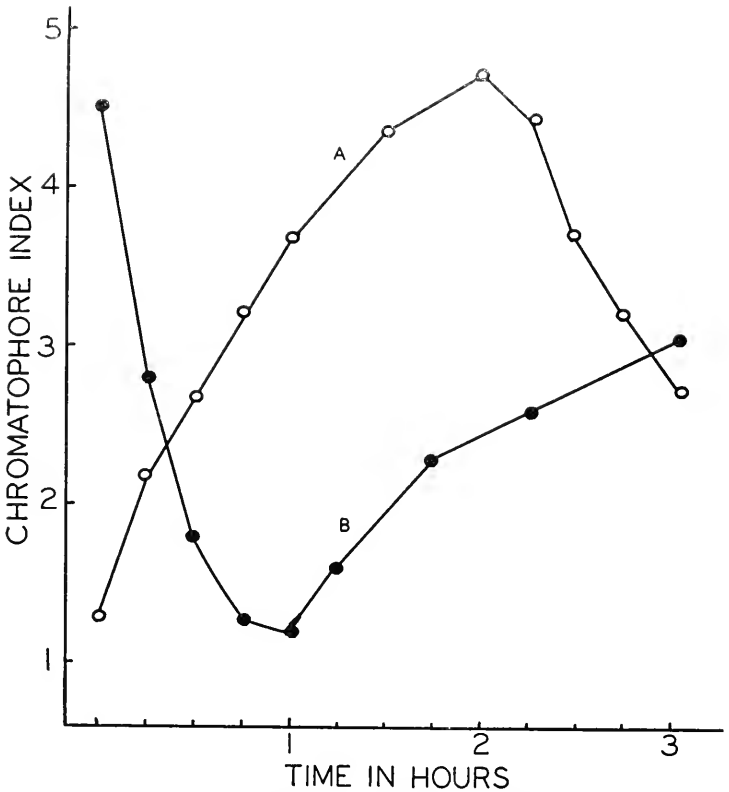

FIGURE 6. Average response of eight young *Cambarus* to injected extracts of (A) sinus glands, optic tracts, and cerebral ganglia in a conc. of 2.5; (B) connectives and thoracic cord in a conc. of 2.5.

of each extract was assayed upon isolated pieces of the carapace of *Cambarus*, the effective concentration included the specified structures from five animals.

An average of the results obtained from six identical experiments are presented in Figure 4. It can be seen that the sinus glands contain a substance which induces a dispersion of the red pigment of *Cambarus*. Whether the weak dispersing action of optic tracts and cerebral ganglia is due to the active production of this factor or merely to a diffusion through the tissue could not be determined by the method employed.

A similar number of experiments using extracts of the connectives or any segment of the thoracic cord demonstrated a concentration of the red pigment (Fig. 4). Since the individual fragments elicted approximately the same responses the substance controlling the concentrating action would seem to be relatively uniform in its distribution throughout the various regions of the cord.

3. Effect of concentration

In order to determine the relative effectiveness of the two chromatophorotropic factors, assays were made of various concentrations of the organs demonstrated

to contain each factor. Since 0.5 cc. was used in each assay, an extract prepared from the structures of a single animal will be designated as having a concentration of 1. Extracts were prepared of the sinus glands, optic tracts and cerebral ganglia in effective concentrations of 15, 10, 5, 2.5, 0.5, and 0.25. Another series of extracts was prepared and included connectives and thoracic cords in effective concentrations of 15, 10, 5, 2.5, and 0.5.

The results obtained from the two series of experiments are shown in Figure 5. It can be seen that above a concentration of 2.5 there is very little increase in the activity of either the dispersing or the concentrating factor. Increasing the concentration above this point results in a slightly increased rate of activity while the magnitude of the response is not appreciably altered in either case. Decreasing the concentration below this point, however, results in a decrease in both the rate and magnitude of response. Although variations in the potency of extracts at low concentrations cannot be completely explained at this time, it is possible that extracts prepared from light-adapted animals contained slightly more of the factor inducing dispersion than did extracts prepared from dark-adapted animals.

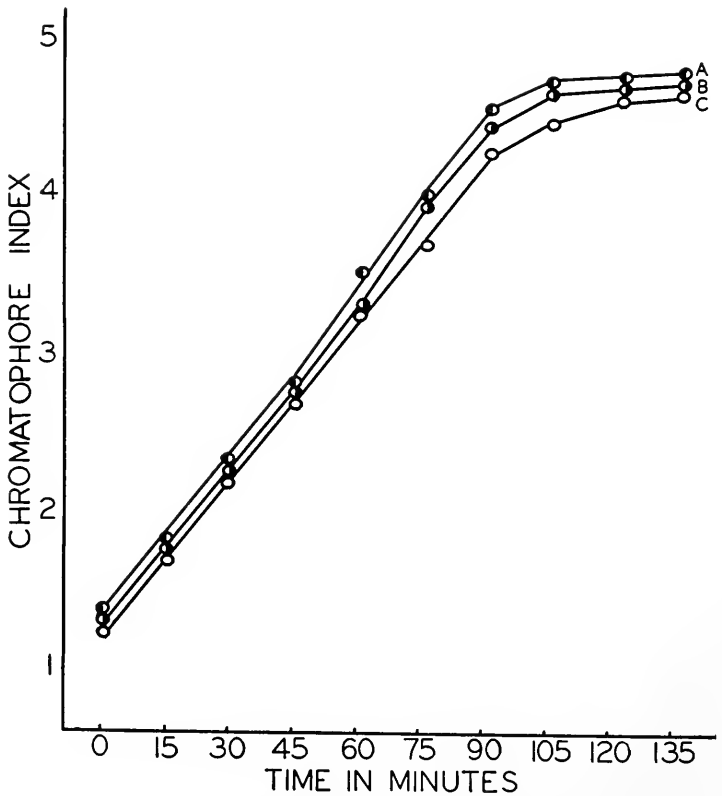

FIGURE 7. Comparative dispersing activity of extracts prepared from: A—sinus glands and entire nervous systems; B—sinus glands, optic tracts and cerebral ganglia; C—sinus glands alone. All extracts were prepared in a concentration of 5.

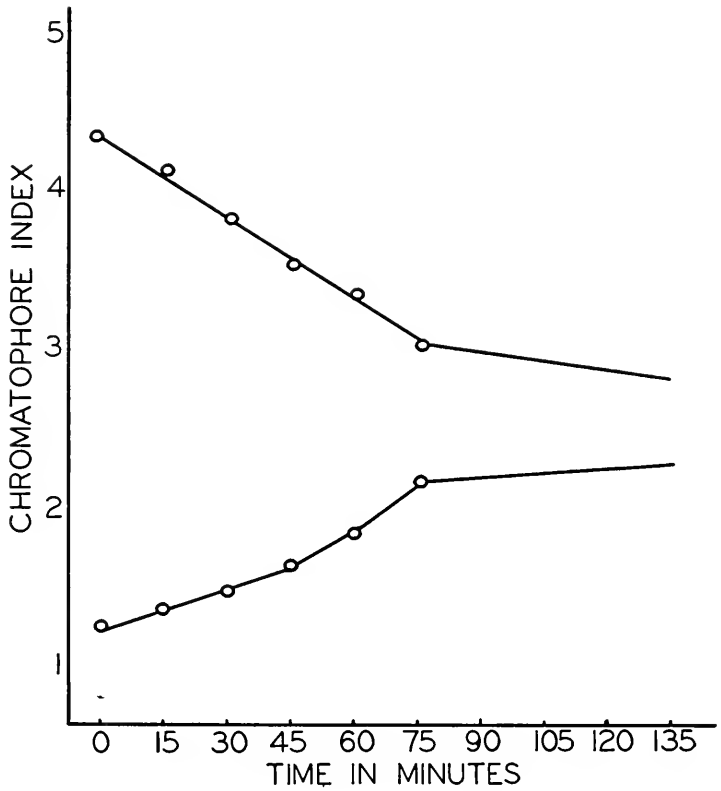

FIGURE 8. Dual response of the red chromatophores of *Cambarus* following perfusion with dilute (conc. 0.25) extracts of sinus glands plus the entire nervous system of *Trachelipus*.

A comparison of the two families of curves obtained (Fig. 5) indicates that the potency of the dispersing factor decreases more rapidly with decreasing concentrations that does that of the concentrating factor. A difference in the rate of activity of the two factors was also noted, since, within the range of concentrations studied, maximum dispersion was never attained in less than 90 minutes while complete concentration could be achieved in less than one hour. These two observations suggest that the factor eliciting concentration is stronger than that inducing dispersion. However, when extracts prepared from the entire nervous system including the sinus glands were assayed, only a dispersion of the red pigment was achieved. These conflicting observations suggest the existence of an antagonistic action between the two factors.

Since these studies, made upon isolated pieces of the branchiostegite, gave no indication of the duration of the responses obtained, eight young intact *Cambarus* were injected. Light-adapted *Cambarus* were injected with an extract of sinus glands, optic tracts and cerebral ganglia, while eyestalkless *Cambarus* were injected with extracts of connectives and thoracic cords. Both extracts were prepared in a concentration of 2.5. The responses obtained (Fig. 6) are of es-

sentially the same rate and magnitude as had previously been attained by the chromatophores of isolated pieces of the carapace. With respect to duration, the concentrating factor appeared to have a slightly more lasting effect than did the dispersing factor.

4. Antagonistic action

In sections 2 and 3 it was demonstrated that extracts of the sinus glands alone, or of the sinus glands, optic tracts and cerebral ganglia, induced only a dispersion of the red chromatophores of *Cambarus* while extracts of the connectives and thoracic cord induced a concentration of the same pigment. It was also shown that extracts of the entire nervous system (including the sinus glands) produced only a dispersing effect. The comparative dispersing action of the three types of extracts containing sinus glands, in a concentration of 5, is shown in Figure 7. These responses indicate that the concentrating factor in the nerve cord is prevented from eliciting a response when in the presence of high titers of the dispersing factor of the sinus gland. It is also apparent that the nervous

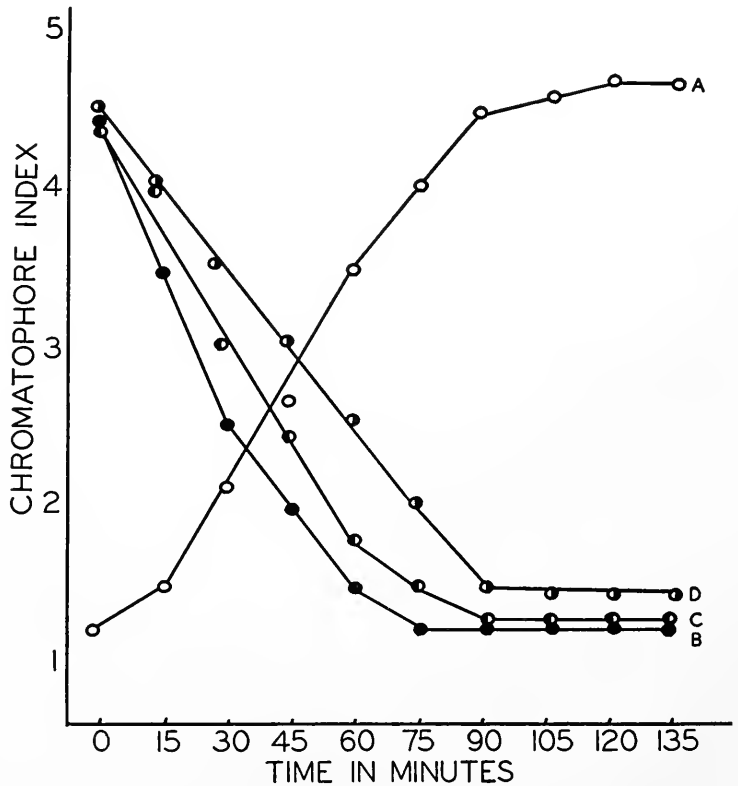

FIGURE 9. Further demonstration of the mutual antagonism existing between two chromatophorotropins of *Trachelipus*. A—dispersing factor alone (conc. 5). B—concentrating factor alone (conc. 5). C—concentrating and dispersing factor in a ratio of 4:1. D—concentrating and dispersing factor in a ratio of 3:2.

system, with the exception of the optic tracts and cerebral ganglia, does not contribute to the dispersing activity to any appreciable degree.

When the effects of very low concentrations of extracts were studied it was found that extracts of sinus glands, optic tracts and cerebral ganglia (conc. 0.25) produced only a dispersing effect, irrespective of the initial state of the chromatophores. On the other hand, extracts of the sinus glands plus the entire nervous system (conc. 0.25) exerted both a concentrating and a dispersing influence (see Fig. 8) dependent upon the initial state of the chromatophores. These results give support to the evidence that two factors are involved, since both types of response were elicited by the same extract when employed in low concentrations. The differential effects observed would not seem to be due to differing concentrations of the same substance.

In an attempt to determine the degree of antagonism existing between the two factors, extracts were prepared in a concentration of 5, of the concentrating factor and of the dispersing factor. These extracts were then mixed in various concentrations (4:1 and 3:2) and their effect determined. Figure 9 illustrates the responses which were obtained. It can be seen that when the concentration of the concentrating factor exceeds that of the dispersing factor, only the effect of the former is evident. The antagonism shown to exist between the two factors demonstrates that high concentrations of either factor will inhibit the effects of the more dilute factor. When both factors are present in small quantities, each produces a weak response and the chromatophores affected reach an intermediate condition.

5. Responses of Trachelipus

Since considerable technical difficulty is associated with the injection of *Trachelipus,* the results obtained from these experiments cannot be considered as conclusive. Due to the decreased viability of the animals and to the difficulty of observation following injection, the responses could not be readily interpreted. In general, the following effects were initiated.

Injection extract	Response of *Trachelipus*
Cambarus sinus glands	darkening
Trachelipus sinus glands	lightening
Trachelipus nerve cords	darkening

Although the results obtained must be considered as inconclusive, they suggest that the responses of the chromatophores of *Trachelipus* are directly opposite to those of the red chromatophores of *Cambarus.*

Discussion

The sinus glands of *Trachelipus* have been shown to contain a substance inducing a dispersion of the red pigment of the decapod *Cambarus,* and possibly a concentration of the melanin of the isopod *Trachelipus.* A dispersion of the red pigment of the decapod *Leander* following injection with extracts of the entire heads of terrestrial isopods *Mesidothea, Porcellio,* and *Oniscus* has previously been reported by Ståhl (1938). On the other hand, concentration of the melanin of

the isopod *Idothea* following injection of head extracts of the terrestrial isopod *Armadillidium* was observed by Okay (1945).

The results presented here indicate that the sinus glands at least of terrestrial isopods, although anatomically very similar to those of decapods, elaborate a substance inducing effects opposite to those elicited by extracts of the eyestalks of *Palaemonetes*-like decapods.

It has been shown by previous work (Koller and Meyer, 1930; Suneson, 1947; Carstam and Suneson, 1949) that eyestalk extracts of decapod Crustacea induce melanin dispersion in certain isopods. Evidence presented in this work, while only suggestive, indicates that the response in *Trachelipus* to *Cambarus* extracts is therefore like that of *Idothea*. In this respect, the isopods examined resemble the brachyuran Crustacea. This suggests an apparent difference in the reactive system as well as in the chromatophorotropins elaborated by the sinus glands of the two groups. However, there would appear to be a functional similarity, since extracts of the sinus glands of either group, when assayed upon their normal reactive system, are found to induce a concentration of the predominant pigment type.

The presence of a second and antagonistic hormone controlling the melanophores of isopods was suggested by Smith (1938) working with *Ligia oceanica*. He concluded that the background responses of *Ligia* are dependent upon the presence of two antagonistic hormones, one inducing a dispersion of the melanin, and the other a concentration of the same pigment.

Extracts of the connectives and thoracic cord of *Trachelipus* were found to induce a concentration of the red pigment of *Cambarus*, and possibly a dispersion of the melanin of *Trachelipus*. This factor, which induces an effect opposite to that produced by extracts of the sinus gland, is almost uniformly distributed throughout the connectives and ventral nerve cord.

A mutual antagonism has been shown to exist between the two chromatophorotropins of *Trachelipus*, at least in respect to their effect upon the red pigment of *Cambarus*. The direction and magnitude of the response is the result not only of the concentration of each factor, but, more directly, of the ratio between these concentrations. In this way a sufficient excess of either factor prevents the more dilute factor from eliciting a response. On the other hand, very dilute extracts known to contain small quantities of both factors were observed to induce responses in either direction dependent upon the initial condition of the chromatophores. The chromatophores perfused with these extracts attained a final condition of intermediate dispersion. Extracts prepared from the entire nervous system, including the sinus glands, have been shown to induce only a dispersing effect at high concentrations, while weak responses in both directions were elicited by dilute extracts. The rate of response is determined to a considerable degree by the concentration of the extract, but again is more closely correlated with the ratio between the two antagonistic factors present within a given extract. In regard to this point, it should be noted that the subesophageal ganglion and the connectives are located within the head of terrestrial isopods and, therefore, extracts prepared from the entire head would be expected to contain both factors. Due to the observed antagonism between the two factors, the weak activity of the optic tracts and cerebral ganglia does not necessarily preclude the possibility that both factors may be present within these tissues.

The effect which these observed properties of the two chromatophorotropins have upon the normal responses of the isopod must remain, for the present, hypothetical. It is interesting to note, however, the close correlation between the two chromatophorotropins of *Trachelipus*. Should this second factor be subsequently shown to exist within the nervous systems of isopods in general, the dispersion of the melanin observed to occur following the stimulation of the dorsal ocelli (Smith, 1938) may be due to the elaboration of high titers of this substance.

The identity or lack of identity of this factor with the *Crago*-darkening hormone found to be uniformly distributed throughout the central nervous system of *Idothea* (Brown and Saigh, 1946) also remains to be determined.

Although the observed chromatic responses of *Trachelipus* were slight, they appear to follow the same pattern as those observed in other species of isopods. However, the slight physiological color changes induced either by alterations in light stimulus or by the injected extracts could not be the sole explanation of the wide variations in color existing among these animals. Morphological color changes must necessarily assume an important role in the coloration of the adult animal. On the other hand, the ability of larval *Trachelipus* to exhibit strong physiological responses to light suggests that the loss of reactivity of the pigmentary system is a function of the progressive fusion of the chromatophore network with age.

In general, it can be stated that the progressive decrease in the reaction of the pigmentary system of *Trachelipus* is not accompanied by a corresponding loss of the chromatophorotropins of the sinus glands and central nervous system. It is possible that the hormones controlling physiological color changes may also be of functional significance in the control of the morphological color changes observed in these animals.

Summary

1. The terrestrial isopod *Trachelipus rathkei* was found capable of exhibiting weak physiological color changes in response to changes in background. Under the stimulus of diffuse light, animals maintained upon a light background became lighter than animals maintained in the dark. Under the stimulus of more intense light, the animals darkened even upon a light background.

2. Structures showing a marked resemblance to the sinus glands of decapods are located at the distal end of each optic tract and agree in position with the sinus glands of other isopods.

3. Extracts of the sinus glands of *Trachelipus* induce a strong dispersion of the red pigment of *Cambarus*.

4. Extracts of the optic tracts or cerebral ganglia induced a weak dispersion of the red pigment of *Cambarus*, while extracts of the connectives or any segment of the thoracic cord induced a strong concentration of the same pigment.

5. A comparison of the influence of change in concentration upon the effectiveness of the two chromatophorotropins indicates that above a minimal concentration, the rate of response is only slightly increased, whereas the magnitude of response remains unaltered. Below this level, both the rate and magnitude of response decline with decreasing concentrations.

6. An antagonistic action was found to exist between the two chromatophorotropins such that high concentrations of either factor prevented the more dilute

factor from exerting its influence. When very dilute extracts known to contain small quantities of both factors were assayed, both responses were elicited, such that the chromatophores assumed a condition of intermediate dispersion.

7. The response of *Trachelipus* to injected extracts proved inconclusive. The initiated responses suggest that the reactions of the pigmentary system of *Trachelipus* to injected extracts are opposite to those of *Cambarus*.

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VARIATIONS IN TEMPERATURE AND LIGHT RESPONSE WITHIN A PLANKTON POPULATION

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Zooplankton tends to concentrate about a particular depth, this depth being characteristic of certain external conditions, of the species concerned, and frequently also of the stage of maturity and brood of the organism. Diurnal and seasonal rhythms in external conditions are frequently reflected in vertical migrations of the animals, and geographical gradients in such factors as light and temperature have been shown to be associated with correlated changes in the vertical distribution of the species concerned. There is an extensive literature on the subject, that part pertaining to the Crustacea having been well reviewed by Cushing (1951). He describes also the experiments on tropisms, taxes, etc., which have been made in an attempt to understand the behavior observed in the field. Although widely differing and sometimes contradictory results have been obtained by different workers, it appears that such differences are characters of particular species rather than of whole groups. So far as it is possible to visualize a typical copepod, etc., it appears that the general pattern of behavior is much the same at least in copepods, chaetognaths, siphonophores and pteropods (Moore *et al.*, 1953). With the exception of larval fishes, which rarely exhibit diurnal migration, it is probable that the principles are generally applicable to such zooplankton as are capable of vertical movements.

Cushing shows how light and temperature are generally accepted as the major environmental factors controlling vertical distribution, with only a minor part played by salinity, CO_2 and other factors. There are many instances where, during most of the 24 hours, the plankton migrates vertically as if it were following a level of optimal illumination. On the other hand, there are instances of a thermocline proving a barrier to the vertical movement of a species, and the upper or lower limits of some species have been shown to follow closely the seasonal movements of particular isotherms (Nikitine, 1929). Vertical movements in response to light and temperature stimuli have been demonstrated experimentally, although the results sometimes appear to conflict with observations of natural behavior. It has also been shown that the nature of the response to light may be modified by the temperature.

Laboratory experiments, at least with marine zooplankton, suffer from the difficulty of reproducing natural conditions. Comparatively few marine zooplankton have been successfully kept for any long period in the laboratory. The ecologist has the uncomfortable feeling that the more successful laboratory species are the least typical, and can be reared only because of this. There are factors such

[1] Contribution No. 756 from Woods Hole Oceanographic Institution; Contribution No. 137 from the Marine Laboratory, University of Miami.

as food, previous treatment, internal rhythms and others which may well modify behavior. On the other hand, any attempt to analyze behavior under natural conditions suffers from the large number of simultaneously varying factors, none of which can be controlled. It has the advantage, though, that the animals may be assumed to be behaving typically, and that, particularly in the tropics, a large number of species may be studied, so that the pecularities of atypical species will not seriously affect the observations. The present paper is a study of the relative responses to temperature and light of some zooplankton in the Florida Current, and how these vary in a vertical section through the plankton population.

Material and Methods

The material was collected in a region of the Florida Current lying from ten to forty miles east of Miami, Florida. This is ocean water with isotherms sloping very steeply across the current so that a wide range of temperatures is available at any depth. Seasonal variations in the current pattern and velocity result in a wide range in conditions at any one station throughout the year. An account of the hydrographic conditions at the ten mile station has already been published by Miller *et al.* (1953) and the methods used in collecting plankton were described in the same paper. The material was drawn from samples, collected for other purposes, in part under a joint program of the Marine Laboratory of the University of Miami and the National Geographic Society, and in part by the Marine Laboratory under contract number NObsr-57146 for the Office of Naval Research. The analyses were carried out under the latter contract and under Bureau of Ships contract number NObsr-43270 at Woods Hole Oceanographic Institution. The siphonophore examination was carried out in part by the author and in part by D. C. Roane (1954). The chaetognaths were examined by H. Owre and her results will be included in a doctorate thesis at the University of Michigan. To all of these the author wishes to express his indebtedness.

The present work is restricted to the period within about two hours either side of noon, when the illumination is not changing rapidly, and it is assumed that the plankton has had time to stabilize at the depth at which it is found. Vertical temperature sections were available for each station. The vertical distribution of illumination was calculated by methods previously used (Moore, 1950) and from these the depths of selected isotherms and isolumes were obtained. For each station, the percentage vertical distribution of each species was calculated as follows. First, the count from each of the oblique net hauls was scaled to the equivalent of a tow of one mile, the factor being obtained from the trace on the depth-distance recorder towed along with the nets. Each count was assumed to be applicable to a column of water reaching from half way between the mid-depth of that tow and the mid-depth of the tow above it to half way to mid-depth of the tow below it. The top tow was assumed to terminate at the surface, and the lowest at the deepest point shown on its trace. Integration of the whole column was performed by multiplying each count by the length of water column to which it applied, and adding the products. By the use of a calculating machine, the levels were found corresponding to 10%, 20%, etc., of this total. These, referred to as the 10% level, etc., are the depths above which this percentage of the species occurred on the particular occasion. Values from 10% to 90% were

calculated, but the 0% and 100% levels were not sufficiently clearly defined to be usable.

The following species, used in previous work, were present in sufficient numbers to be significant. It should be noted that, with the inclusion of more stations, some of the regressions obtained differ from ones previously quoted.

SIPHONOPHORA

Chelophyes appendiculata (Esch.)
Diphyes bojani (Esch.)
D. dispar Chamisso & Eysenhardt
Eudoxoides mitra (Huxley)
E. spiralis (Bigelow)
Abylopsis eschscholtzii Huxley
A. tetragona Otto.
Bassia bassensis (Quoy & Gaimard)

CHAETOGNATHA

Sagitta enflata Grassi
S. hexaptera D'Orb.
S. lyra Krohn
S. bipunctata Quoy & Gaimard
S. serratodentata Krohn
S. minima Grassi
S. decipiens Fowler
Pterosagitta draco (Krohn)
Krohnitta subtilis (Grassi)

The various species characteristically occupy different levels. In order that the data for the various species could be combined, the mean 10%, 20%, etc., levels were calculated for each species, these being the means of all stations. For each station, the results were then expressed as deviations from the mean on that occasion, a positive value being deeper, and a negative shallower. The deviation of a typical siphonophore on that occasion was the mean deviation for the eight species available, and that of a typical chaetognath was the mean for nine species. For comparison, the levels of the 15° C. isotherm and of the 10^{-2} isolume were similarly expressed as deviation from their respective means.

After testing for correlations between the deviations of the plankton and the isolume and isotherm, and finding that these were significant, partial regressions were calculated with the results shown in Figures 1 and 2. The regression coefficients show the extent of vertical movement of the plankton in relation to a one-meter shift in the 15° C. isotherm or 10^{-2} isolume. In all cases the coefficients are positive, that is to say, the plankton movement is in the same direction as the movements of the isotherm and isolume.

For simplicity it may be assumed that the temperature and light graphs in the above figures are straight lines. If the regressions of plankton movements on isotherm and isolume movements are referred to as temperature and light responses, then the regressions of the responses on percentage level can be calculated. From these, the levels occupied by the typical siphonophore or chaetognath may be predicted for any given hydrographic conditions for this region. As a verification of the goodness of fit between such predicted levels and those actually observed, six stations were selected which offered a particularly wide range of hydrographic conditions. Figures 3 and 4 show the comparison of the values from the 10, 50 and 90% levels as predicted and as observed. These cover a more than five-fold range of depth under different conditions, and the agreement appears to be good.

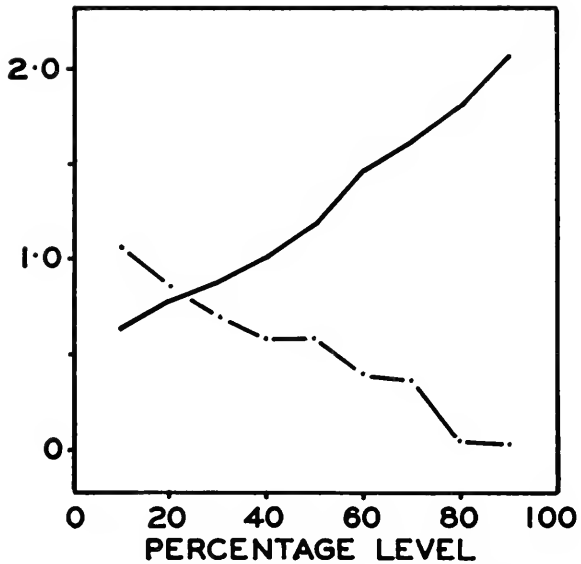

FIGURE 1. Typical siphonophores. Partial regression of plankton movement on movement of the 15° C. isotherm (whole line) and of the 10^{-2} isolume (broken line), and the variation of these regressions with percentage level; 0% represents the top, and 100% the bottom of the plankton column.

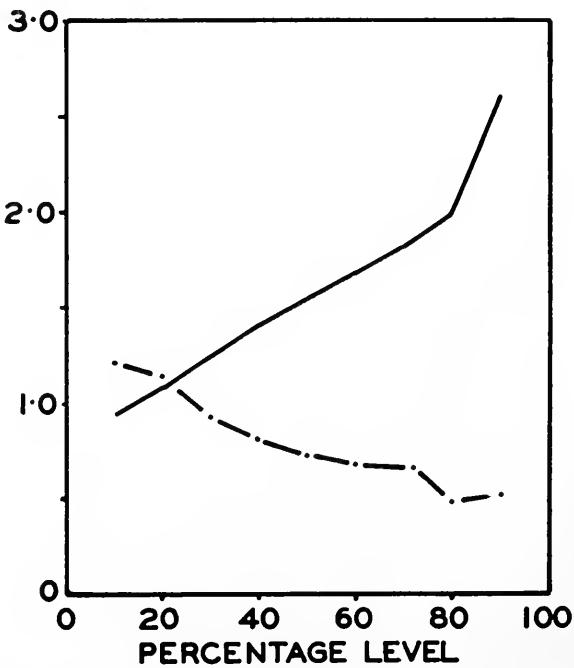

FIGURE 2. Typical chaetognath regressions indicated as in Figure 1.

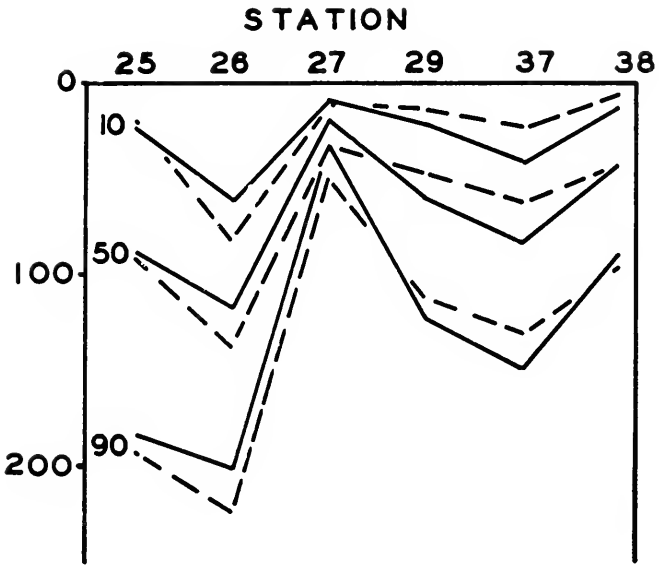

FIGURE 3. Typical siphonophore. Comparison of the observed depths, in meters, of the 10%, 50% and 90% levels at six stations (whole lines) with the depths predicted by the regressions referred to in the text (broken lines).

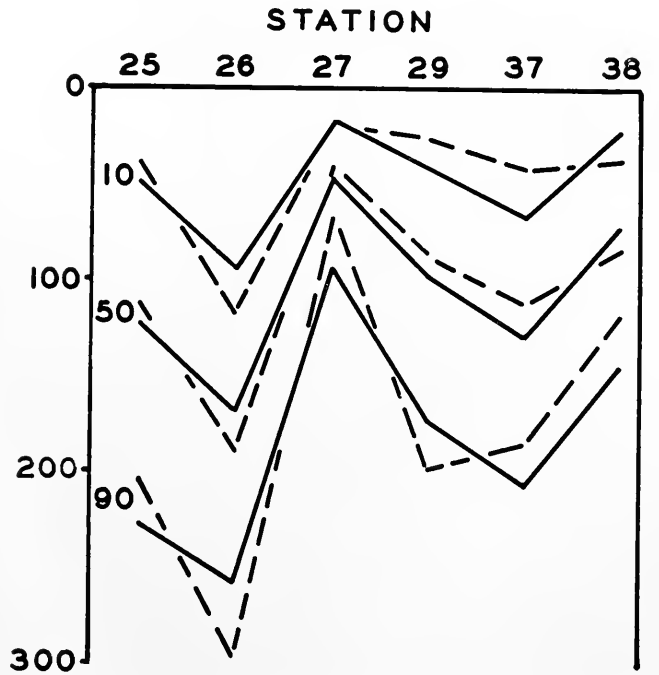

FIGURE 4. Typical chaetognath comparisons indicated as in Figure 3.

Discussion

Cushing (1951) summarizes many examples of plankton whose day level varies on different occasions. Where these variations have been correlated with changes in an environmental factor, this has usually been illumination. The Florida Current provides an unusually wide range of temperature variation at depth, and so may be particularly advantageous for demonstrating the role of temperature. Further, most previous workers have been concerned with the level of maximum concentration of the plankton and have not differentiated between the behaviors of the shallower and deeper members of the population of a given species. In an earlier study (Moore, 1953; Moore *et al.*, 1953) in which considerably fewer data were available than in the present work it was shown that there was an increase in temperature response and a decrease in light response in the lower levels of the plankton as a whole. The same trend was indicated in the four separate groups studied, but many of the values could not be considered statistically significant. J. B. Lewis (in press) has studied the euphausids of this area, and shown that illumination plays the major part in controlling both day level and vertical diffuseness, with little or no demonstrable effect of temperature. Roane (1954) compared the average day level of a group of siphonophores at two stations in the Florida Current and found these to be 79 and 253 meters. The corresponding extinction coefficients were 0.062 and 0.051 while the depths of the 15° C. isotherms were 151 and 619 meters, respectively. It seemed probable that the great range in depth of the animals was associated with both illumination and temperature differences. The present work shows that the observed vertical distributions can be accounted for if both temperature and light play a part in regulation of level, and if it is assumed that their effects are additive.

Two characteristics of the graphs in Figures 1 and 2 call for explanation. The temperature and light responses change more or less linearly with percentage level, and the regressions of plankton movement on isotherm and isolume movement may have values well in excess of unity. The author has, so far, been able to find little information on how the nature or strength of a reaction varies among the individuals in an invertebrate population. The frequency distribution may perhaps be expected to follow either a symmetrical or a skewed normal curve. The latter is true in what is perhaps a parallel case, the variations in rate of hemolysis of red cells in a sample (Ponder, 1930, 1932). Too much reliance cannot be placed on the exact form of the present curves, and unfortunately the ends of the curves are lacking, even the 10% and 90% values probably being based on too small counts to have much significance. It seems clear, though, that they cannot represent a frequency distribution rising to a marked peak at any particular value. Subsequent work on single species suggests that they may, with more data available, prove to be flat-peaked curves. Another explanation of their shape lies in the probability that, as shown below, temperature and light are not sufficient alone to account for the observed behaviors, and a further environmental factor will have to be considered.

The extent to which the regression coefficients may exceed unity can hardly be attributed to error due to inadequate data. They indicate that the animals may respond to a change in depth of an isotherm or isolume by moving considerably further. In other words, although the movements of the animal are in such direc-

tion as could maintain them at a constant temperature or illumination, they do not, in fact congregate at the depth corresponding to such a constant value. Finally, very much poorer correlations are obtained if an attempt is made to relate the depth at which the animals are found to *in situ* temperatures and illuminations, than if the correlations are made between the depth changes of the animals, the isotherms and the isolumes. All this suggests that a third factor, related to depth, and which we tentatively suggest may be pressure, must be included in the complex. Although pressure has received much less consideration in this connection that the other two factors, there is some evidence that it may affect vertical movement of zooplankton (Hardy and Bainbridge, 1951).

The similarity of Figures 1 and 2 gives support to the statement already made that there may be a pattern of depth control mechanism typical of widely different animals. Within the species which we have grouped together, specific differences may be expected both in the strengths of the responses and in the relative response to light and temperature. Such would be in agreement with the very different behavior patterns observed in the many forms which have been worked with. We have found a marked difference in response in different parts of a population. There is no evidence as to whether this represents permanent differences among the individuals or whether it represents a sorting, resulting from varying responses, of individuals whose responses fluctuate continually. Differences between individuals may be expected, but so may changes in the reactions of a single individual. The latter might be more or less random, they might vary in relation to previous condition or to some control such as a feeding cycle, or they might vary with an inherent diurnal or other rhythm. Whatever may prove to be the case, a significant point seems to emerge in connection with the interpretation of experiments with plankton. Firstly, if the experimental animals are not always collected from the same part of the plankton column then differences may be expected in the nature of the experimental behavior. Secondly, experiments made with animals taken from the top or bottom of the population may yield results which are not applicable to the main concentration of the population.

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MITOCHONDRIAL STRUCTURE IN PARAMECIUM AS REVEALED BY ELECTRON MICROSCOPY

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The structure of the mitochondria as revealed by electron microscopy has received considerable attention recently, and from these studies there have emerged several general descriptions of the ultrastructure of these cell particulates. Common to all the descriptions is the belief that mitochondria possess a membrane which is distinct from the internal matrix and which constitutes the external edge of the structure. The structures found in the interior of mitochondria have been variably interpreted as having ridges arising from the membrane projecting into a central matrix (Palade, 1953), parallel plates or membranes extending from side to side (Sjöstrand and Rhodin, 1953), concentrically arranged lamellae (Beams and Tahmisian, 1954), or parallel rods (Glimstedt and Lagerstedt, 1953). Our study of mitochondrial structure in *Paramecium,* based upon sections manipulated in a manner slightly different from those in most of the above studies, leads us to conclusions concerning structure which are different from any presented up to this time. A preliminary report of these has appeared (Powers, Ehret and Roth, 1954).

Materials and Methods

1. Organisms

Clonal cultures of two species of *Paramecium* were employed. The principal stocks examined were 51 VIII kk (Sonneborn) of *P. aurelia* and So 6 C (Chen) of *P. bursaria.* The organisms generally were used near the end of a growth cycle, very few food vacuoles being present in the cells at the time of fixation.

2. Fixation

The organisms, after having been concentrated by slow filtration through sintered glass, were fixed in 1% OsO_4, buffered at pH 7.4 with 0.045 *M* citrate-phosphate buffer (McIlvaine's), for 30 to 60 minutes at about 26° C. At the end of the fixation period the animals were carried through a series of fluids (without centrifugation) as follows: distilled water, 2 changes, 5 minutes each; 50% ethyl alcohol, 15 minutes; 75% ethyl alcohol, 30 minutes; one part absolute alcohol, one part methacrylate mixture, 30 minutes; methacrylate mixture, 3 changes, one hour each. The methacrylate mixture was 40% ethyl methacrylate and 60% n-butyl methacrylate with the hydroquinone inhibitor removed. After the third change, the plastic was irradiated with ultra-violet light for 12–16 hours at 40–45° C.; this treatment produced complete polymerization without the use of chemical catalysts.

3. Sectioning and mounting

Sections were cut with a glass knife in an International Minot thin-sectioning microtome equipped with a worm gear mechanism allowing a minimum thickness of 1/40 μ. Sections in the range 1/10 to 1/40 μ were used for observation. The solution used to float the sections was either 20% dioxane or 20% ethyl alochol. The sections were allowed to float on the solution for 10–20 minutes at 30–40° C. before being mounted on formvar-covered grids. Some of the sections were studied with no further preparations. Others were soaked briefly in toluene to remove the plastic. This treatment consisted of immersing the grid with the membrane-supported section for 20–30 minutes at room temperature.

4. Microscopy

An RCA type EMU-2A electron microscope was used. The microscope was equipped with the standard objective pole piece using a 60 μ objective aperture or with the wide-angle pole piece using rear focal plane objective aperture. The micrographs were taken at magnifications of 900–9200 diameters, further magnification being accomplished photographically.

Results

Identity of the particles

The particles studied here conform, in chemical characteristics, to descriptions of mitochondria given by other authors: they are osmiophilic and under certain physiological conditions they stain blue-green after Janus Green B, and pink after 2,3,5 triphenyl tetrazolium chloride. In addition they have been shown by Thomson (personal communication) to be associated with several oxidative enzymatic activities ascribed to mitochondria in other forms.

Size and shape

In crushed unfixed preparations under phase optics, the mitochondria appear as small spheroids about 0.7 μ in diameter. In 10% sucrose solution they also appear as spheroids. However, sections of mitochondria (Figs. 4–17) after osmic fixation appear to have been derived from cylinders with rounded ends, as well as from spheroids.

We believe that the spheroid bodies seen under phase optics in freshly crushed preparations may present the geometric shape of many of the particles in the living condition, because, as well as can be seen, the particles beneath the pellicle of the intact cell just before crushing appear spheroidal. If the spheroidal shape predominates in the living material, it is apparent that processing has caused a change in the shape of many of the particles from spheroids into cylinders and hyperboloids-of-one-sheet, with rounded ends, and with lengths about twice their widths. The volumes of the two structures are about the same (a cylinder 0.5 $\mu \times$ 1.0 μ, compared to a sphere 0.70–0.75 μ in diameter).

It is certain that fixation with OsO_4 results in some changes in size of organelles within these cells. The trichocysts, evident in Figures 2, 3, 4, and others, are larger than they are in the living cell. This fact is demonstrated by a comparison of Figure 1 with Figure 2. Figure 1 shows a group of trichocysts just

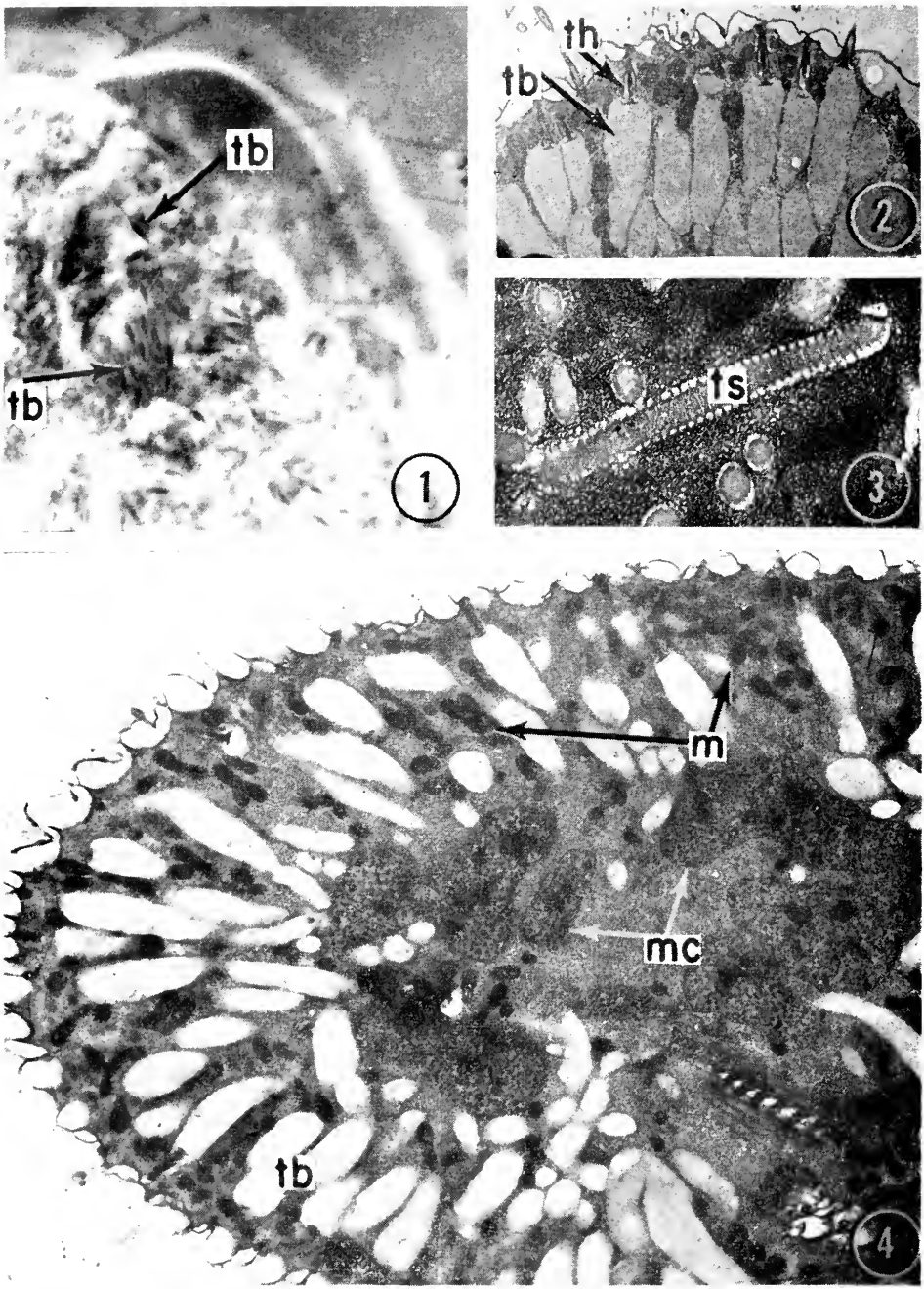


FIGURE 1. Phase contrast photograph of surface of compressed specimen of *P. aurelia* showing size of body of trichocyst relative to head in living condition. 1000 ×.

m, mitochondrion; mc, macronucleus; mcm, macronuclear membrane; mt, mitochondrial tubule; n, nucleolus; p, pore of mitochondrial tubule; pl, pellicle; pt, plastic; s, peritubular space; tb, trichocyst body; tc, trichocyst cap; th, trichocyst head; ts, trichocyst shaft ("exploded").

beneath the pellicle in a freshly crushed preparation. The tip length : body length ratio in this preparation, and in all others like it, is about 2 : 3. In the electron micrograph of sections of osmium-fixed material, the tip appears to retain its live dimensions, but the body, in all the preparations we have examined, is enlarged with a resulting ratio of 2 : 5 or more. In a few preparations complete shaft formation is seen even within the cell boundaries. So, as far as these structures are concerned, the fixation procedure is evidently not the best. In regard to others, however, the fixation procedure appears to be very good. The close adherence of the cytoplasm to the macronuclear membranes (Figs. 4, 5, 9) is in contrast to the situation observed after fixation of *Paramecium* by an agent such as warm Schaudinn's solution, which causes the appearance of an artificial vacuolar space separating the macronucleus from the cytoplasm. In regard to the mitochondria, it is probable that, in addition to the change in shape, some swelling of the structures occurred also, *i.e.*, in the undisturbed state the bodies may be slightly more compact.

Distribution

Figure 5 demonstrates that mitochondria in *P. aurelia* are, with certain exceptions, most abundant near the pellicle of the animal. The relationship between distribution of mitochondria and the reproductive stage of the cell will be discussed in a subsequent report.

Structure

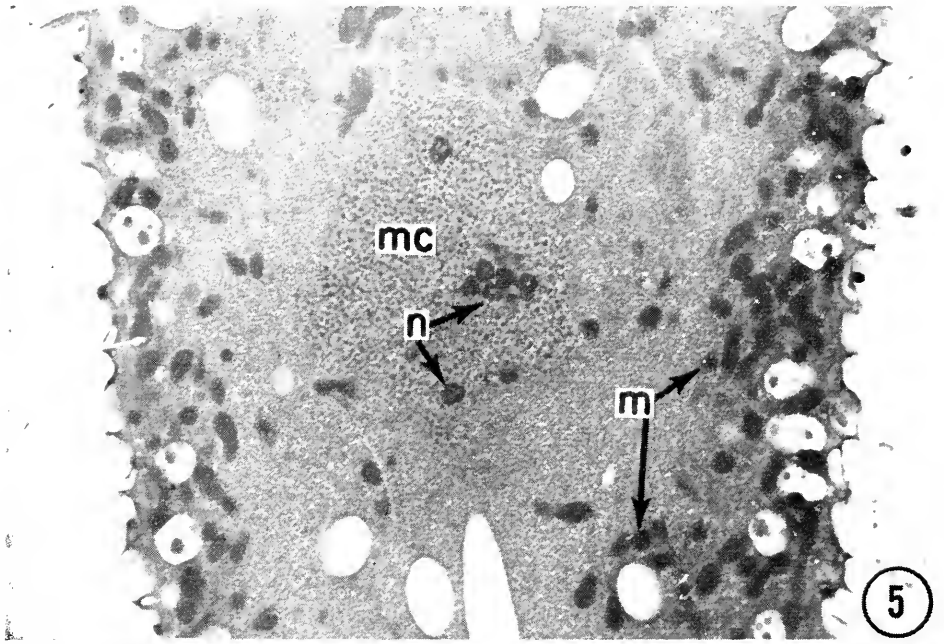
Figures 6–17 present the evidence for our idea of mitochondrial structure in *Paramecium*. Figure 6 is typical of the appearance of sections which are not soaked in toluene before examination. It may appear from this photograph that the mitochondrion is a sac bounded by a continuous membrane as postulated by others for metazoan and protozoan mitochondria. The central area (the "mitochondrial matrix" of other authors) appears clear and is approximately equivalent in density to the structureless ground substance representing the cytoplasm in these sections. Against this background, osmiophilic structures (the "microvilli" of Sedar and Porter, 1954) are found. Also in this section it is to be noted that the pellicle appears to be a double structure, and that the cytoplasm is homogeneous except for widely scattered dense granules.

Figure 7, from a section in which the plastic remains, demonstrates the same mitochondrial structure. It is presented to show in addition the appearance of the tip of the trichocyst and the surrounding cap when viewed in section with the plastic-in. This is to be compared with Figure 8, an adjacent section which was soaked in toluene for 30 minutes before examination. The tip of the trichocyst and the surrounding cap are sharply defined, in contrast to the fuzzy, ill-defined representation in Figure 7. Furthermore, no detail is visible in the space between the tip and the cap when the plastic is in, although threadlike structures extending between the tip and the cap are clearly visible after toluene treatment of the sec-

FIGURE 2. EMG (electron micrograph) of section of *P. aurelia*, demonstrating enlargement of trichocyst body relative to head after fixation with OsO_4. 5000 × Plastic removed.

FIGURE 3. EMG of fully extended trichocyst in cytoplasm. 10,000 ×. Plastic removed.

FIGURE 4. EMG of section of *P. aurelia*. Autogamous animal showing distribution of mitochondrial relative to the trichocysts and the macronuclear fragments. 4000 ×. Plastic removed.



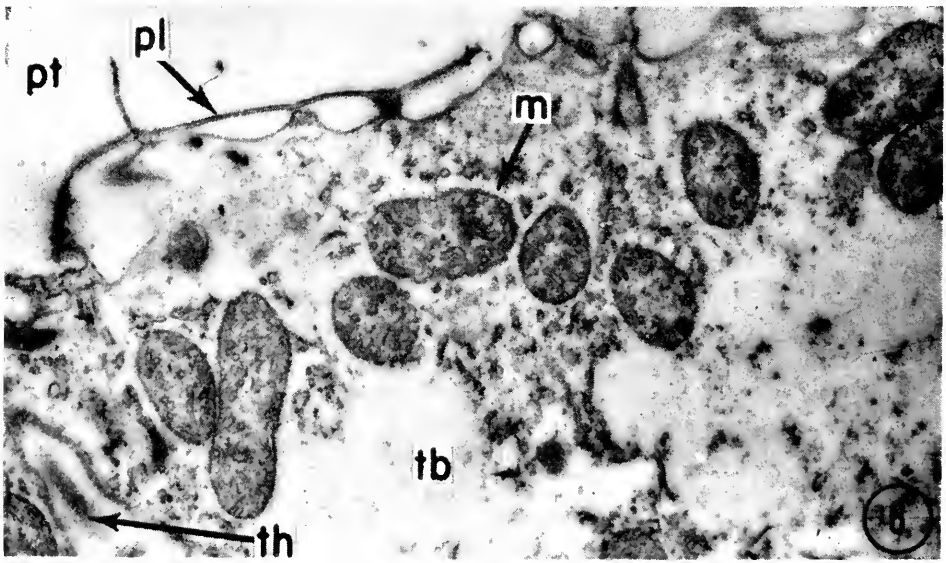


FIGURE 5. EMG of section of *P. aurelia* showing peripheral distribution of mitochondria in cytoplasm. Macronucleus containing nucleoli visible in center of section. 5000 ×. Plastic removed.

FIGURES 6 AND 7. EMGs of sections of *P. aurelia* with plastic remaining in. The cap, head, and body of the trichocyst are to be compared with corresponding structures in Figure 8. Note that the body of the trichocyst is hardly distinguishable from the plastic outside the pellicle. 20,000 × and 26,000 ×.

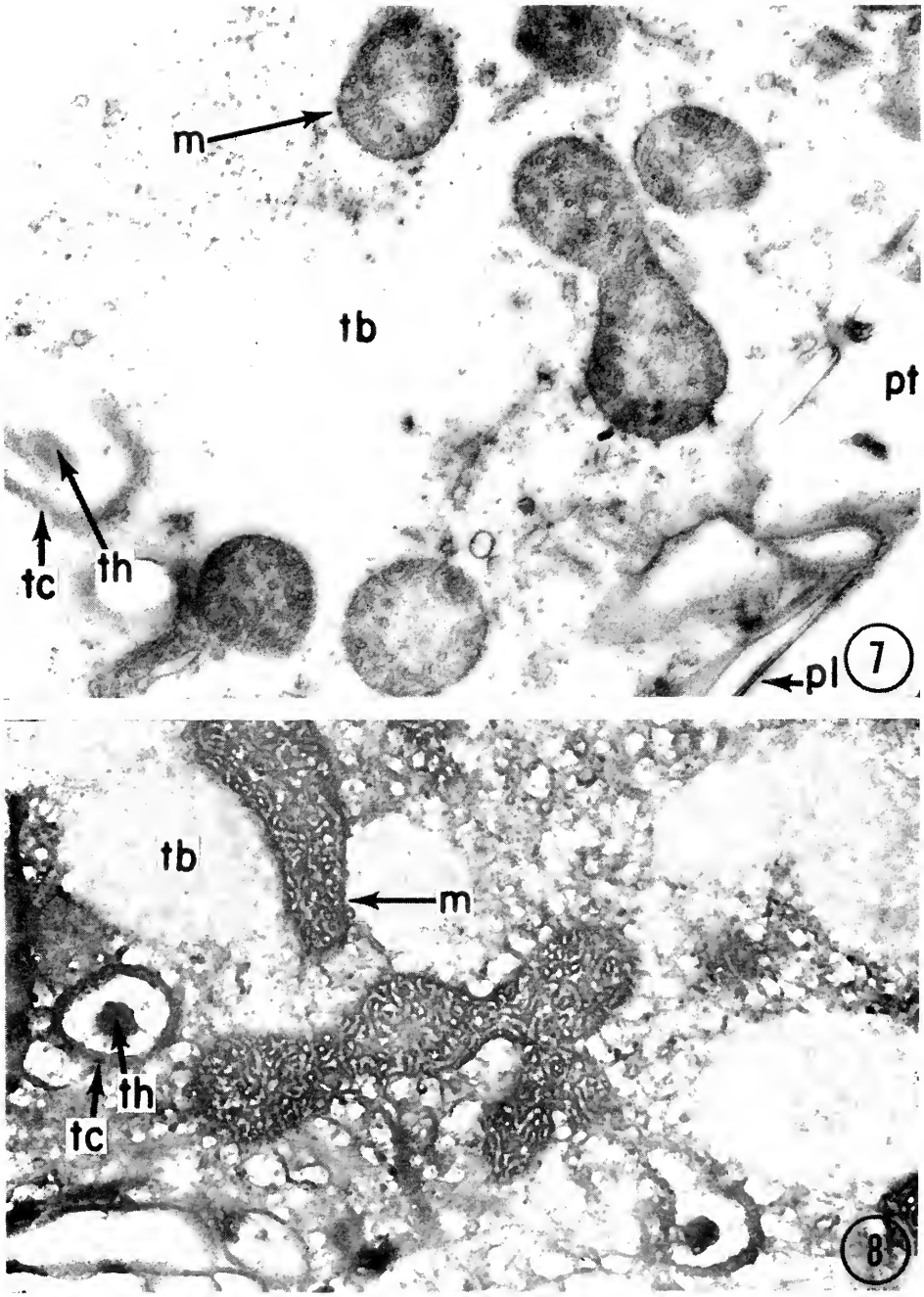


FIGURE 8. Section similar to those in Figures 6 and 7, except that toluene treatment preceded examination. This section came from the same block as those in the two previous figures. 26,000 ×. Plastic removed.

tion. The body of the trichocyst in Figure 7 is homogeneous and structureless; in Figure 8 the body is seen to have a grainy appearance. The ground substance of the cytoplasm in Figure 8, in contrast to Figure 7, is net-like, vacuolated, and contains particles of varying density (or osmium content). The frequency of occurrence of the densest particles indicates that they are identical with the cytoplasmic particles seen in Figure 7, but the net-like, less dense structures cannot be seen in Figure 7 because of masking by the plastic.

The mitochondria in Figure 8 are seen to consist of an osmiophilic material that is interrupted by numerous circular and elliptical interspaces. There is no external membrane distinct from this material. There is no clear central area but, rather, material that is less dense than some portions of the "outside edge" and material surrounding many of the interspaces. The more dense material about the spaces is continuous with the less dense material surrounding it.

Figures 9–17 are additional examples of sections through mitochondria demonstrating their ultrastructures. All the sections present essentially the same picture. There is an osmiophilic substance constituting the mass of the particle. This substance appears to be continuous throughout all sections except for very small interspaces. The interspaces appear as sharply defined circles, ellipses, or straight or contorted canals. Arrangements of the interspaces are not altogether random, for patterns are evident in the form of apparent concatenation of circles (Fig. 11), and whorled (Fig. 9) and anastomosing (Figs. 9, 10, 17) canals. All the sections show that the mitochondrion consists of this osmiophilic substance permeated by numerous small canals, irregularly but definitely arranged. The mitochondria of *P. bursaria* (Figs. 12, 13) appear more compact than those of *P. aurelia*, but otherwise show the same general structure.

The size of these interspaces was measured from greatly enlarged pictures. In a series of 30 circles, which were considered cross-sections of the interspaces, the mean diameter was 15 mμ with a standard deviation of 0.9 mμ. The range was 7.9 mμ to 25.2 mμ.

At first glance some of the sections (Figs. 16, 17) appear to show that the mitochondrion is surrounded by a membrane distinct from the interior, resembling the membrane of the macronucleus (Fig. 9). Closer examinations reveal, however, that the thick edge is clearly continuous with the material within the mitochondrion.

In many of the sections it can be seen that the interspaces are continuous with the cytoplasm, *i.e.*, an opening exists in the edge of the mitochondrion at that point. These holes are infrequent; they cannot be observed in most sections. However, numerous examples of them can be found and some are offered in Figures 14–17. Even when the plastic remains in the section, the structures visible in the matrix are occasionally seen to open to the outside (Fig. 7).

Discussion

The additional treatment of soaking the sections in toluene, which removes most of the plastic, reveals structure of the mitochondrion invisible in sections not so treated. On the basis of Roth's (1954) study of the effects of methacrylate on appearance of structure in sections, it is clear that the effect of the presence of the plastic is to mask certain detail in all parts of the cell, including the mito-

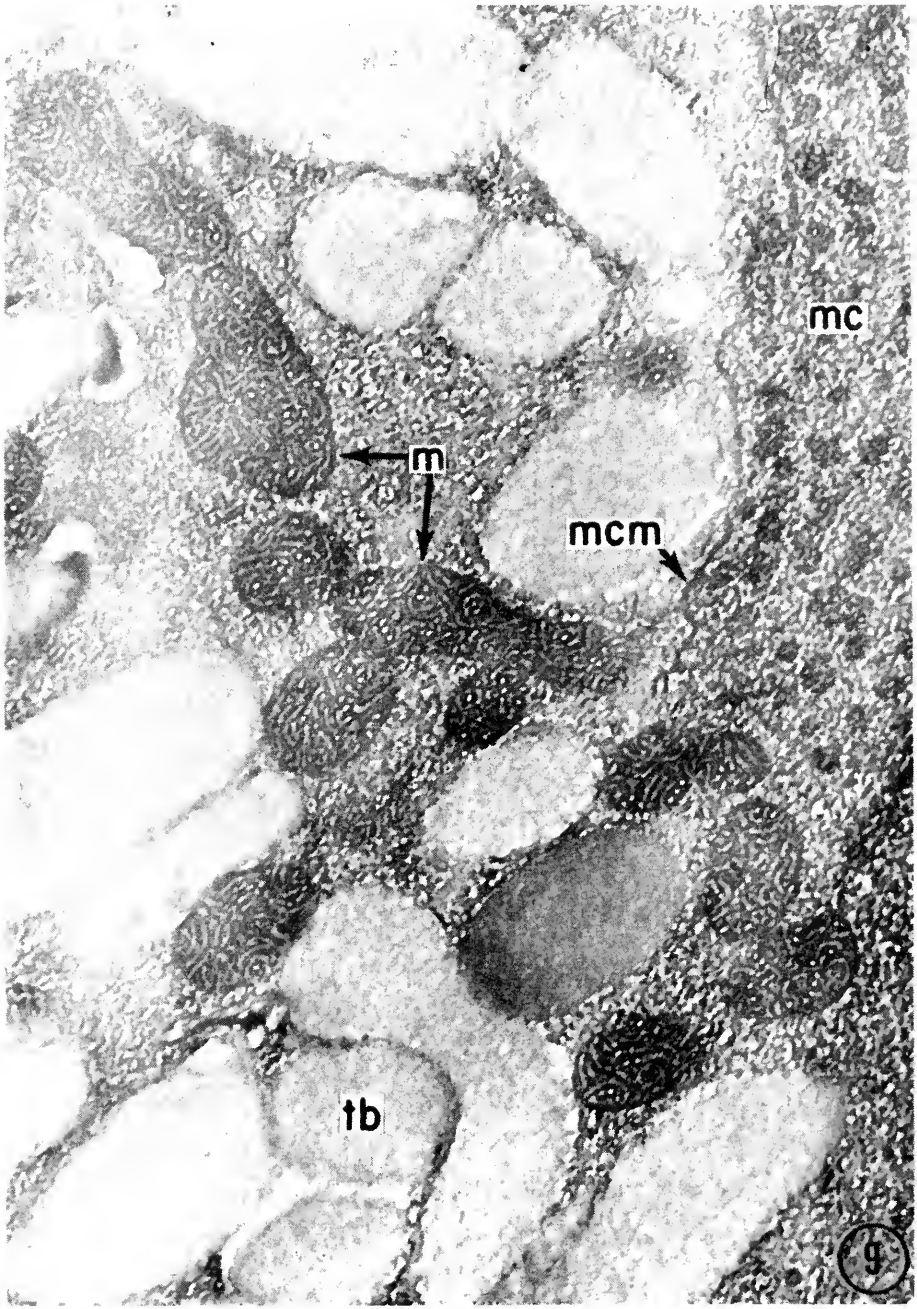


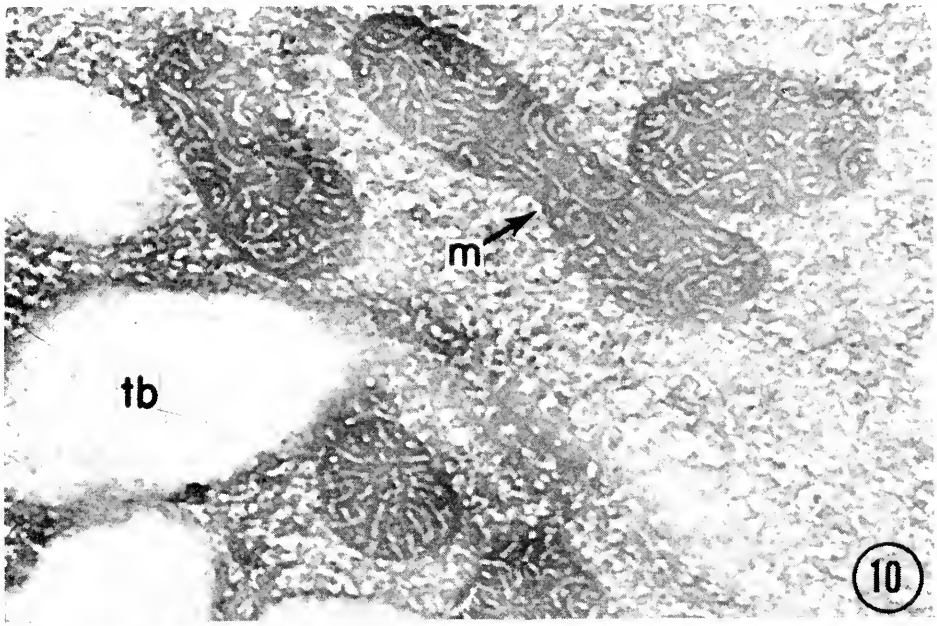
FIGURE 9. EMG of section of *P. aurelia*. On the right side is the macronucleus with its intact membrane. The details of mitochondrial structure are typical for *P. aurelia*. 42,000 ×. Plastic removed.

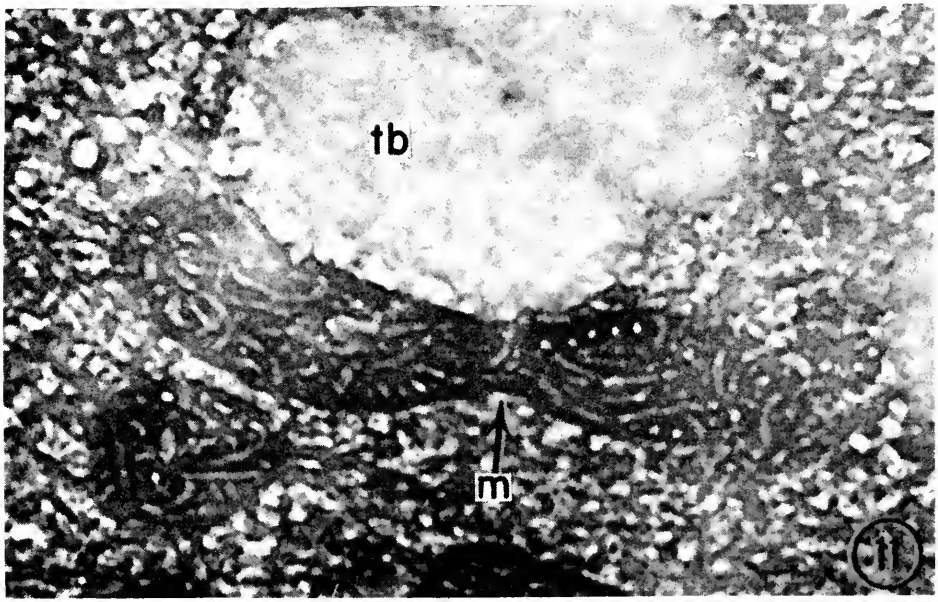
chondrion. All material of low electron scattering power ("density") is obscured by the plastic because it does not allow distinction between this low density material and those areas which appear as spaces in the sections prepared in the other manner. At the same time, the difference in effective density between the high density material and the low density material is greater, so that these areas are in sufficient contrast against the "structureless" background to be visible in the photograph (*e.g.*, some of the material immediately bordering the canals and the very dense granules of the cytoplasm). The result is that we see thin-walled "tubules" in the mitochondria and discrete, well-separated granules in the cytoplasm. The knowledge, then, that areas of low density are not distinguishable from those of no density and that areas of great density are the only ones seen when the plastic remains in the sections reconciles differences in appearance by the two techniques.

While removal of plastic may cause some displacement of ultrastructure (Hillier and Gettner, 1950), we propose that the structure of the mitochondrion is revealed only under these conditions. Removal of plastic from these sections results in cross-sections of cilia which seem collapsed to a slight extent because the double structure of the individual fibers reported by others (Fawcett and Porter, 1954) is recognized only with difficulty. But it should be noted that the change brought about by the removal of the plastic is a slight movement of structure, and this kind of change hardly accounts for the architecture observed in the sections of mitochondria after plastic removal. It is difficult to conceive that structure of such high degree of organization (comparable in all the details visible in sections before plastic removal) is the result of changes brought about by solvent action.

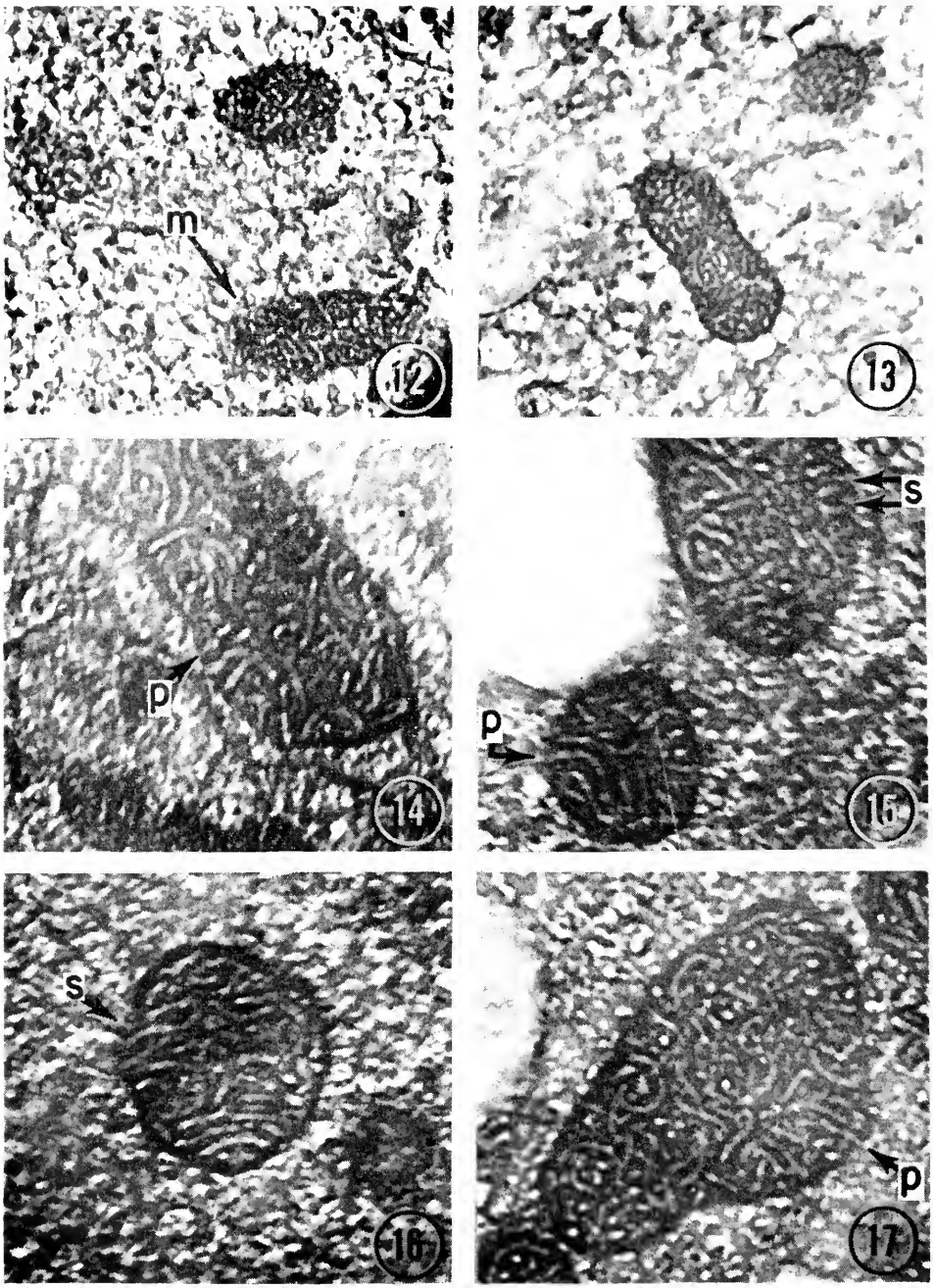
There are two other reports of structure of mitochondria of protozoa as revealed by electron microscopy, one in *Euglena* (Wolken and Palade, 1953) and the other in *Paramecium multimicronucleatum* (Sedar and Porter, 1954). These authors, using the generally accepted method of leaving the plastic in during examination, conclude that the mitochondria possess external membranes. In *Euglena* numerous ridges (called *cristae*) project from the "membranes" into an internal structureless matrix. The authors point out that this mitochondrion has a differentiated membrane similar to that observed in mitochondria of other animal cells. In *P. multimicronucleatum*, it is said, there are villi projecting from the external "membrane" into the internal matrix. The latter report is reconcilable with our observations in all respects, for, prior to being soaked in toluene, the sections show the same structure reported by these authors. In regard to the observation in *Euglena* (Wolken and Palade, 1953), we suspect that removal of the plastic might reveal that the matrix has real structure, and that this structure may have the same relationship to the "membrane" as that observed in our studies.

In other studies (Palade, 1953; Sjöstrand and Rhodin, 1953) mitochondrial membranes are described from sections of mammalian tissues in which the plastic remains during examination. Very clearly defined dense lines are visible about the particles, but close examination of many of the photographs shows that the membranes are frequently discontinuous and vaguely defined, and sometimes bear unexpected (for membranes) relationships to the structures found internally. In view of this and in view of our demonstration that plastic removal reveals





FIGURES 10 AND 11. EMGs of mitochondria of *P. aurelia* at high magnification showing typical structure. 69,000× and 84,000×. Plastic removed.



FIGURES 12 AND 13. EMGs of sections of *P. bursaria* showing the same general mitochondrial structure as observed in *P. aurelia*. 26,000 ×. Plastic removed.

FIGURES 14–17. EMGs of sections of *P. aurelia*. Pores of mitochondrial tubules and peritubular space are shown. Figure 17 is an enlarged mitochondrion from Figure 9. 67,000 ×, 67,000 ×, 74,000 ×, and 67,000 ×. Plastic removed.

many details of structural interrelations not visible in other sections, the generalization that mitochondria have differentiated membranes is open to question.

Two general kinds of interpretation of the structure of the mitochondrion can be made on the basis of the observations reported here. The particle can be considered a continuum which is interrupted by numerous canals, the canals bearing the same relation to the substance of the particle as does the space made by a pin in a lump of clay to the clay itself. Or, it can be considered to be made up of a tightly packed collection of tubules (like hollow macaroni), the walls of which in some places fuse to form a continuum of osmiophilic substance. In other areas the tubular characteristic is retained.

Examination of the figures presented, especially in the regions of looser structure, leads us to favor the tubular interpretation. In this interpretation, the basic structure is a tubule with a lumen approximately 15 mμ in diameter. The innermost portion of the tubule wall appears very dense after osmium fixation; the outermost portion of the wall, except at the edge of the particle, is frequently less dense. These portions often are continuous with corresponding parts of adjacent tubules setting up a continuum not separable into component tubules. In places the tubules are evidently not continuous, forming what might be called the peritubular (or intertubular) space. At the edge of the particle this continuum is often dense. Infrequently, the tubules are seen to open through the dense edge of the particle, the lumen being continuous with the surrounding cytoplasm. The lumen itself is represented by a space in the photographs. Of course, this does not mean that this lumen is empty in the living condition; it does mean that any substance occurring there must be different from that of the wall of the tubule which reduces OsO_4 and becomes relatively dense.

This interpretation accounts for all details visible in both types of sections. With plastic in, the frequently dense edge and the dense linings of the tubules apparently constitute the entire structure. When the plastic is removed, the external "membrane" is seen to be a dense peripheral portion of tubule wall and the tubules are seen to consist of both dense and less dense material. A sac-like membrane,[1] such as seen in Figures 4, 5, 9, surrounding the macronucleus, cannot be demonstrated, although the presence of the macronuclear membrane itself indicates that toluene treatment did not remove any structure like it.

The relation of this tubular interpretation of mitochondrial structure in *Paramecium* to the descriptions given for certain other organisms is not direct, although certain similarities are recognizable. Glimstedt and Lagerstedt (1953) conclude from studies of isolated particles that in rat liver the mitochondrion consists of a collection of granules arranged in cords; and although there is no evidence that the cords are hollow (tubular), the method of preparation would not permit observation of a cavity even if it does exist, and these structures may, in fact, be quite similar to those described for *Paramecium*. In the case of the mitochondrion of the germ cell of *Helix* (Beams and Tahmisian, 1954), which consists of a group of concentrically arranged lamellae, the relationship is even less clear and direct. However, the development of a lamellar structure from tubular precursors is suggested by Leyon (1954) for the development of the grana of the choroplast in *Aspidistra* from the primary grana. A process like this (phylogenetically or

[1] We are distinguishing between boundary interfaces and the kind of structure demonstrated by cell and nuclear membranes.

ontogenetically) could unite conceptually such seemingly diverse mitochondrial microanatomy as that seen in *Paramecium, Helix,* and the rat. On the other hand, it is recognized that real morphological differences in mitochondrial structure may exist; for instance, the mitochondrion of the proximal convoluted tubule as interpreted by Sjöstrand and Rhodin (1953) might suggest another basic structure.

The observations of Weinstein (1954) of the structure of particulates called sarcosomes in sections of chicken heart muscle are directly comparable to those reported here. The sections are quite similar to those of *Paramecium* mitochondria from which the plastic was not removed. Longitudinal and cross-sections of tubules within these particles in the muscle tissue are easily observed in the photographs. Although Weinstein (as well as Leyon) interprets his photographs as showing sac-like membranes, they can as easily be interpreted as not demonstrating the presence of a membrane on the external surface. This particle of the chicken heart, then, becomes nearly identical in structure to the mitochondrion of *Paramecium.*

Based on chemical and physical observations made on the mitochondria of several organisms, certain inferences concerning structure appear in the literature. One opinion is that the behavior of mitochondria in hypertonic and hypotonic solutions, together with some information on permeability of sarcosomes, indicate the presence of a differentially permeable membrane about the mitochondrion. This opinion was most recently presented by Lindberg and Ernster (1954) who feel that the recent morphological evidence as presented by Palade (1953) and Sjöstrand and Rhodin (1953) is complete confirmation of the membrane theory, and is so final as to make any contrary opinion "hardly maintainable today." Another view (Harman, 1950; Green, 1952) is that the biochemical and biophysical evidence is not of such a nature as to require that mitochondria possess membranes. It had been proposed that a complex protein can exhibit the osmotic behavior observed with no unique requirement for fluid interiors surrounded by differentiated membranes. The evidence presented in this paper supports this suggestion, in that the mitochondrion is shown to consist of an almost continuous material which is not separated from the exterior by a membrane different from it. One other characteristic, not predictable from the biochemical evidence, is revealed—the continuous material is the substance of the walls of tubules, the lumens of which are sometimes seen to open to the outside.

The way in which the mitochondrion functions physiologically may only be guessed from the morphological evidence. The most simple thought is that reaction of enzyme and substrate occurs at the external surface of the mitochondrion. However, the existence of the tubules and their openings to the cytoplasm suggests that these reactions may occur within the lumens of the tubules. In this instance, the surface available for reaction is very much greater than in the former case; and if in addition the peritubular spaces are involved, the surface reaction area is even greater yet.

Summary

1. An electron microscopical study of the mitochondria in *Paramecium aurelia* and *P. bursaria* has been made from thin sections of cells imbedded in methacrylate

plastic. Some of these sections were studied directly (plastic-in), and others were soaked in toluene before examination (plastic-out).

2. Plastic removal reveals structural detail not visible when the plastic remains in the section. At the same time, the relationship between the newly observed structures and those seen in the other sections is accounted for.

3. The undifferentiated mitochondrion in *Paramecium* is interpreted to consist of a compact mass of twisted tubules, the walls of which are made up of at least two kinds of substances (based on density differences in the photographs). The lumen of the mitochondrial tubule is about 15 mμ in diameter and is seen at times to be continuous with the cytoplasm. A distinct feature of this mitochondrion is the lack of a demonstrable membrane distinct from the material of the walls of the tubules in contrast to some interpretations of mitochondrial structure in many other organisms.

4. The basic tubular structure of the mitochondrion in *Paramecium* is recognizable in the cytoplasmic inclusions (mitochondria, primary grana) of several phylogenetically distant organisms.

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THE FATES OF SEGMENTS FROM TUBULARIA PRIMORDIA[1]

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During the reconstitution of decapitated *Tubularia* stems, a conspicuous pigment precedes morphological differentiation in the primordial region. Accordingly, it was very early postulated, by Loeb (1892) and by Driesch (1899), that the pigment itself, as a "formative substance," may initiate or control differentiation of the hydranth. Recently, Goldman (1953) showed that at least a part of the pigment is synthesized within the primordium during reconstitution. Meanwhile, Davidson and Berrill's (1948) experiments with mutilated primordia showed that the pigment distribution can be used as a landmark by which primordia can be sectioned to give stems and fragments of abnormal but predictable behavior. That work suggested the possibility of clarifying the role of pigment in determination, by removing various pigmented and non-pigmented regions of primordia and allowing them to differentiate in isolation.

Material and Methods

For each experiment, a single colony of *Tubularia crocea,* from the same area off Woods Hole, Massachusetts, was used. Stems were selected for uniformity of diameter and appearance, and decapitated one to three millimeters below the hydranth. The proximal (aboral) ends were cut to give stems 7 to 10 mm. in length, and the stems were left in fresh sea water at 18° to 22.5° C. until a reasonable number had developed to the desired stage. Those stems were isolated and divided evenly into lots representing control and experimental groups. The desired segments were then removed with scissors under a binocular microscope, and isolated. Control groups consisted of unoperated stems. The water in which all stems and segments were kept was changed at least once a day. All data recorded represent the form of the hydranth at the time of emergence from the perisarc. Frequently, the regenerates were unable to emerge because of the absence of a point of attachment to the perisarc; in those cases, the regenerate was removed with dissecting needles for observation.

All data are recorded in tabular form, each table or figure headed by explanatory diagrams. The first vertical column at the left of each figure contains a diagram representing the stage worked with. Pigment distribution, where pigment is present, is indicated by stippling. The second vertical column consists of a series of diagrams showing the places from which segments were cut, such segments being indicated by diagonal lines. The horizontal row at the top of each figure contains diagrams representing the form of the regenerate at the time of

[1] This study was undertaken at the Marine Biological Laboratory on a fellowship grant from the University of Illinois.

emergence. A description of each of the types of diagrams appears in Figure 1. In each square of the following figures the number of individuals appears in standard lettering, while the percentage of the total is italicized.

Results

1. The development of random segments of varying length.

Pieces of primordia are, of course, considerably smaller than stem pieces customarily used in differentiation studies, and so it was necessary first to determine what effect small size itself might have on reconstitution. Accordingly, a number of segments of varying lengths were cut at random from normal stems, sorted into three size groups, and allowed to develop independently. Segments of about 0.25 mm. were never observed to differentiate, but always either died or rounded permanently into undifferentiated cellular clumps. Segments 0.5 to 0.75 mm. in length did better: of 60 cases studied, 8 (13.3%) formed complete hydranths, 3 (5%) formed bipolar hydranths, 2 (3.3%) lacked, or possessed reduced, proximal tentacles, and 47 (78%) either died or failed to differentiate. Of 34 segments approximately one millimeter in length, 8 (23.6%) formed complete hydranths, 3 (8.8%) formed bipolars, and 23 (67.7%) died or failed to differentiate. The forms described are diagrammed in Figure 1. Driesch (1897), following the development of short stem pieces apparently one to three millimeters in length, reported that 6.1% were unipolar, 31.7% bipolar, and 62.2% complete hydranths. In the experiment reported here, the percentages are 8.3, 25.0, and 66.7, respectively, if dead and undifferentiated segments are excluded from the calculations.

It is therefore evident that segments of small size as a rule form either complete hydranths, none at all, or bipolar hydranths. Hydranths in which a set of tentacles is missing ("unipolar hydranths") without being replaced with a duplicate of the other set are rare. Hence, small size tends largely to inhibit reconstitution completely, or to effect the production of bipolar hydranths.

2. The development of segments from the prepigmentation stage (stage II)

Figure 2 shows the development of segments removed from stems two hours after decapitation, and before any pigmentation could be observed. The terminal

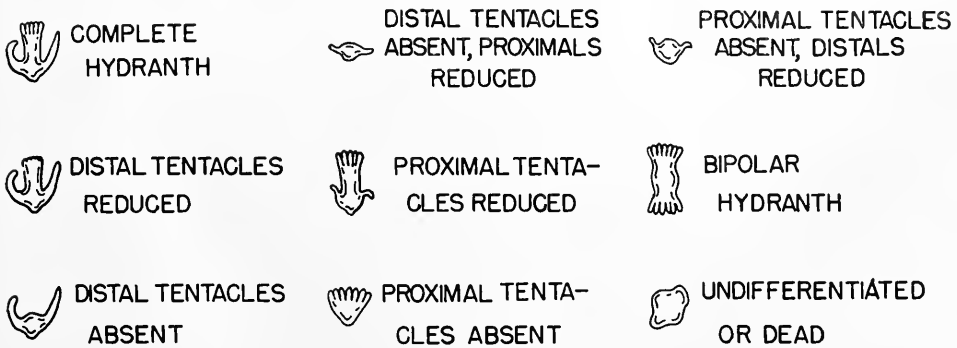

Figure 1. An explanation of the symbols used in the figures.

STAGE	SITE OF SEGMENT	SEG-MENT	NUM-BER				
A B		A	74	0	1 1%	24 32%	49 67%
		B	73	73 100%	0	0	0
		CON-TROL	72	68 94%	0	0	4 6%

FIGURE 2. The development of segments from the pre-pigmentation stage.

segments, 0.5 to 1.0 mm. in length, showed virtually no regulatory ability with respect to proximal tentacle formation, two proximal tentacles being formed in the case of a single segment. Regulation with respect to distal tentacle formation was complete in both distal and stem pieces.

3. The development of segments from the stage of diffuse pigmentation (stage III)

Figure 3 illustrates the development of segments from stems in which the pigment had begun to collect in the first three millimeters of the stems, but in which there was no sharp boundary between pigmented and unpigmented regions, one grading into the other. In stage III, as in stage II, no morphological differentiation is apparent. Segments representing the terminal 0.5 mm., which was not pigmented, failed to develop in 90% of the cases, evidently because of their small size. The development of distal tentacles in 18 individuals (10% of the

STAGE	SITE OF SEGMENT	SEG-MENT	NUM-BER							
A B C D		A	182	0	0	0	1† .5%	17† 9%	0	164 90%
		B	20	0	0	0	0	9 45%	7 35%	4 20%
		C	77	23* 29%	5 7%	7 9%	3 4%	3 4%	8 10%	28 36%
		D	177	171 97%	1 .5%	0	0	0	0	5 3%
		AB	56	1 2%	0	0	0	26 46%	2 4%	27 48%
		BC	161	66 41%	2 1%	0	1 .5%	28 17%	46 29%	18 11%
		CON-TROL	228	219 96%	0	0	0	0	0	9 4%

FIGURE 3. The development of segments from the diffuse pigmentation stage. † Occurred in one experiment only and could not be repeated. * A 72-hour time lag in emergence was observed.

total) occurred in one experiment only, as noted by the dagger in Figure 3, and could not be repeated. The development of segments from other levels indicated that the ability to form distal tentacles resides in more proximal parts at this stage, and it is probable that in the erratic experiments cited the proximal cuts were made farther from the tip than intended, thus including some of the region of distal tentacle potency. Segments representing approximately the distal half of the pigmented region (B segments) formed distal, but not proximal, tentacles, while segments representing the proximal half of the pigment (C) formed an array of types that included distal and proximal tentacles in all combinations, as did segments comprising the entire pigmented region (BC segments). Note that a 72-hour time lag in emergence of the C segments occurred, and that 29% had produced complete hydranths by that time. The delay appears to represent a reversal of development, stimulated by cutting, and resulting in reorganization to form complete hydranths in those cases.

Segments comprising the distal half of the pigmented region plus the stem tip (AB segments) developed in much the same way as the B segments alone, indicating that the unpigmented stem tip is not important in reconstitution. The behavior of the BC segments, in forming no proximal tentacles 17% of the time, and of the C segments in forming unipolar distals in 3 cases, is taken to mean that the pigmented region of stage III ends at a region of transition between areas of differing abilities for proximal tentacle formation.

Stems from which all of the pigmented area was removed (D) formed complete hydranths in 97% of the cases.

4. The development of segments from the stage of defined pigmentation (stage IV)

Figure 4 illustrates the development of segments removed from primordia in which the pigment had become sharply defined at its proximal limit. As in the preceding stages, no morphological differentiation had occurred by that time. In addition to the large pigment band, which ended about 2.5 mm. from the tip of the stem, many stems also possessed a more narrow, darkly pigmented band which occupied the distal 0.25 mm. of the larger band. In order to determine any effect of the band on the development of the segments, some cuts were made so as to include it with the terminal, or A, segments: those are designated A+. B segments represent the distal half of the pigmented area, including the narrow band. B-C segments represent the entire pigmented area minus the narrow band, and the B-CD segments represent stems from which only the unpigmented tip and the narrow band were removed.

As in stage III, unpigmented tip segments showed very poor reconstitution, but tip segments that included the narrow band (A + segments) developed distal tentacles in 54% of the cases, and in 79% of the cases in which they contained the entire distal pigmented half (AB segments). However, the B-CD segments developed distal tentacles in 95% of the cases, and the B-C segments in 61% of the cases. The C and CD segments, which also lacked the narrow band, also developed distal tentacles, in 62% and 82% of the cases, respectively. Therefore, the distal, narrow band, which probably marks the area in which the distal tentacles will form, evidently is not necessary for distal tentacle formation: more proximal

stem levels can also form distal tentacles if called upon to do so, and with no loss of time. It will be seen later that this is a general ability that applies to all stages.

The development of the A, A+, AB, B, B-C and C segments, which represent various combinations of the pigmented and tip regions, indicated that, surprisingly, proximal tentacles were unable to form in any part of the pigmented region. Of 166 individuals represented by those segments, only two showed proximal tentacle formation. On the other hand, all pieces that included the stem proper, that is, pieces 7 to 10 mm. in length (B-CD, CD and D segments), of course developed proximal tentacles if they differentiated at all.

5. The development of segments from the tentacle band stage (stage V)

Figure 5 represents the development of segments removed from primordia that had developed to the stage in which the pigment was sharply distributed between two bands occupying the terminal 3.0 mm. of stem. Unpigmented tip segments (A segments) showed their typical inability to form tentacles. As shown by the figures for the B and BC+ segments, all of the segments that contained the distal pigment band formed distal tentacles if they developed at all. Of the segments that contained only the proximal band plus the proximal half of the unpigmented interband region (C-D), only 8 of 33 developed, and of those only one lacked distal tentacles. However, stems to which the proximal band and interband regions were left attached (CDE) developed in 10 of 14 cases, and of those, half lacked distal ten-

STAGE	SITE OF SEGMENT	SEGMENT	NUMBER							
		A	29	0	0	0	0	1 3%	0	28 97%
		A+	33	1 3%	0	0	14 42%	3 9%	0	15 46%
		B	29	0	0	0	22 76%	0	0	7 24%
		C	29	0	0	0	17 59%	0	1 3%	11 38%
		D	29	27 93%	0	0	0	0	0	2 7%
		AB	33	1 3%	0	0	22 67%	0	3 9%	7 21%
		B-C	13	0	0	0	6 46%	0	2 15%	5 39%
		CD	17	12 70%	2 12%	3 18%	0	0	0	0
		B-CD	20	14 70%	5 25%	0	0	0	0	1 5%
		CON-TROL	43	43 100%	0	0	0	0	0	0

FIGURE 4. The development of segments from the defined pigmentation stage.

STAGE	SITE OF SEGMENT	SEGMENT	NUMBER						
A-E		A	53	0	0	0	0	1 2%	52 98%
		B	11	0	0	0	11 100%	0	0
		BC+	34	7 20%	0	0	24 71%	0	3 9%
		C-D	33	5 15%	0	1 3%	2 6%	0	25 76%
		CDE	14	5 36%	3 21%	2 14%	0	0	4 29%
		E	38	28 73%	4 11%	0	0	0	6 16%
		CONTROL	65	64 98%	0	0	0	0	1 2%

FIGURE 5. The development of segments from the tentacle band stage.

tacles or possessed rudimentary ones. It was repeatedly observed during the experiments that any segment, from any stage, can eventually form distal tentacles if it can differentiate at all. If the distal tentacles are absent or rudimentary at the time of emergence, it indicates that the process of distal tentacle formation has been arrested or delayed in starting.

The limitation of proximal tentacle potency, as the above data show, was more clear by stage V. In the case of the C-D segments, which consisted of the proximal band plus the proximal half of the interband region, of the 8 that developed, 6 possessed proximal tentacles. In the BC+ segments, however, proximal tentacles formed in 20% of the cases, even though they possessed no part of the proximal band, but only the distal band and half of the interband region. It therefore appears that the region of proximal tentacle potency extends a short distance distal to the limit of the stage V proximal band.

6. The development of segments from the proximal striation stage (stage VI)

When segments are isolated from advanced primordia in which the proximal band has developed tentacle ridges, proximal tentacles can develop only in segments which contain a part of the proximal anlage. A tendency in the same direction also holds true for the distal tentacles, since the absence of the distal band leads to the absence of distal tentacles from the regenerate. Usually, however, within another day, the incomplete regenerate belatedly begins to form a tiny set of distal tentacles. In the case of the proximal tentacles, however, once the hydranth has differentiated without them, they can never subsequently be re-formed. Thus, even *Tubularia* is not totipotent in all situations. This ability of mutilated primordia to reconstitute missing distal, but not proximal, tentacles was first observed several years ago, by Davidson and Berrill (1948).

Since primordia of stage VI showed the same range of regenerative abilities that the adult hydranth shows, data from the experiments are not included here.

Discussion

Ordinary stem segments seldom give rise to unipolar hydranths, whereas segments removed from primordia in various stages of reconstitution often do. The data cited above indicate that the nature of the affected structures and the degree of the effect depend upon the positions in the primordia of the regions incorporated into the segments, and upon the time at which the cuts were made. A regional change in regenerative potency occurs within two hours after removal of the hydranth from the stem, since a short segment that would ordinarily form a complete hydranth or a bipolar instead forms a unipolar hydranth possessing only distal tentacles, and no proximals. Thus, two hours after decapitation, the distal millimeter of stem can no longer support proximal tentacle formation. The single exception to this behavior, indicated in Figure 2, can be explained most reasonably by assuming that in that case the cut was made at a point of transition between areas of differing abilities for the support of proximal tentacle formation.

As Figure 3 shows, by the time the stage of diffuse pigmentation develops, further changes in the regenerative potential of the first three mm. of the stem have occurred: proximal tentacles can no longer form from the distal half of the pigmented region, while the proximal pigment halves can produce both distal and proximal tentacles in any combination of number or quality, depending upon the particular segment. It is reasonable to assume that the cuts, in those cases, fall at levels at which rather abrupt changes have developed along the stem with respect to the ability to form tentacles. On the other hand, cuts through the transition zones of later stages often lead to the production of fewer or rudimentary tentacles. Thus, although the transition from a determined to a non-determined zone is rather abrupt, there nevertheless appear to be short zones of gradual change along the axis, and the inclusion of small amounts of such gradient regions in a segment can thereby lead to quantitative manifestations in the hydranth. However, hydranths possessing such rudimentary tentacles ordinarily go on to form tentacles normal in both size and number in a few days. It is as if the adult hydranth can later furnish some missing quantity if a little is present in the operated segment.

The C segments of Figure 3 are interesting in that they indicate that the cutting operation may sometimes result in delayed emergence, accompanied by reorganization to form complete hydranths. Note that similar segments from stage IV (Fig. 4) were not delayed in emergence, and did not produce complete hydranths.

Since the distal pigmented halves of stage III (Fig. 3) do not form proximal tentacles, while the proximal halves may or may not, it appears that the critical cuts, which determine whether a particular segment will be able to form proximal tentacles, are those that separate the pigmented from the unpigmented parts of the stem. Since those cuts were made as precisely as possible to coincide with the limit of pigmentation, and since they produced a spectrum of tentacle types, it appears that the pigment limit of stage III marks a zone of transition with respect to proximal tentacle determination: proximal tentacles form only when the

cut happens to include enough of the presumably unpigmented proximal tentacle area. That area, therefore, appears to be immediately behind the pigmented region of stage III. Moreover, stage III stems from which all of the pigmented region has been removed go on to produce normal, complete hydranths, which emerge at the same time that the controls do. Meanwhile, the distal tentacle region, which is surely removed by such a cut, must be able to re-form during that time. The distal tentacle area is only beginning to be laid out by stage III, and can be duplicated in time for normal emergence.

There is never any region in which distal tentacles cannot form if enough time is allowed, as observed by Davidson and Berrill (1948), Peebles (1900), Driesch (1897), and during the course of these experiments. Peebles, using stems which had reconstituted to the stage in which both distal and proximal tentacle striations were present, made cuts through the proximal striations. The terminal segments never developed proximal tentacles, with one exception in more than 100 cases. Driesch, using stems of the same stage, made cuts midway between the two striated regions, and never observed proximal regeneration of the terminal piece, while the stem piece was always able to produce a complete hydranth. Finally, Gilchrist (1937) showed that a similar situation occurs in polyps of *Aurelia*. These have only a single tentacle group; however, fragments removed from the body of the polyp can always form the tentacles and a hypostome, whereas fragments of the hypostome, which is distal to the tentacles, cannot form tentacles unless some of the cells from the base of a tentacle are included. It may therefore be a rule that fragments from polyps and polyp primordia can reconstitute structures that are normally distal to the original location of the fragments, but not more proximal structures. Such a rule would not apply, however, to something like the gonophores of *Tubularia*, which Davidson and Berrill (1948) showed can form from a very broad region of the primordium.

By the time the pigment band of stage IV has formed the two bands of stage V, the region of proximal tentacle potency is restricted to the region occupied by the proximal band plus a part of the interband region, while a less sharp tendency toward the restriction of distal tentacle potency to the distal band is indicated. Figure 5 shows that segments that do not contain the distal band, or a part of it, tend not to form distal tentacles by the time of emergence, or else emerge with rudimentary distal tentacles.

When proximal tentacle striations have formed (stage VI), proximal tentacles apparently cannot form from any piece of the primordium distal to the striated region. Segments removed from between the pigment bands of this and earlier stages are of interest because they produce a spectrum of tentacle types. Evidently, the exact levels of the distal and proximal cuts determine whether the interband segments will be able to form distal or proximal tentacles, or both, or neither.

It is of interest to compare the extent of pigmentation with the extent of the regions in which tentacles can form, since these do not coincide in all stages. In stage II, no pigment concentration can be observed, and yet determination has already progressed to the point where proximal tentacles cannot form from the distal 0.5 to 1.0 mm. of stem. By stage III, the pigment ends about 3 mm. from the stem tip, and proximal tentacles can no longer develop from the terminal 2.5

to 3.0 mm. of stem. The region of proximal tentacle potency remains permanently in that position, while the limit of pigment moves distally, so that, by stage IV, it occupies only about the terminal 2.5 mm., and does not occur at all in the region in which the proximal tentacles are destined to form. In stage V, the pigmented region gives rise to two bands, with the proximal half of the pigment moving back so that it secondarily coincides with the region in which the proximal tentacles will develop. The earliest shrinking of the region in which proximal tentacles can form therefore appears to be one which limits the proximal tentacle potential to an unpigmented region.

Stevens (1901) has discussed the possible significance of the pigment in reconstitution, as a result of her studies of reconstituting *Tubularia* stems. The studies led her to conclude that the pigment is accumulated by the primordium from the circulating fluid of the gastrovascular cavity, into which it is released by breakdown of the endodermal ridges. She also stated that much of the pigment is expelled by the newly emerged hydranth (something not observed during these experiments) and, discussing the "formative substance" of Driesch and Loeb, said (p. 414), "The indication is that the red granules, far from being 'formative substance,' are in fact the only part of the cell material from the disintegrated endodermal ridges, which the animal cannot make use of in regenerating a hydranth, and therefore rejects at the first opportunity." Although there is no reason why a "formative substance" could not be ejected after it was no longer useful, it now appears that the pigment of *Tubularia*, at least in visible concentrations, does not contribute to reconstitution.

Cohen (1952) showed conclusively that some of the pigment that appears in the primordium is collected by the cells of the primordium from the circulating gastrovascular fluid, while Goldman (1953) showed equally conclusively that the rest of it is actually synthesized within the primordium. None of this information, of course, clarifies the role of the pigment in determination itself. However, as stated above, proximal tentacle determination can, and normally does, take place in the absence of discernible pigment. It is also significant that, during these experiments, occasional stems would never produce any visible pigment, even though they were perfectly able to reconstitute, producing colorless hydranths. Occasionally, the laboratory collectors brought in whole colonies of *Tubularia* in which very little pigment was discernible, and stems from those colonies reconstituted as well as stems from deeply pigmented colonies. The conclusion therefore follows that the concentrated pigment that accumulates during reconstitution is a secondary result, and not a cause, of tentacle determination.

Summary and Conclusions

The primordia from five stages in reconstitution of *Tubularia crocea* were cut into short segments, using the distribution of pigment as a landmark, and the segments were allowed to develop in isolation from the rest of the primordia. When the differentiated segments were ready to emerge, the completeness of reconstitution was observed. It was hoped in this way to determine what relationship exists between the characteristic pink pigment that accumulates during reconstitution, and distal and proximal tentacle formation, and, also to learn something

of the time sequence and extent of determination. The following conclusions are based on the experiments.

1. Stem segments less than one millimeter in length tend to form undifferentiated clumps of cells, or bipolar hydranths which have two sets of distal tentacles and may or may not have proximal tentacles. Unipolar hydranths (one set of distal tentacles and no proximals) are rare.

2. Two hours after decapitation, the distal millimeter of stem can no longer form proximal tentacles, but distal tentacles can form from any fragment of appreciable size.

3. At stage III, in which the pigment is vaguely concentrated in about the distal three millimeters of stem, proximal tentacles cannot form in fragments from the distal 2.5 to 3.0 mm., and distal tentacle formation shows a tendency to occur less frequently in the proximal half of the pigmented area than in the distal half.

4. At stage IV, when the pigment is sharply confined to about the distal 2.5 mm. of stem, the region of proximal tentacle potency occupies the same region as in stage III, which, in stage IV, is no longer pigmented.

5. At stage V, in which two pigment bands exist, the distal band appears necessary for the formation of distal tentacles in time for emergence, while the proximal band represents the only region that regularly can give rise to proximal tentacles.

6. At stage VI, in which the proximal pigment band is striated, there is an increased tendency toward the restriction of distal tentacle potency to the distal band.

7. The earliest disappearance of the ability to form proximal tentacles occurs in the pigmented region, when the proximal tentacles can form only in regions lacking visible pigment.

8. The pigment of *Tubularia* appears to play no causal role in the progressive disappearance of regenerative ability. It seems most likely, therefore, to be a by-product of the activity that accompanies the reconstitutive process.

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INFECTION OF COCKROACHES WITH HERPOMYCES (LABOULBENIALES). I. LIFE HISTORY STUDIES[1, 2]

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Largely by chance it was discovered that our laboratory colony of the oriental cockroach (*Blatta orientalis* L.) was infected with *Herpomyces stylopygae* Spegazzini. Subsequently, some of our boxes of the german cockroach (*Blattella germanica* L.) were found infected with *H. ectobiae* Thaxter. How long these infections have been present in our colonies is not known. More recently we introduced a colony of a giant tropical cockroach (*Blaberus craniifer* Burm.) from Key West, Florida; on arrival these specimens were found infected with *H. tricuspidatus* Thaxter. Since most past studies of the Laboulbeniales, and all of those on cockroach-inhabiting species, have been by taxonomic mycologists, we decided to examine the etiology and the host-parasite relationship from an entomological point of view. Incidentally, some points of strictly mycological interest were noted and will be recorded.

Although we have utilized all three of the above species of *Herpomyces,* the majority of our data deal with *H. stylopygae* on oriental cockroaches. The data are sufficiently voluminous that they are being presented as a series of papers. In the present paper we are presenting data on the life history of the fungus, its transmission, and some notes on the structure of asci and ascospores. In another paper (Richards and Smith, 1955a) we are presenting our histological and histopathological work. A separate note (Richards, 1954) treats histochemical differentiation of the fungal walls. Subsequent papers are planned covering our experimental work on host specificity, location on the host specificity, germination and development.

Due to the tremendous variability within the Laboulbeniales, one should remember that all of our data deal with the single genus *Herpomyces* found only on cockroaches, and most of our data deal with the single species *H. stylopygae.* The majority of the approximately 1500 described species (Benjamin and Shanor, 1950) of Laboulbeniales occur on species of Diptera and Coleoptera (flies and beetles). Morphology and habits are quite diverse. For instance, there are a number of records of spores germinating while still within the perithecium

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(*Laboulbenia*, *Dioicomyces*, *Dimorphomyces*, etc.); with thousands of specimens examined we have never seen this phenomenon in *Herpomyces*. Generalization seems dangerous at the present stage of knowledge of this group.

The Validity of Herpomyces stylopygae Spegazzini

Thaxter (1908) described the species *H. periplanetae* as occurring on both the american (*Periplaneta americana* L.) and the oriental (*Blatta*, = *Stylopyga*, *orientalis* L.) cockroach. Subsequently, Spegazzini (1917) separated the form found on the oriental cockroach on the basis of relatively slight differences: larger perithecia, a blunter shield over the secondary receptacle, a blackened base to this shield (Fig. 3) and a few other minor points. Thaxter (1931) questioned this separation on such slight differences, but we have had to conclude that the two are distinct (whether species or strains) because we have been unable to obtain development of the form from oriental cockroaches on *Periplaneta americana*, *P. australasiae* or *P. brunnea*, the listed hosts of *H. periplanetae* (details will be given in Richards and Smith, 1955b). Yet there is no difficulty in transferring the infection in the laboratory from one oriental cockroach to another.

Distribution of H. stylopygae on Blatta orientalis

The infection is most commonly limited to the antennae (Fig. 1). Of 50 infected cockroaches taken at random from the colony and examined carefully, 38 (75%) had plants only on the antennae, 11 (22%) had heavy antennal infections plus some plants on the maxillary palpi, and a single specimen (2%) had a heavy antennal infection plus some plants on both the maxillary and labial palpi. No plants were found elsewhere on the body. However, with hundreds of roaches handled (but not searched) subsequently we have found several males with a few plants on the cerci, and one male with four plants growing close to one another on the ventral surface of the right metathoracic femur. In no case have plants been found elsewhere on the body except when there was a very heavy infection on the antennae. And nowhere on the body except on the antennae do the infections become so heavy they almost hide the surface of the infected part. The antenna shown in Figure 1 represents only a moderate infection; heavy infections so cover the antennae that they photograph less well.

The long antennae of cockroaches are their investigative organs. In a colony, and in nature, they are waved about in all directions making repeated contact with their substrate and their neighbors. When under crowded conditions, common in nature as well as in culture boxes, cockroaches are continually making contact with the antennae of other individuals. The contact involves all exposed surfaces, even the ventral surface, as one cockroach runs over another. As shown in Figures 1 and 2, mature perithecia commonly have a spore or spore group protruding from the subterminal aperture for 80–85% of its length. Such spores are dislodged by a touch or jarring. Considering the habits of cockroaches and the presence of such easily dislodged spores, it is obvious that the antennae must serve as efficient spore brushes and spore dusters. Further, it seems obvious that under crowded conditions spores should become placed on all portions of the body. It is simple to demonstrate that this is indeed true; areas of the cockroach body wall

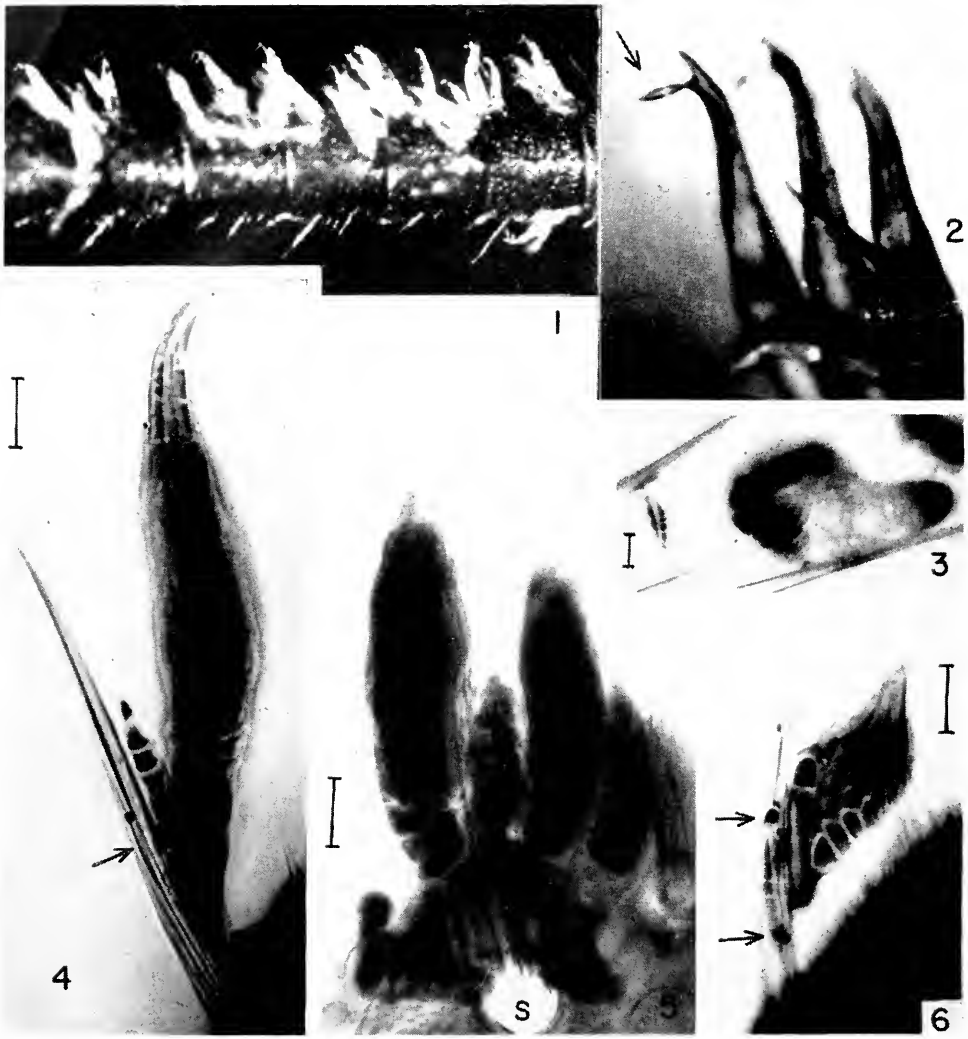

FIGURE 1. A small portion of an antenna of *Blatta orientalis* moderately infected with *Herpomyces stylopygae* (living). There are fifteen plants on these six antennal segments. Note spores protruding from the subterminal apertures of many of the perithecia.

FIGURE 2. Higher magnification of mature, living perithecia showing a spore or pair of spores (arrow) protruding from each perithecium (only one in clear focus).

FIGURE 3. Basal shield of *H. stylopygae* showing naturally blackened base and stained apex (cotton-blue). Note also pair of spores on cuticle surface (one in 2-cell stage, other in 4-cell stage. Bar 10 μ.

FIGURE 4. Young mature female plant of *H. ectobiae* on antennal seta of *Blattella germanica*. Note basal holdfast of spore (arrow) and basal prolongation of plant to setal base. Cotton-blue stain. Bar 10 μ.

FIGURE 5. Larger female plant of *H. ectobiae* spreading across antennal surface of *B. germanica* after reaching base of seta (S). Cotton-blue stain. Bar 10 μ.

FIGURE 6. Young male plant of *H. ectobiae* on antennal seta of *B. germanica*. Note spore in 4-cell stage alongside this plant on seta (holdfasts indicated by arrows). Cotton-blue stain. Bar 10 μ.

excised, stained with cotton-blue in lacto-phenol and examined under the microscope show spores all over the body surface. Clearly the hypothesis that the specific locations of infections are due to transfer of spores to only those locations, as suggested by Thaxter (1896) and preferred by Benjamin and Shanor (1952) for *Laboulbenia* species, cannot hold for infections of *H. stylopygae*. We hope at a later date to discuss this question of development to maturity only on certain areas, but we might mention here, in passing, that in single insect species histochemical differences in the cuticle surface for different areas of the body have recently been reported (Richards, 1952).

At frequent intervals, cockroaches clean their antennae by bending them down and pulling the shaft of the antenna through the mouth parts, thus masticating and scraping them. Likely this is the source of infections developing on palpi though infection from another individual does not seem impossible. It is tempting to speculate that the lower incidence of infection on palpi may be due to the antennal infection having to mature first but the very low incidence on all parts of the body except the antennae makes such a speculation decidedly questionable.

On the antennae plants may be located anywhere but are seldom seen on the most basal segments. On the average there are about one hundred segments in each antenna of *B. orientalis*. Approximately equal numbers of plants grow to maturity on segments of the apical 90% of the antennae, more local distribution in individual cases being shown by actual counts of spores and plants to be correlated with and hence due to corresponding irregularities in the chance distribution of spores.

Plants may be found growing on hard sclerite areas or on soft membrane. Correlated with the fact that most of the surface of the antenna is sclerotized, most of the plants are found on sclerites. Commonly they are associated with setae and penetrate through the setal sockets but also, commonly, they penetrate through sclerite where no seta is located (see Richards and Smith, 1955a).

Infections develop much more heavily on males than on females. Rough estimates from 24 ♂ and 24 ♀ taken from infected colonies show that there are on the average more than twice as many plants on infected males. Infections on nymphs are never very heavy but infected individuals of all instars can be found. In a specific test where 4 ♂, 4 ♀ and 4 nymphs were exposed to infection from 8 adult males (in a pint jar) for just one day and then isolated, 117 mature plants were found on the males, 62 on the females but only 11 on the nymphs at about two weeks later. For these counts, both antennae were amputated at the base, stained with cotton-blue in lacto-phenol and the number of spores and plants tabulated for every antennal segment. It was found that there were 416 spores or plants on the females and 525 on the males, *i.e.*, about 50 per antenna, but only 119, *i.e.*, 15 per antenna, on the nymphs. Since infection was from adult males, it follows that infected males distribute approximately equal numbers of spores to adults of either sex but significantly less to nymphs. Of more interest is the fact that exposure to infection was on a known single day and examination was made at a known subsequent interval (11–14 days). If one assumes all spores will germinate and grow on an antenna, all of these should have been reaching maturity at the time of examination. Tabulation was made under four headings: 2-cell spores, 4-cell spores, immature plants, and mature plants. Of the total of 1060 spores, only 16 were still in the 2-cell stage, but 502 had not progressed past

the 4-cell stage. This growth failure of almost 50% was not uniformly distributed. Tabulating the values:

on nymphs (119 spores): 29% past 4-cell stage, 9% mature, at 13–14 days.
on females (416 spores): 44% past 4-cell stage, 15% mature, at 12–13 days.
on males (525 spores): 67% past 4-cell stage, 22% mature, at 11–12 days.

Clearly, either the per cent growing on males > on females > on nymphs, or there is a delay at the 4-cell stage with sporadic initiation of subsequent development being most frequent on males and least frequent on nymphs. At this 11–14 day time almost exactly one-third of those which had passed the 4-cell stage had mature perithecia in each group. Perhaps this can be interpreted as favoring the second alternative. Such a difference in frequency of initiation of later development would give the observed point that, both in colonies and after known infections, about twice as many *mature* plants are seen on males as on females.

We have succeeded in inducing growth of *H. stylopygae* only on live cockroaches. Plants will not grow past an early germination stage on exuviae (shed skins) or on excised and explanted pieces of abdominal cuticle or integument floating on broth in a humid atmosphere; they will not grow on amputated antennae even at 100% relative humidity; and if the cockroach dies the plants wither within a few days. However, plants are active for some time after infected antennae are amputated. An amputated antenna makes a good spore brush for experimental infections. After one use it may be held for a few hours in moist air and used again. Actually this is illustrated by Figure 1. In mounting this amputated antenna for photographing, all the spores were dislodged; the mount was held in moist air and this photograph taken some hours later.

Following infection with spores, mature *H. stylopygae* are found on *Blatta orientalis* in a little less than two weeks (25–27° C.). About the same length of time is required for *H. ectobiae* on *Blattella germanica*. Somewhat longer, perhaps three weeks, is required for *H. tricuspidatus* on *Blaberus*.

Egg capsules from heavily infected *B. orientalis* females give batches of nymphs which do not develop infection. Also spores dusted or brushed onto egg capsules or exposed eggs do not develop into visible plants. Finally, surgical implantation of infected antennal fragments into various parts of the body cavity of oriental cockroaches gave entirely negative results.

In conclusion, infection is readily obtained by direct contact placing the spores on an appropriate external surface but, in our experience, by no other means. It is reasonable to suppose, as Thaxter (1896) did, that infection might also be indirect by deposition of a spore on any surface from which another insect by chance collects it onto itself. Lindroth (1948) has recently reported such indirect transmission in certain *Laboulbenia* spp. where apparently spores may survive in soil for some weeks before being picked up by a passing beetle. We have not attempted to check this possibility but to judge from our experience with glass needles (Table I) we would suspect that while many spores are doubtless dropped, few would ever subsequently become transferred to a passing insect.

Notes on Asci and Spores of H. stylopygae

Having only dry material at his disposal, Thaxter was unable to be sure how many spores were in an ascus. Most Laboulbeniales have four spores per ascus

but some have eight. Clearly there are eight in *H. stylopygae* (Figs. 7 and 8). The asci are a fat cigar-shape and measure about 12×60 μ, those used for the photographs seeming to be broader because somewhat flattened by cover-glass pressure to bring the spores into one focal plane.

An apparently new point is that in this species the asci do not disintegrate to liberate spores within the perithecia. Perithecia of living plants teased in a drop of water liberate both spore-filled and empty asci (Figs. 8 and 9). The spore-filled asci were sometimes seen to liberate their spores one after another through

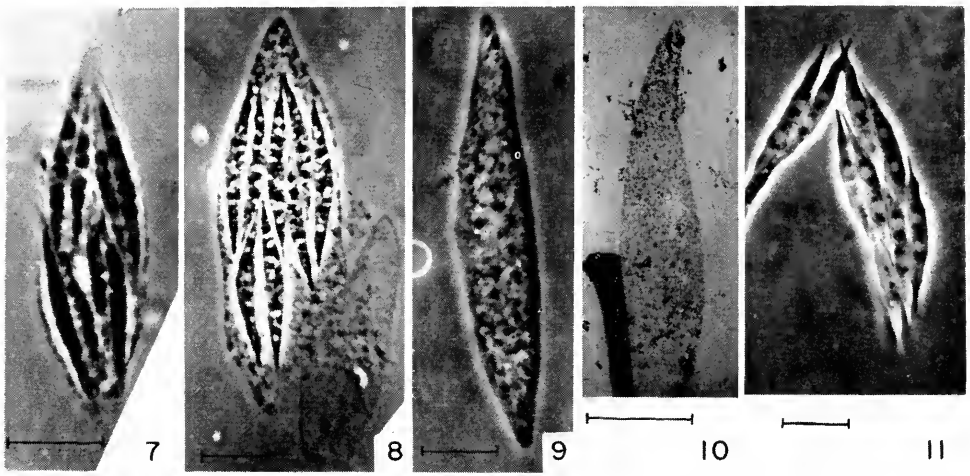

FIGURE 7. Mature ascus with its eight spores after staining with the gram stain. From perithecium of *H. stylopygae*. Note that both spores and the ascus wall are gram positive. Bar 10 μ.

FIGURE 8. Mature ascus of *H. stylopygae* in water; somewhat compressed by coverglass pressure. Dark phase-contrast photomicrograph of living material. The pressure used to get all the spores into one focal plane has resulted in a rupture of the ascus wall and extrusion of material at the lower right of the figure (such rupture is not normal). Bar 10 μ.

FIGURE 9. Empty ascus or ascus ghost of *H. stylopygae* in water. Dark phase-contrast photomicrograph (not stained). Note contents, absence of any indication of rupturing or disintegration (spores released through terminal pore), absence of any operculum, and apparently unitunicate condition. Bar 10 μ.

FIGURE 10. Electron micrograph of empty ascus ghost of *H. stylopygae*. Shows extreme thinness of dry ascus wall, and hence small amount of solid material therein, and no indication of more than a single layer. Bar 10 μ.

FIGURE 11. Living spores of *H. stylopygae* in water; teased from fresh perithecium. Note clustering of the spores. Bar 10 μ.

an aperture in the end as drying of the water began to exert cover-glass pressure on the ascus. No rupture of the ascus wall was evident, and the terminal hole is not visible after the spores have been discharged (Fig. 9). Although the observed liberation of spores was under unnatural condition, the presence of numerous empty asci within partially emptied perithecia implies that liberation of the spores is normally via a perforation (presumably at the distal end) rather than by disintegration. As Figures 9 and 10 show, the asci are inoperculate and unitunicate (see Luttrell, 1951).

The spores seem to be always liberated from the asci while within the perithecia. At least we have never seen asci ejected, the canal of the perithecium is too small for an intact ascus, unless the ascus were compressed, and we obtained only spores, never asci, in picking spore groups off the tip of the perithecium (Table I).

After liberation of the spores, the empty ascus is left as a ghost containing many highly refringent granules and droplets (Fig. 9). Commonly, active Brownian movement is evident. Dr. Benjamin suggests that these granules and droplets represent epiplasm, *i.e.*, protoplasm remaining after the aggregation of most of the material into spores (by "free cell formation"). The interior of mature perithecia contains gram-negative, bipolar-staining, short-rod, bacteria. In stained smears these are particularly abundant around empty or torn asci.

The ascus wall, like the spores, is gram-positive, and of the order of 0.1 μ thick. On electron microscope grids the empty asci dry down to give ghosts of about 9×43 μ; correcting for the flattening this gives about 6×40 μ. The electron micrographs show only that the ascus wall is exceedingly thin after drying, not over a few hundredths of a micron thick, and that it is heavily contaminated with debris, presumably from the granule-containing fluid within the empty ascus (Fig. 10).

It is generally stated that spores are discharged in pairs throughout the Laboulbeniales although various published drawings of asci do not show such association of spores in pairs. Dr. R. K. Benjamin tells us that there is good evidence to believe that this generality holds for many genera because the dioecious plants are invariably found growing in pairs even though widely scattered on the host (*Dioicomyces, Aporomyces, etc.*).[3] However, Thaxter (1931) has already remarked on the commonness of single plants in *Herpomyces*, and it became evident early in our studies that this was partly or perhaps entirely due to the discharge of single spores. Single plants could also originate from the loss of one member of a spore pair; we do not know whether this occurs naturally but in lacto-phenol mounts one sometimes sees two sets of holdfasts but only a single spore (Fig. 13), indicating the loss of one member of the pair either before or during preparation of the slide.

To determine the actual number of spores discharged at one time, a census was made of the spore clusters protruding from the perithecia of 128 ($= 2^7$) plants. For this purpose, infected oriental cockroaches were observed under high power of a dissecting binocular microscope, protruding spore groups picked off individual perithecia by touching the group with the tip of a fine glass microneedle, and then transferring the spore group to a droplet of cotton-blue stain in lacto-phenol. Subsequently the coverglasses bearing rows of stain droplets were inverted on a depression slide and each droplet carefully searched, with the final check on spore count being made under oil immersion. Validation of the technique was observational: the protruding spore groups could be seen to be removed;

[3] This opinion assumes that all spores germinate and grow into readily located plants. An alternative possibility is that plants mature only when in pairs. Some reason for holding the second possibility in mind until it is disproven comes from data on the germination of *Herpomyces* on hosts on which they will not mature. These data will be presented subsequently. Also we have occasionally seen single plants of *H. ectobiae* alongside an ungerminated spore; these could have arisen from growth of only one of a spore pair (Fig. 6).

they could be seen on the microneedle, and the microneedle was clean following immersion in the stain droplet.

The data may be tabulated in various ways (Table I). There is some question as to whether these particular numbers could be reproduced because the one-two dozen cockroaches used were not truly randomized and we noted that one individual would yield only single and paired spores whereas the next one might give a high percentage of multiple groups. Perhaps such individual differences are due to the age of the plant or (more likely, we think) due to the length of the time interval since protruding spores were last brushed off. However, the important points are independent of the above uncertainty. These are:

(1) The spore groups protruding from perithecia may be sufficiently adherent to remain together as a compact cluster in the stain droplet, or they may dissociate into several (up to 4) smaller groups, even to single spores.[4] In column

TABLE I

Tabulation of data from 128 protruding spore groups picked off the tips of perithecia (columns 1 plus 2), and from the 188 spore groups into which these separated in the stain droplets (columns 5 plus 6). Total number of spores 362

Number of spores per group	From 128 perithecia		Total spores per group		From 188 adhering groups		Total spores per group	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
1	14	11	14	4	75	40	75	21
2	62	48	124	34	82	44	164	45
3	10	8	30	8	10	5	30	8
4	32	25	128	35	16	9	64	18
5	5	4	25	7	3	1.6	15	4
6	1	0.8	6	1.7	1	0.5	6	1.7
7	1	0.8	7	2	0	0	0	0
8	2	1.6	16	4.4	1	0.5	8	2.2
12	1	0.8	12	3.3	0	0	0	0

1, the numbers found per stain droplet (= per perithecium) are listed; in column 5 the numbers found per association group are listed. The 128 perithecia actually gave 188 groups in the stain. Perhaps a similar dissociation could occur on transference to the surface of another host (see below).

(2) The ejection of single spores is common.

(3) Most of the spore groups consisted of 1–4 spores but higher numbers up to 12 were occasionally found.

(4) The values shown in columns 1–4 of the table do not represent any common form of distribution; they are probably the product of several ejections preceding dislocation of the group (see below). In contrast, the values in columns 5–6 (listings for 188 adhering groups) do represent a skew-form distribution

[4] Sets of two were usually pairs but commonly two singles; sets of three were either 3 or 2 + 1 or 1 + 1 + 1; sets of four were 50% tetrads but sometimes 2 + 2 or less often 2 + 1 + 1 or 1 + 1 + 1 + 1; sets of five consisted of 5 or 3 + 1 + 1 or 2 + 2 + 1 or 2 + 1 + 1 + 1; the set of six consisted of 4 + 2; the set of seven was 4 + 2 + 1; the sets of eight were one set of 8 and one of 3 + 2 + 2 + 1; and the set of twelve was 6 + 5 + 1.

curve commonly encountered in biology; they also give a closer approximation to what one can see on the surface of cockroaches from infected colonies.

Another important point not shown by the table is that the aperture in the perithecium is not of sufficient diameter to permit the simultaneous passage of groups of 8 or 12 spores, and is questionably adequate for the simultaneous passage of a tetrad. Probably the answer is to be deduced from the observation that perithecia under coverglass pressure in a drop of water may be seen to eject spores like bullets coming out of a machine gun. They are ejected too rapidly for accurate determination of how many emerge at once but under these conditions they seem to come out singly. The important point, however, is that they are all ejected for about the same distance and hence align to form a cluster which to visual inspection may appear to be as tight a pack of adhering spores as if they had been ejected

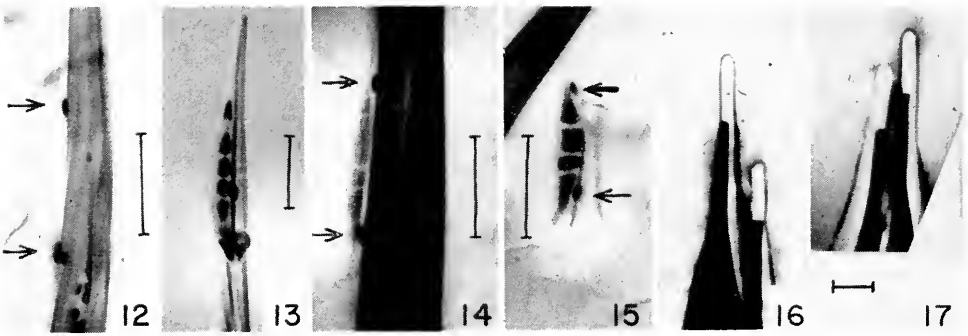

FIGURE 12. Part of a setal shaft from antenna of *Blatta orientalis* showing the two spore holdfasts (arrows) of *H. stylopygae* after dislodgment of the spore during preparation of slide. Cotton-blue stain. Bar 10 μ.

FIGURE 13. Spore of *H. ectobiae* in 4-cell stage on antennal seta of *Blattella germanica*. Note that there is a pair of basal holdfasts; presumably one spore of a pair became dislodged. Cotton-blue stain. Bar 10 μ.

FIGURE 14. Spore of *H. stylopygae* in 4-cell stage on an antennal seta of *Blatta orientalis*. Shows basal and apical holdfasts (arrows) but the sheath is almost invisible. Bar 10 μ.

FIGURE 15. Germinating spore of *H. stylopygae* on surface of antenna of *Blatta orientalis*. Note characteristic method of growing over the basal holdfast. Cotton-blue stain. Bar 10 μ.

FIGURES 16 AND 17. Electron micrograph of ends of two spores of *H. stylopygae*. The line in the underlying formvar membrane shows the original position and shape of the end of the spores. Under electron bombardment the spores have shrunk back and developed blunt ends (opaque). Bar 1 μ.

as a group. We suspect that ejection from the perithecia of *H. stylopygae* is of single spores and that groups of pairs or more represent two or more ejections which adhere because of the sticky external coat (said to be "gelatinous").

Examination of amputated antennae or other areas of the body wall, or of shed skins (exuvia), stained and cleared with cotton-blue in lacto-phenol and examined as whole mounts show large numbers of single and paired spores both on setae (Figs. 13–14) and on the body wall proper (Figs. 3 and 15). Larger groups are uncommon but occasionally seen. These data suggest that the numerous single plants arise from the development of single spores, and that at least some of the large plant clusters commonly seen arise from the development of multiple spore clusters.

Incidentally, both male and female plants develop to maturity in isolation; presumably the crowding permits fertilization of such female plants by nearby males. Single and paired spores (Fig. 13) as well as single (Figs. 4 and 6) and paired (Fig. 5) plants were also found with *H. ectobiae*.

Spores are normally and probably always discharged in the 2-cell stage. All of the 362 spores of Table I were 2-celled. Suspended in distilled water (Fig. 11) they measure 2–3 × 20–30 μ. Likely these spores are somewhat enlarged by uptake of water. A dried spore examined in the electron microscope measured 1.4 × 25 μ with the bluntly rounded tips (Figs. 16–17) measuring 0.3 × 2.5 μ. These spores are colorless when ejected and have a thin wall. Both nuclei and cytoplasm are acidophilic in staining and gram-positive (Fig. 7). In the living state the clear nuclei are about 2 μ in diameter and are surrounded by small granules a small fraction of a micron in diameter (Fig. 11). The birefringent walls of the spore show in electron micrographs microfibers a few hundreths of a micron in diameter—a common microfiber diameter for chitinous membranes (Richards, 1951).

On the host the spores quickly become 4-celled and then usually show a brown or black spot at each end (Figs. 12–15). There is little or no visible difference between the two ends of the spores in either the 2-cell or 4-cell stages. That the brown or black spots at each tip serve as holdfasts, not previously reported to be present in any species of Laboulbeniales, is shown by the fact that when a spore becomes dislodged during preparation of a lacto-phenol mount these blobs may remain stuck firmly to the body wall or seta (Fig. 12). In the latter part of the 4-cell stage the germinating spores do show a longitudinal differentiation (Fig. 15).

Quite possibly the spores always emerge from the perithecium in one orientation but it does not seem reasonable to assume that with a brushing or dusting dissemination they always become applied to setae or the general body wall in one particular orientation. On setae they are always parallel to the longitudinal axis of the seta, as would be dictated by the action of surface forces, but on the general body wall they are oriented in all directions parallel to the surface. Yet on setae the plants always develop with the base at the proximal end of the seta, and on the body wall the apices of the plant project distally in relation to the antenna. It seems necessary to assume that either the orientation of the developing plant is somehow imposed by its position on the insect's cuticle, or that only those plants appropriately oriented develop (those in reverse orientation not developing and so contributing to the number of ungerminated 4-cell spores always present). Incidentally, artificially germinated spores also show visible differentiation of the two ends shortly after the 4-cell stage, as will be treated in a subsequent paper.

Volume Increase During Growth of H. stylopygae

Assigning appropriate geometric figures to the various stages one can calculate approximate volumes and hence volume increase during growth. Cigar-shaped spores, 2 × 25 μ, have a volume of approximately 25 cubic microns. Mature female plants calculated as a combination of cones, cylinders and elliptical spheroids have a volume 90,000 to 400,000 cubic microns, depending primarily on the number and size of the perithecia. The most gigantic female plants with haustorial bulbs 40 × 100 μ would have a volume of approximately 500,000 cubic

microns. The portion of the plant visible on the surface of the insect, *i.e.*, plant minus haustorium, is considerably smaller, averaging 75,000 to 350,000 cubic microns. (Details of the structure of haustoria are given in part 2 of this series.)

The volume of a female plant is, then, 3500 to 20,000 times that of the spore from which it grows. Male plants are much smaller. Rough calculations from illustrations and measurements given in Thaxter's monographs show that other species of Laboulbeniales increase by 300 × to > 100,000 × the spore size (not including the volume of haustoria presumably present). Obviously a considerable amount of nourishment must be obtained from the host even if we assume that the average density of the spore is considerably greater than that of the mature plant.

Loss of the Infection During Molting

In most cases (Diptera, Coleoptera, Hymenoptera), Laboulbeniales have been described from adult insects which do not molt. But in cockroaches they are found both on adults and the similar-appearing cohabiting nymphs. In adults the infections seem to survive until the insect dies, and in some cases Thaxter has described what he considered to be age changes in certain species. In nymphs of both *Blatta* and *Blattella* the infection is completely lost at ecdysis (molting). The external portions of the plant are visible on the exuvia (shed skin) and appear normal until they dry up.

In a direct test, 18 nymphs of *B. orientalis* were caught in the act of molting. The exuviae were checked and the infections mapped. The individuals were isolated and checked at intervals for 22 days (> 50% longer than the usual life cycle of the fungus). In no case did any infection develop. Many more have been tested indirectly by our finding that the most convenient method for getting known-to-be-uninfected cockroaches is to segregate into a clean container specimens by chance caught molting in our cultures. To the best of our knowledge this phenomenon of the complete loss of infection by molting has not previously been reported.

The failure to regenerate an infection after the thalli have been lost at molting shows that the large haustorial bulbs (see part 2) which must be left behind in the cockroach's epidermis cannot regenerate the lost plants. We are inclined to correlate this with the absence of nuclei from the haustoria but obviously other factors could be involved.

We have never seen any indication of the development of resistance to infection. Infected individuals freed of their infection by molting seem to be just as readily infected as ones not previously exposed to the fungus. In fact, in heavily infested colonies, most of the individuals have been re-infected in each instar and yet the really heavy infections develop on adults. Also, the re-infection may occur immediately because we have obtained some infected individuals after segregating specimens of *B. orientalis* which had molted so recently that their cuticles were still white (*i.e.*, < one hour after molting).

Notes on H. ectobiae Thaxter

H. ectobiae growing on *Blattella* (= *Ectobia*) *germanica* has not been studied as intensively. Some differences from the habits of *H. stylopygae* have been noted.

Infections with *H. ectobiae* seem always to start on setae (Figs. 4–6). Usually they grow to the setal base and make an additional haustorial penetration there (Figs. 4–5). When they spread across the antennal surface larger plants result (Fig. 5). Of the hundreds we have examined no one has been seen in such a position that it could not have started development on a seta. We have noticed that male plants are more likely to reach large size without attaining the setal base than female plants are (Fig. 6). Female plants seem always to attain the setal base prior to maturing perithecia (Figs. 4–5).

Unlike *H. stylopygae, H. ectobiae* develops on any part of the surface of its host though nowhere attaining the density of heavy infections on antennae of *Blatta* and *Blaberus*. There is no evident pattern to the infection and no obvious differences correlated with sex of the host. Systematic survey with tabulation of infected areas on 34 individuals of *B. germanica* showed mature plants on antennae, maxillary and labial palpi, frons, coxae, femora, tibiae and tarsi of all pairs of legs, wings, thorax, abdomen (dorsal, lateral and ventral), and cerci. Some individuals had plants growing simultaneously on most of the above regions. Nymphs of *Blattella* commonly develop heavier infections than nymphs of *Blatta* do.

The spores of *H. ectobiae* regularly have a brown or black spot at each end in both the 2-cell and the 4-cell stage (Fig. 13); at least the proximal one of these appears to serve as a holdfast.

Summary

1. *Herpomyces stylopygae* Speg. is shown to be distinct from *H. periplanetae* Th. by host specificity tests.

2. Spores of *H. stylopygae* are found all over the surface of oriental cockroaches but mature plants are mostly found on the antennae, seldom on palpi and only rarely elsewhere. They grow on setae or on hard or soft cuticle but only on a living cockroach. Infections are heavier on males and on adults and experiments show the infection is disseminated by contact.

3. The ascus contains 8 spores which it liberates within the perithecium through a terminal perforation, leaving the ascus as a fluid-filled ghost.

4. Spores are ejected from the perithecia in various numbers, not just in pairs (Table I). Mostly the groups protruding from the subterminal apertures of the perithecia (Fig. 2) consist of 1–4 spores but groups as large as 12 spores were found. The presence of single, paired and multiple spore groups protruding from perithecia and found on the surface of hosts is correlated with the presence of single, paired and multiple plants on infected cockroaches.

5. Antennae of infected cockroaches serve as efficient spore brushes and dusters.

6. Spores become firmly attached to the cockroach's cuticle by holdfasts developed at both ends.

7. Development from spore to mature perithecia takes nearly two weeks.

8. The volume of a female plant is 3500–20,000 times that of a spore. So much material cannot be obtained from a minute volume of cuticle. A tubular haustorium through the cockroach's cuticle was found to expand into a large bulb in the epidermal cell layer.

9. Infections on adults persist but infections on nymphs were found to be completely lost when the nymph molts. The fungus plants are found intact on the shed skin.

10. There appears to be no development of resistance since individuals freed of infection by molting can be readily reinfected.

11. Some notes are given on spore structure, and on differences shown by *H. ectobiae*.

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CELL MOVEMENT, RATE OF REGENERATION, AND THE AXIAL GRADIENT IN TUBULARIA [1]

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One of the great unifying concepts in the field of development has been that of the axial gradient. As Child envisions the development of polarity in an organism, there is a quantitative gradient in metabolic rate which marks the primary axis, this gradient being responsible for the establishment of qualitative differences along the axis during the course of development (Child, 1941). Such gradients have been demonstrated in a wide variety of plants and animals, among which is the extensively investigated hydroid, *Tubularia*. In this animal the axial gradient is manifested physiologically by a decreasing rate of hydranth regeneration as one proceeds from distal (apical) toward proximal (basal) levels of the hydrocaulus or "stem." This phenomenon was first observed by Loeb (1892), who did not consider the differences in rate to be significant, but confirmation by a host of other workers (Driesch, 1899; Morgan, 1905; Child, 1907a; Hyman, 1920; Barth, 1938, 1940; Spratt, unpublished) leaves no doubt concerning its reality.

During a recent investigation of the mechanism of physiological dominance in *Tubularia* (Steinberg, 1954) it became necessary to measure the rates of regeneration of distal and proximal ends of stems ligated in the middle. In such stems the distal end regenerates considerably faster than the proximal end (Morgan and Stevens, 1904; Peebles, 1931; Barth, 1938). It was observed that once the primordium of the regenerating hydranth was established as a thickened zone near the cut end, differentiation seemed to proceed at about the same rate at both the distal and the proximal ends. The increased time for proximal regeneration apparently was due to a delay in the establishment of the primordium rather than to a slower rate of its differentiation into a fully formed hydranth. Since the experiment was set up with another purpose in mind, the measurements necessary to test the validity of this observation were not made, but other considerations seemed to lend weight to this view.

The axial gradient in *Tubularia* is manifested not only by differences in rate of regeneration, but also by a gradual diminution in size of the primordium formed as pieces are taken from increasingly basal regions of the stem (Driesch, 1899; Hyman, 1926; Barth, 1938). There is also a direct correlation between the length of a piece and the size of the primordium which it develops (Driesch, 1899; Child, 1907b; Peebles, 1931; Spratt, unpublished), contrary to the results of Hyman (1926), longer pieces giving rise to larger primordia. It was found by Peebles (1931) that regeneration could be delayed and the size of the as yet unformed

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primordium of a long stem reduced by removing a large piece from the proximal end of the stem only if the piece was removed before 13 hours of the regeneration period had elapsed. These observations were confirmed by the author (Steinberg, 1954), in whose material, however, a period of 8 hours sufficed to produce the effects obtained by Peebles only after 13 hours. It was shown in addition that the period preceding the appearance of the primordium is one during which the tissue shifts toward the distal end of the perisarc, providing additional cells for incorporation into the primordium, and that rate of regeneration and the size of the regenerant are both functions of the number of cells available. By these experiments it is shown that both rate of regeneration and the size of the regenerant are fixed before the primordium appears. Certain factors, such as the tissue movement, which affect both of these parameters of regeneration, appear to operate exclusively before primordium formation. The evidence presented above applies to systemic factors which affect the rate of regeneration and the size of the primordium at a given level of the stem. It does not, however, exclude the possibility that the rate of differentiation of the primordium into a completed hydranth may vary from level to level or as a function of a large number of other natural variables affecting regeneration rate.

Rate of regeneration in *Tubularia* varies as a function of the following systemic parameters: length of the piece, width of the piece (Hyman, 1926), apico-basal level from which the piece is taken, and distal versus proximal regeneration site on the piece. These variables were chosen for investigation in the present study, the purpose of which was to ascertain whether the differences in rate of regeneration which exist between long versus short stems, narrow versus wide stems, the distal versus the proximal ends of ligated stems, and finally apical versus basal stem levels all lie in the period of tissue movement, or whether under the various conditions mentioned the differentiation process itself proceeds at different rates.

Materials and Methods

Straight, clean, unbranched stems from freshly collected colonies of *Tubularia crocea* were used exclusively. The proximal cut was always made slightly oblique in order to distinguish between distal and proximal ends. Regenerating stems were kept individually in labelled stender dishes of two-inch outside diameter containing ⅜ of an inch of sea water and maintained at a fairly constant temperature on a sea water table over which fresh sea water was constantly flowing. The time of cutting of each stem was noted, and at intervals of a few hours until regeneration in all groups was complete, each stem was inspected and its regeneration stage and the time recorded. To maintain objectivity, the previous history of a stem was never consulted in making a new recording of its regeneration stage.

The animals were divided into 5 pairs of groups as follows, with 20 stems in each group.

I (20° C.)
- Group A. 8-mm. stems with the distal end taken two mm. below the hydranth.
- Group B. 8-mm. stems with the distal end taken 30 mm. below the hydranth, and from the same stems as Group A.

II (16.5–18° C.)
- Group C. 2-mm. stems with the distal end taken two mm. below the hydranth.
- Group D. 15-mm. stems taken just below the 2-mm. stems of Group C.

III (16.5–18° C.)
- Group E. 5-mm. stems of very small diameter, taken two mm. below the hydranth.
- Group F. 5-mm. stems of very large diameter, taken two mm. below the hydranth and from the same colony as Group E.

IV (16.5–18° C.)
- Group G. The distal ends of 10-mm. stems taken two mm. below the hydranth and ligated in the middle with nylon thread.
- Group H. The proximal ends of the stems in Group G.

V (19–20° C.)
- Group I. The same as Group G, but taken from a different colony.
- Group J. The proximal ends of the stems in Group I.

After all of the stems had regenerated, the period of regeneration of each stem was divided into two parts: (1) the length of time between the cutting of the stem and the appearance of the thickening which is the primordium of the regenerating hydranth (= movement time), and (2) the length of time between the appearance of the thickening and the appearance of the constriction which marks the base of the regenerated hydranth (= differentiation time). (For drawings of the stages see Steinberg, 1954.) Since the object of the experiment is to distinguish between the time required for movement and the time required for differentiation, the onset of the "thickened" stage was chosen as the division between the two periods because (1) the only known process preceding it (except for the breakdown, near the two cut ends, of the endodermal ridges which traverse the length of the stem) is the tissue movement, and (2) Davidson and Berrill (1948) have shown that this is the stage at which one finds the first evidence of determination by the test of isolation of parts of the thickening, followed by self-differentiation. It should be mentioned that determination here is only with respect to the replacement of missing proximal structures. Missing distal structures are always replaced in *Tubularia,* even by the adult hydranth.

Experimental Results

1. The basis of the axial gradient and differences in rate of regeneration

The results of the experiments are summarized in Table I. The experiment (II) in which short and long pieces are compared is not satisfactory because 14 of the 20 two-mm. stems which comprised Group C regenerated as partial bipolar forms. In these forms, common in short pieces, the entire tissue of the piece transforms into a pair of hydranths fused at their bases. Such bipolars lack proximal tentacles and in some cases gonophores as well. It is impossible to get regeneration rates in such stems because the constriction which marks the base of a regenerated hydranth and the formation of which is used as the end point of regeneration never appears. As Table I shows, no significant differences were

found, either in the movement or the differentiation phase, between groups C and D, probably because of the small number of cases in Group C. However, in every other pair of experiments there is a large difference in movement time between the two groups of the pair, the group regenerating more slowly always being the one in which the tissue movement occupies more time. This is not the case with respect to differentiation, however. In experiments III, IV and V the differentiation time of both groups of the pair is the same. Experiment I presents a different picture. Group B differentiated *more rapidly* than Group A. This is indeed remarkable, since B is the more slowly regenerating group of the pair,

TABLE I

Mean time required, with the standard error of the mean, for the entire regeneration process and for its two component parts, tissue movement and differentiation, in the various paired experimental groups; p indicates the probability that the difference between the two groups of a pair is due to chance alone. Standard errors and probabilities were calculated by the use of Student's "t" test

Experiment	Group	Description	No of stems	Hours for regeneration	Hours for movement	*p*	Hours for differentiation	*p*
I	A	Distal piece	19	22.6±0.3	14.1±0.2	≪0.001	8.5±0.2	≪0.001
	B	Proximal piece	19	27.9±0.4	21.6±0.2		6.3±0.3	
II	C	Short piece	6	68.3±7.9	32.0±4.4	≃0.1	36.3±6.5	≃0.4
	D	Long piece	16	53.8±3.7	24.2±2.1		29.6±3.0	
III	E	Narrow piece	16	48.5±4.6	22.5±2.2	≃0.005	26.0±4.0	≃0.4
	F	Wide piece	16	59.1±3.1	32.7±2.1		26.4±2.2	
IV	G	Distal end of piece ligated in middle	17	33.3±1.0	21.4±0.7	≪0.001	11.9±0.7	≃0.1
	H	Proximal end of stems in Group G	18	46.5±1.0	33.0±0.8		13.5±0.7	
V	I	See G	19	41.2±2.9	25.8±2.8	<0.001	15.4±0.8	>0.9
	J	See H	15	75.2±5.3	59.9±5.0		15.3±1.7	

being composed of pieces taken from the proximal region of long, unbranched stems, while the pieces of Group A are taken from the distal region. Thus in the case of distal versus proximal pieces of long stems, the slower rate of regeneration of the proximal pieces is entirely due to a slower rate of distad movement of the tissue, preceding the establishment of the hydranth primordium. This means that the axial gradient, as manifested by graded differences in rate of regeneration, is in reality a gradient in rate of tissue movement rather than a gradient in general "metabolic rate" prevailing during the entire period of regeneration. That the physiological processes occurring during the differentiation of a new hydranth

are certainly not slower in the more proximal regions is clearly shown by the fact that in these experiments the differentiation process itself is actually more rapid in proximal than in distal regions. In the other experiments, also, dealing with two groups regenerating at different rates, the difference in rate of regeneration between the two groups is in every case due to a difference in the time occupied by the tissue movement. In no case is it due to a difference in the rate of differentiation.

2. Reverse movement, rate of differentiation, and dedifferentiation

Table I shows that groups taken from different colonies may regenerate at markedly different rates. (Each experiment was performed with stems from a separate colony from which both groups within the experiment were taken.) Individuals *within* a colony may also regenerate at widely different rates. Table II illustrates the amount of variation in regeneration rate which may exist between comparable pieces from stems taken from a single colony. This particular sample

TABLE II

An example, drawn from the protocols of Group F, to show the amount of variation in rate of regeneration which may exist among comparable stems from the same colony. The figures indicate the number of stems in each stage at the times given

Time in hours	Nothing	Optical density gradient	Distal thickening	Proximal ridges	Distal ridges	Constricted or emerged
16.5	15	5	0	0	0	0
21.0	12	4	4	0	0	0
25.5	11	4	3	2	0	0
36.0	2	1	12	3	2	0
40.0	1	0	9	7	1	2
47.0	1	0	4	8	5	2
55.0	1	0	3	4	4	8
71.0	0	0	0	2	1	17

(Group F) was selected because it possessed the greatest amount of variation of any of the groups in these experiments. Not only does rate of regeneration considered as a whole vary, but the rate of differentiation also varies widely from colony to colony, as shown in Table I, and between different individuals from the same colony, as shown by the fairly sizeable standard errors for differentiation in some groups.

The protocols of the experiments show that neither the tissue movement nor the differentiation is always continuously progressive. In a large number of cases the tissue movement has been found to stop and then to reverse itself. In fact, regeneration at the proximal end of a piece ligated in the middle or of a long piece in which dominance does not act is dependent upon such a reversal in the direction of movement of the tissue (Steinberg, 1954). This is usually true in *T. crocea,* with which the present experiments were performed. However it is invariably true in the species (probably never identified) used for the experiments just referred to, because in that species the tissue movement is much more pronounced than it is in *T. crocea.* In yet a third species (referred to by Mr. Gray

of the Supply Department of the Marine Biological Laboratory as "the long, white kind") the movement is equally pronounced, while in a fourth species (referred to as "the short, brick-red kind," and probably *T. tenella*) the movement does not occur—a fact which has marked effects upon the phenomenon of dominance. In addition to reverses in the direction of movement, many cases have also been observed in which the differentiation process came to a standstill and then reversed. In the most extreme case a stem in the distal tentacle ridge stage, on the verge of constricting, began to dedifferentiate and continued this process until all traces of differentiation disappeared, including the thickening which had marked the primordium of the regenerating hydranth. At the same time it was observed that the tissue was moving back toward the proximal end of the piece. Much later a new primordium appeared and this time regeneration was completed. Dedifferentiation in *Tubularia* has been described before (Driesch, 1897, 1908; Peebles, 1900), but only as one possible aftermath to the cutting of the differentiating primordium. In the present cases it appears as a not infrequent occurrence in normal regeneration.

The observation that during the dedifferentiation of one primordium the tissue was undergoing a shift back toward the proximal end suggested that the many cases of dedifferentiation might be attributable to such movements. This seemed reasonable because (1) differentiation does not begin until a certain minimal concentration of cells has been accumulated at the presumptive regeneration site, and (2) the degree of regulation within a one- or two-mm. piece is also dependent upon cell number (Steinberg, 1954). This suggests that the continued differentiation of the primordium might be very sensitive to a decrease in its cell density, such as would occur if the tissue of the stem were to move away from it during the course of its differentiation. A direct test of this hypothesis by observation is not always possible because only rather gross movements of the tissue can be detected. However a rough test can be made by plotting the mean time for differentiation in a group against the number of reverses per stem in the movement phase. There are difficulties in the preparation of such a graph. It is not easy to decide in what cases a reversal in the direction of movement as noted in the protocols is truly a reversal and in what cases it merely reflects the fair amount of subjectivity involved in such observations. As has been mentioned before, in order to preserve as much objectivity as possible, the previous recordings for a regenerating stem were never looked at when a new recording was being made. As a result a considerable number of borderline cases are placed on one side of the line in one recording and on the other side in the next. Quite obviously these cases cannot be considered as true reverses in the movement of the tissue. Therefore the following three alternative criteria were established for the validity of reverse recordings. (1) Both the original stage and the subsequent reverse in the movement must be established by at least two consecutive similar recordings for each stage; (2) there must be at least two consecutive recordings which progress in the reverse direction; or (3) the recorded reverse in the movement must be of such magnitude that it could not be attributed to a difference in judgment. When these criteria are rigidly applied, the number of reverses is reduced so drastically that the maximum number in any group is four (Group D), with other groups yielding two reverses (Groups C, F and J), others yielding only one (Groups E, H and I), and the remaining groups yielding none (Groups A, B and G). These

criteria, although they are the best that could be devised, are for two reasons not nearly adequate for the establishment of an accurate correlation between reverse movements and the mean rate of differentiation. (1) In some groups there is a very large number of reverse recordings which do not meet any of the above criteria for significance, while in other groups there are none or very few. This

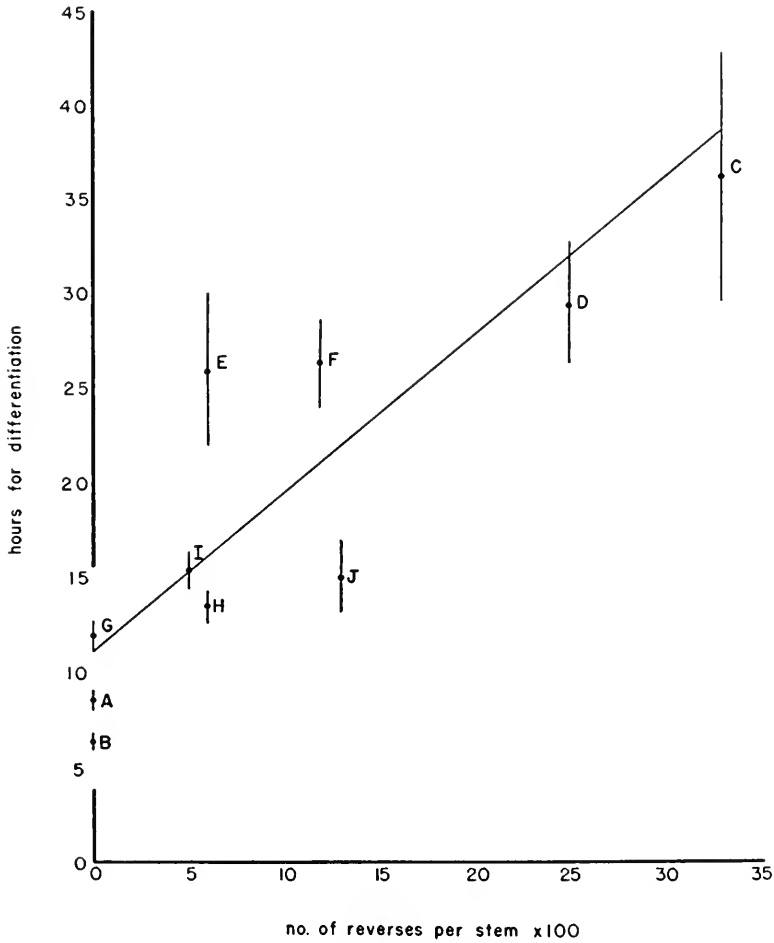

FIGURE 1. Correlation between the number of reverses during the movement phase and the time required for differentiation in the various groups. The line was arrived at by the Method of Least Squares.

can only mean that many reverse movements of short duration occur in some groups but cannot be distinguished from reverse recordings which are attributable to differences in judgment. (2) The number of reverses that meet one of the three criteria for significance is so small that it is bound to introduce large errors in the calculations. Nevertheless a very rough correlation between reverse move-

ments and rate of differentiation should be found if such movements really act to retard or reverse the differentiation process. That this rough correlation does indeed exist is shown in Figure 1. There is a general tendency for groups showing a higher percentage of reverses in the movement phase to show a slower mean rate of differentiation.

From the above we see that at least part of the differences in rate of differentiation which exist between colonies and between individual stems from the same colony are due to retardations and reversals in the differentiation caused by a movement of the tissue away from the differentiating primordium. The knowledge of such a correlation opens the way for a more quantitative treatment of the relationship between

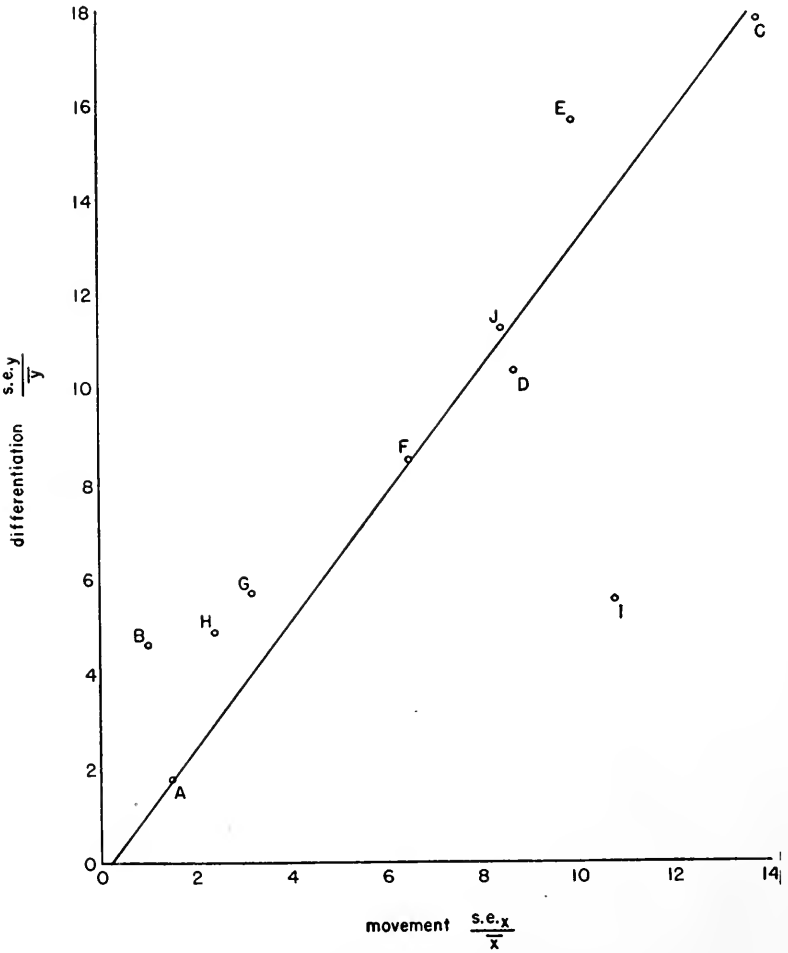

FIGURE 2. Correlation between the variation in time required for movement and the variation in time required for differentiation in the various groups. $\bar{x}$ = mean time for tissue movement; S.E.$_x$ = standard error of the mean for movement; $\bar{y}$ = mean time for differentiation; S.E.$_y$ = standard error of the mean for differentiation. The values on both axes are multiplied by 100. The line was arrived at by the Method of Least Squares.

variations in tissue movement and variations in rate of differentiation among similar stems from the *same* colony. The variation in tissue movement among the stems in a group can be numerically expressed by the figure $S.E._x/\bar{x}$, where $\bar{x}$ is the mean time for movement and $S.E._x$ is the standard error of the mean. Similarly the variation in rate of differentiation in a group can be expressed by $S.E._y/\bar{y}$, where $\bar{y}$ is the mean time for differentiation and $S.E._y$ is again the standard error of the mean. Since the variation in rate of differentiation has been shown to be caused, at least in part, by variations in tissue movement, the relationship between the two can be expressed by plotting the values of $S.E._x/\bar{x}$ against the values of $S.E._y/\bar{y}$ for the various groups and fitting the best possible straight line to the resulting points. This involves the assumption that the tendency of the stems in a group to undergo variations in movement is constant throughout the entire regeneration period. Should a particular group by chance show more variation in movement during one phase than during the other, the calculated value of $S.E._x/x$ for that group will be correspondingly high or low. Nevertheless, as far as can be determined, such fluctuations should occur equally in both directions, so that the over-all accuracy of the plotted line should not be seriously affected. Figure 2 shows the resulting graph. The fact that the line, obtained by the Method of Least Squares, passes almost directly through the origin indicates that differences in the movement of the tissue probably represent the *only* systemic factor contributing to variations in rate of differentiation among similar stems from the same colony. It does not, however, exclude the possibility that other factors may contribute to the large differences in rate of differentiation between *different* colonies. The slope of the line (1.35) indicates that the differentiating primordium is quite sensitive to slight movements of the tissue, an increase of 1 in $S.E._x/\bar{x}$ being responsible, on the average, for an increase of 1.35 in $S.E._y/\bar{y}$. This constitutes evidence that the differentiation process is continuously dependent upon a critical cell density in the primordium, and that when the cell density falls below this critical level the process of differentiation stops and even reverses.

3. The nature of the tissue movement

The movement of the tissue toward the distal end of the stem is due to an apparent contraction of the tissue as a whole in the distal part of the stem, accompanied by a stretching of the tissue in the proximal part (Steinberg, 1954). The result is the appearance of a gradient in optical density of the stem, the optical density being greatest distally, where the cells are the most numerous, and progressively decreasing proximally. It was considered likely, in view of a number of observations, that the tissue movement is of an amoeboid nature. Amoeboid movements of two ectodermal cell types of gymnoblasts, the cnidoblasts and the interstitial cells, have been described by Hadzi (1909), Kuchner (1934), Moore (1952), and Spratt (unpublished). The author has observed the amoeboid activity of the ectoderm of *Cordylophora, Eudendrium* and *Pennaria,* but has found no evidence of amoeboid movement of the endoderm. This situation seems to be reversed in *Hydra* (Papenfuss, 1934; Lehn, 1953), but seems to be rather general among the gymnoblasts.

In order to determine whether the distad movement of the tissue is of an amoeboid nature, the following experiment was performed. Twenty-five 7½-mm. stems were cut and all of the tissue was extruded from the perisarc with the ex-

ception of a band of tissue 1½ mm. long in the middle of the stem. If the movement of the tissue is of an amoeboid nature, the entire length of tissue should move toward the distal cut end of the perisarc. In 20 of the 25 stems the tissue did indeed move toward the distal cut end of the perisarc, traversing the three-mm. distance in the course of about 6 hours. In two stems the tissue moved in both directions, completely occupying the 7½-mm. length of perisarc in approximately the same length of time, while in only one case did the tissue fail completely to move. The tissue of the remaining two stems underwent incomplete distad movement. In many of the stems an optical density gradient appeared during the course of the movement. Two attempts were made to repeat this experiment, 5 stems being used each time, for the purpose of obtaining a photographic record of the movement, but each time the tissue moved toward both ends of the perisarc equally. The most likely explanation of this is that in both of the latter attempts colonies were used which had been in the laboratory for two or three days, such colonies giving aberrant results in many different types of experiments. An examination of the advancing edge of the tissue showed that it was composed of a very thin sheet of cells moving on the inner wall of the perisarc. At some points it could be seen that the extreme margin was a single layer of ectoderm cells, the endoderm terminating a few cells behind the advancing edge.

It is concluded that the movement of the tissue during the first phase of regeneration in *Tubularia* is an amoeboid movement of the ectoderm, the endoderm presumably being carried along passively because of its firm adhesion, by means of the mesoglea, to the ectoderm. Whether all of the cells of the ectoderm are amoeboid or whether the movement is due to the activity of certain specific cell types cannot be said at this time. It follows that the axial gradient in *Tubularia,* as manifested by graded differences in rate of regeneration, is a reflection of a gradient in the rate of movement of the amoeboid cells of the ectoderm.

Discussion

It is claimed by Tardent (1952, 1954) that the interstitial cells of hydroids are of great importance in the regeneration process and that the observed apico-basal gradient in the number of interstitial cells is the basis of the axial gradient in rate of regeneration. In the first paper it is stated that there is no longer any doubt that these cells migrate toward the cut surface where they form the "blastema." The author presents no observations to support his statement, relying upon claims to this effect in the literature. Perhaps the experiment most suggestive of such a phenomenon is that of Evlakhova (1946), in which the basal halves of normal individuals of *Hydra attenuata* were grafted to the apical halves of x-irradiated individuals of *Pelmatohydra oligactis* which are incapable of regeneration and tend to disintegrate. Regeneration followed amputation through the irradiated *Pelmatohydra,* the interstitial cells and cnidoblasts of the latter now being found to be those of *H. attenuata.* This, however, does not constitute evidence that the interstitial cells *formed* the regenerant. It is well known that the presence of non-irradiated tissue can vitiate the effects of x-irradiation elsewhere in the system, possibly by the contribution of -SH compounds (Barron, 1946; Jacobson *et al.,* 1949; Chapman *et al.,* 1949; Patt *et al.,* 1949; Abrams and Kaplan, 1951; Allen, 1951; Gershon-Cohen *et al.,* 1951). In his second paper Tardent acknowledges

(p. 628) that in *Tubularia* there is no such thing as a regeneration blastema and briefly considers the possibility that the other cells of the tissue may also contribute to the regenerant. However if one isolates a one-mm. length of stem, the entire piece transforms directly and *completely* into hydranth structures. This would appear to be a clear demonstration that the process of regeneration involves *all* of the cells of the regenerating region. The postulate that the axial gradient in rate of regeneration is due to a gradient of the cellular precursors of the regenerant is clearly inconsistent with the above observation.

The only existing theory up to the present time which attempts to explain the determination of the size of the hydranth primordium and the time required for regeneration is that of Barth (1944). This author points to the fact that both primordium size and the regeneration rate can be decreased by exposure to low oxygen tensions during the course of regeneration. He shows further that stems consume less oxygen at lower oxygen tensions and concludes that since energy production would be a function of oxygen consumption, both primordium size and regeneration rate are determined by the rate of energy production. In the light of the new facts concerning amoeboid movement of the tissue during regeneration and its importance in determining regeneration rate, a reinterpretation of Barth's data becomes possible.

In Barth's paper we read (p. 361), "During from 5 to 22 hours of exposure to N_2 sea water the stems appear to undergo no regeneration, since, when they are returned to O_2 sea water, they take the same time for regeneration as freshly cut stems." From this we can conclude that the amoeboid movement of the tissue cannot proceed anaerobically. Considering this, plus the fact that both primordium length and regeneration rate are determined during the movement phase of the regeneration process, it would appear that the action of low oxygen tension in reducing the primordium length and the regeneration rate is upon the amoeboid movement of the tissue, which would slow down and eventually stop as the oxygen tension is decreased. Barth also points to Peebles' finding (1931) that after 15 hours of regeneration the as yet unformed primordium could no longer be reduced in size by removing a large piece from the proximal end of the stem, that between 6 and 13 hours some of the primordia could be reduced in size, and that from two to four hours the size of all of the primordia could be reduced. He correlates this with his own finding that exposure to low oxygen tensions for various periods during the course of regeneration can decrease the size of the primordium, and concludes (p. 364) that "The results show that determination of the length of the primordium is progressive with time. There is no evidence of a sudden determination at some definite time. On the contrary, it is possible to change the length of the primordium during most of the period of regeneration. This implies that the distal parts of the hydranth are determined first and the more proximal parts later, for the changes in length of the primordium mean a change in the amount of stem derived from the proximal regions. Obviously, this proximal region forming the base of the hydranth must remain undetermined until the final few hours of regeneration." Since it has been found that the length of the primordium (and the rate of regeneration) is determined during the movement phase of regeneration and *some time before* the primordium is established, while determination of the parts of the regenerating hydranth cannot be detected until *after*

the primordium is established, it would seem more fruitful to consider these as two separate processes. If this is done, we can no longer consider that regional functional determination within the primordium proceeds basipetally or that the basal region of the hydranth remains undetermined until regeneration is nearly complete.

The statement that the length of the primordium can be changed during most of the period of regeneration is open to question and might best be discussed in connection with another experiment in Barth's paper. Stems were cut and divided into four groups. The first group, which served as a control, was allowed to regenerate under normal conditions. The second, third and fourth groups were treated with 0.07 *M* urethane for the periods from 0–10, 10–20, and 20–30 hours after cutting, respectively. The results show that regeneration in these groups was delayed 13.7 hours, 17.4 hours, and 4.4 hours, respectively, while the primordium length was also decreased, although not in proportion to the delay in regeneration. This latter fact is used as evidence that primordium length and regeneration rate are controlled by factors operating at different times. This conclusion is at variance with other findings already presented, but it might be pointed out in addition that when stems regenerate at low temperatures, regeneration rate is decreased (Moore, 1910; Moog, 1941) while primordium length is increased (Moog, 1941). It may be that at low temperatures the initiation of differentiation is delayed more than the tissue movement, so that more cells have a chance to enter the region in which reconstitution will occur before the differentiation begins, resulting in an increase in the size of the primordium. A similar effect may explain the lack of parallelism between regeneration rate and primordium length in Barth's urethane experiment.

An explanation is needed for the fact that Barth has reported an effect of low oxygen tension and of urethane on the length of the primordium well beyond the time at which both Peebles and the author have found the length to be no longer alterable by the removal of a large proximal piece of the stem. The following discussion may serve to bring these apparently contradictory findings into closer agreement. In Barth's experiments the stems were treated with urethane during specific intervals of time rather than during specific stages in their regeneration. Table II shows the wide spectrum of stages in which similar stems from the same colony may be at any given time. Admittedly the variation shown in Table II is the largest found in any of the groups in the present experiments, but the fact remains that there may be a considerable spread in regeneration rate among the stems of an experimental series. Treatment during specific intervals of time, then, may result in different effects upon different stems, depending upon their stage. This would not be revealed in Barth's data because he presents his results as averages. If there were a very specific stage at which the treatment is effective, the effect would be manifested as one which gradually appears and then gradually disappears, due to the use of averaged data and specific time intervals rather than specific stages. This is actually shown by the results. Finally, the time intervals during which the animals were treated are referred back to the time period for normal regeneration and used as a measure of the sensitivity of the controls to urethane treatment. However, if stems treated for the period between 10 and 20 hours after cutting are delayed an average of 17.4 hours, the treatment has not only stopped all regeneration at around 10 hours, but has even occasioned

the necessity of a mean recovery period of about 7.4 hours. It can be inferred from these results that the control stems were highly susceptible to the treatment at 10 hours, but no inference can be drawn concerning their susceptibility at, say, 15 or 20 hours because the treated stems did not pass through a comparable stage until some time after the cessation of the treatment. It would appear, then, that the effects of oxygen and of urethane upon regeneration rate and primordium length may be interpreted as having terminated at an earlier time than would at first be suspected. "Determination" of the length of the primordium would be expected to proceed gradually after the tissue movement has begun, and to terminate at the time when a certain critical concentration of cells has been reached at the presumptive regeneration site. It may be stated in summary that all existing evidence is consistent with the view that both the regeneration rate and the primordium length are determined during the phase of tissue movement and before the appearance of the primordium. One qualification should be added, however. We have seen that a reverse movement during the course of differentiation may slow down, stop, or even reverse the differentiation process. This being the case, the determination that we speak of is at best a labile one, and in the very strictest sense it would not be true to say that the regeneration rate of a particular stem is fixed when the primordium makes its appearance. This does not, of course, alter the fact that differences in regeneration rate between two *groups* of stems from the same colony, such as have been studied in the present experiments, are entirely due to differences in the time occupied by the movement (pre-differentiation) phase of the regeneration process.

There appears to be good evidence that there is a gradient in oxygen consumption, high distally and decreasing proximally, in the tubularian stem. Hyman (1926), Barth (1940), and Sze (1953), using a modified Winkler method, a Warburg respirometer, and the Cartesian diver method, respectively, have all found this to be true. Certain objections can be raised to these experiments, such as the number of approximations made by Hyman, the inclusion by Barth and Sze of the perisarc, which is thicker proximally, when taking dry weights, and the unusually small number of cases (14 stems divided among three experiments) in the experiments of Sze. Nevertheless, even allowing for these objections, it still appears that a respiratory gradient exists. If respiratory rate can be assumed to be a good measure of the physiological processes occurring, what explanation can be offered for the lack of an apico-basal gradient in rate of differentiation? In the present experiments a gradient in rate of differentiation has been found, but it goes in precisely the opposite direction. A possible explanation is to be found in the measurements made by Barth (1940) on oxygen consumption during the course of regeneration. This author finds (p. 369) that "The (respiratory) rate is highest from about 7 to 16 hours and it is during this period that the size of the primordium is determined (Peebles, '31). These changes in rate are observed only in the case of short (2–4 mm.) stems. The S-shape of the curve is lost or almost lost when the data from long (8–15 mm.) stems are plotted." It has been found (Steinberg, 1954, and the protocols of the present experiments) that in short stems the tissue moves toward the distal end and then ceases movement, and that after a hydranth regenerates distally it produces an inhibitor which prevents further movement. In long stems the tissue continues to move, even after the regeneration of a hydranth distally, presumably because of the dilution

of the inhibitor below an effective concentration by the increased volume of the coelenteric fluid. These observations correlate perfectly with the observations of Barth quoted above if one assumes that the differences in oxygen consumption which appear are measurements of the differences in the movement of the tissue. It has already been inferred that the tissue movement is a process which requires oxygen. Thus we see that oxygen consumption is higher distally than proximally, and the tissue moves more rapidly distally than proximally; oxygen consumption is highest in short stems from about 7 to 16 hours after cutting, and the tissue movement is occurring in short stems from the average colony at this very time; the above peak in oxygen consumption is not found in long stems, and the tissue does not stop moving in long stems. It would appear, then, that the gradient in oxygen consumption is in reality a measure of the rate of tissue movement in the different levels of the stem. However the question of what causes the cells from basal regions to move more slowly than those from apical regions must still remain a subject for future investigation.

Summary

1. The regeneration process in *Tubularia* is divisible into two phases. There is a period of tissue movement, during which extra cells are provided to the presumptive regeneration site, followed directly by the period of differentiation of the new hydranth.

2. Differences in rate of regeneration between narrow versus wide stems, the distal versus the proximal ends of stems ligated in the middle, and apical versus basal stem levels are all due to differences in the time occupied by the tissue movement.

3. Narrow and wide stems differentiate at the same rate, as do the distal and proximal ends of stems ligated in the middle. However, pieces from *basal* stem levels differentiate, in these experiments, at a significantly *greater* rate than pieces from *apical* stem levels.

4. Both rate of regeneration considered as a whole and rate of differentiation vary from colony to colony and between comparable stems taken from the same colony.

5. Neither the tissue movement nor the process of differentiation is always continuously progressive. Reverses in the direction of movement and spontaneous dedifferentiation of the differentiating primordium have both been observed.

6. The differentiation process is continuously dependent upon a critical cell density in the primordium. Evidence is presented that differences between stems in the movement of the tissue during the differentiation process represent the only systemic factor responsible for variations in differentiation rate among similar stems from the same colony.

7. The tissue movement is due to the movement of the amoeboid cells of the ectoderm, and the axial gradient, as manifested by an apico-basal decrease in rate of regeneration, is a reflection of the apico-basal gradient in the rate of tissue movement.

8. Both the apico-basal gradient in oxygen consumption and differences in oxygen consumption during regeneration correlate perfectly with the rates of tissue movement at different levels of the stem and with the period during which

the movement occurs. It is inferred that differences in oxygen consumption indirectly measure the differences in tissue movement, which is an aerobic process.

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INDUCTION OF MOLTING IN THE CRAYFISH, CAMBARUS, BY MODIFICATION OF DAILY PHOTOPERIOD[1]

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Several investigators have been concerned with the modifications in reproductive phenomena in the Crustacea which are induced by subjecting the animals to various daily light rations. Thus Stephens (1952) studied the modifications in oöcyte maturation in the crayfish, *Cambarus*, induced by exposure to various daily photoperiods and postulated a definite role of light in the control of normal reproductive cycles in these animals. Paris and Jenner (1952) extended the observation of photoperiodism in the Crustacea to the fresh water prawn, *Palaemonetes*, and also demonstrated that administration of the daily light ration in a discontinuous fashion may permit stimulation by daily rations which are not effective when administered continuously. Stephens (1953) provided evidence for an influence of day-length on the rate of development of secondary sex characteristics of the crayfish, *Cambarus*.

Hormonal influences on the crustacean sex cycle were first demonstrated by Panouse (1943, 1944, 1946) for the shrimp, *Leander*, and the accelerating effect of eyestalk removal in ovarian development was subsequently confirmed for *Cambarus* and the fiddler crab, *Uca*, by Brown and Jones (1947, 1948). Carlisle (1953) has recently demonstrated an inhibitory factor controlling sex reversal in *Lysmata*. The presence of a molt-inhibiting principle in the sinus gland-X-organ complex has been verified in a number of laboratories (reviewed in Brown, 1952) although this principle may not always be present (Carlisle, 1954) and Echalier (1954) provides evidence suggesting the occurrence of a molt-accelerating principle in the Y-organ of *Carcinus*.

Finally, Scudamore (1948) presents evidence for a functional interrelation of the processes of egg-laying and molt. In view of the hormonal influences on the molting process, the intimate relation between the molting cycle and the reproductive cycle, and the influence of light as an exteroceptive factor controlling reproduction, the possibility of the modification of molting by treatment with various light periods is of considerable interest.

The results of experiments involving exposure of *Cambarus* to various daily periods of illumination which had been undertaken for other purposes suggested that molt frequency might be influenced by this treatment. However, the numbers of animals involved were small and the results inconclusive so that it was thought desirable to conduct observations on a larger group in order to obtain a more reliable indication of a possible influence on molting.

[1] The author wishes to express his sincere appreciation of the interest and encouragement of Dr. F. A. Brown, Jr. of Northwestern University. This work was initiated under a predoctorate research fellowship of the National Institutes of Health.

Material and Methods

The animals employed in the experiment to be described were *Cambarus virilis* collected in an artificial mud-bottom lake north of Chicago, Illinois. They were obtained in mid-September and maintained in the laboratory until early November in a large tank supplied with running water. During this time the tank was shielded with black cloth so that all of the animals were maintained in constant darkness. The animals were rather small (average carapace length about 2 cm.) and were probably immature yearlings. The oöcytes of the females were small and showed no sign of yolk deposition in contrast to the normal situation in mature *C. virilis* females at this season. Also the gonopods of the males were uniformly juvenile in form (form II) while those of mature males are typically form I (sexually active) at this time (Scudamore, 1948).

Early in November, these animals were divided into three groups and maintained under the following lighting conditions:

Group 1 consisted of one hundred and thirty animals which were placed in a tank shielded with black cloth to provide constant darkness.

Group 2 consisted of one hundred and ten animals subjected to normal day-length for the local latitude and season. This day-length varied from an initial maximum of ten hours and thirteen minutes through a minimum of nine hours and seven minutes on December 21st (data from the American Ephemerix and Nautical Almanac).

Group 3 consisted of sixty-five animals receiving twenty hours of light per day from 2 A.M. to 10 P.M. Lighting was provided by two 25-watt, unfrosted incandescent bulbs suspended from a wooden frame approximately ten inches above the water level. This produced an illumination of from 8 to 50 foot candles at the water surface. All tanks were covered with black cloth to prevent the access of undesired illumination and the lights were controlled by automatic time clocks.

The temperature in each tank was taken daily and the water flow was adjusted so that the temperatures in the three tanks were equal. Each tank was examined daily for dead animals and cast exoskeletons. As animals died they were autopsied and the gastroliths, if any, removed and weighed after drying. For some days preceding molt in crayfishes calcium is stored in the form of gastroliths in the anterior wall of the stomach. Scudamore (1947) has studied this cycle in animals induced to molt by removal of the eyestalks and demonstrates an increase in gastrolith weight as these animals approach the time of molt. Consequently gastroliths were weighed to provide some information concerning the position of the animals in the molt cycle. In order to compensate for variation in size of the animals a gastrolith factor was calculated and the results are recorded in this form in Table I. This gastrolith factor consists of the weight of the gastroliths in milligrams divided by the cube of the carapace length in centimeters. This quotient is multiplied by 100 for convenience.

Results

The animals in the three groups showed clear differences with respect to molt and gastrolith formation, and mortality.

Group 1, which was composed of thirty-seven females and eighty-three males, was maintained in constant darkness. This group showed the lowest mortality

and the least tendency toward gastrolith formation. The average temperature in this tank for the experimental period was 15.2° C. Seventeen animals died during the sixty-four-day experimental period which is a mortality of 13%. Only one animal of the seventeen which died showed any trace of gastrolith formation. This was a male which died sixty-three days after the beginning of the experiment and had a gastrolith factor of 24. No molts occurred in this group.

TABLE I

Record of the time of death or molt and the gastrolith factors for animals of group 2 (normal daylength) and group 3 (20-hour light)

Day	Group 2 (normal daylength)		Group 3 (20-hour light)	
	Sex	Gastrolith factor	Sex	Gastrolith factor
4	female	0		
6			male	0
8			male	0
9			male	0
			female	63
11			male	0
13	male	0	female	0
	male	0		
15	male	5	male	0
			female	0
16			male	0
			male	49
17	female	0	female	0
	male	0	female	trace*
	male	0	male	3
19	female	0	female	0
	male	0	male	0
	male	0		
20			female	131
			female	52
21			female	8
			female	18
23	male	0	male	0
	male	0		
	male	1		
24	male	2	female	0
27	male	0	male	0
	male	trace*		
	male	10		
	female	6		

* Too small to be isolated and weighed.

TABLE I—*Continued*

Day	Group 2 (normal daylength)		Group 3 (20-hour light)	
	Sex	Gastrolith factor	Sex	Gastrolith factor
29	male	0	male	0
			male	25
30			female	0
			male	trace*
32	male	0	male	0
			male	32
34	male	0	female	0
	male	14		
35			female	0
			female	56
38	male	6		
	male	21		
42	male	trace*	male	6
			male	35
44	male	successful molt		
	male	0		
	female	0		
	female	22		
48			male	successful molt
50			female	successful molt
			male	3
53	male	0		
55			female	0
59			male	successful molt
			male	(post-molt)
			male	38
			male	14
			female	0
61	male	15	male	successful molt
			female	successful molt
			male	1
64	male	0	male	successful molt

Group 2, a group of thirty females and eighty males, was exposed to normal day-length under the conditions described. The average temperature in this tank during the period of observations was 15.2° C. Thirty-one of these animals died during the experimental period which is a mortality of 28%. Twelve of the animals which died showed some development of gastroliths and one male molted forty-four days after the beginning of the experiment. Thus 40.6% (thirteen of thirty-two) of the animals examined showed some indication of molt or its successful completion. The average gastrolith factor for those animals which ex-

hibited gastroliths at the time of death, sex, and gastrolith factor or record of molt for the animals in this group are recorded in Table I.

Group 3, composed of twenty-five females and forty males, was exposed to twenty hours of light per day from 2 A.M. to 10 P.M. The average temperature in this tank was 15.3° C. Forty of these animals died during the observation period which is a mortality of 62%. Eighteen of the animals which died showed some sign of gastrolith formation and one of them died after having successfully molted. Six animals molted successfully and survived during the course of these observations. Hence 54.4% (twenty-five of forty-six) of the animals observed molted or showed an indication of the initiation of molt. The average gastrolith factor for those animals which exhibited gastroliths at the time of death was 29.5. The time of death, sex, and gastrolith factor or molt record for the animals in this group are listed in Table I.

The difference in behavior between the animals maintained in constant darkness (group 1) and groups 2 and 3 appears significant on inspection. This is verified statistically when one considers the percentage of the animals observed which show an indication of molt in the three groups. The difference between group 1 and groups 2 and 3 is more than twice the standard error of the difference. However, this does not suffice to demonstrate the significance of the difference between group 2 and group 3 (difference is 1.2 sigma). Applying Student's t test after assigning a reasonable gastrolith factor to the successful molts, the probability that groups 2 and 3 are not different is less than 0.01. The factor value of 150 which was assigned to animals which molted is probably somewhat low; higher values would emphasize the difference between the two groups.

Concerning the differences in mortality of the three groups, all the differences are significant (greater than twice the standard error of the differences) provided one includes the animals which showed gastroliths at the time of death. However, after these animals are excluded, the death rate of the animals exposed to twenty hours of light per day is still significantly higher than that of the animals in groups 1 and 2.

Discussion

It can be concluded on the basis of these results that exposure of *Cambarus virilis* to rather low light intensities during the winter months is capable of inducing molt in animals which would fail to molt if maintained in constant darkness. It is interesting to note that apparently rather short day-lengths are competent to produce this effect. Light periods to which the group in normal day-length, group 2, was exposed varied from a maximum of ten hours and thirteen minutes to a minimum of nine hours and seven minutes. This is in contrast to the results obtained by Paris and Jenner (1952) where a light period of 11 hours per day failed to induce ovarian development in *Palaemonetes*. These authors conclude that the response of these animals is in many respects analogous to the flowering response in long-day plants. On the other hand, Stephens (1952) observed modifications of yolk deposition and oöcyte maturation induced by rather short day-lengths although the situation is somewhat complicated by the fact that her initial exposures were longer (twelve hours) and decreased gradually.

In the results presented here, it would seem that the difference between the response of the animals to short days and twenty-hour days is quantitative rather

than qualitative. If there is a threshold for this response, it lies below ten hours and thirteen minutes.

It does not seem likely that photoperiodic responses can provide a complete explanation for the timing of the events in the molt cycle but it does appear that they would contribute in this regard. The typical molt cycle in *Cambarus* consists of a variable number of molts in the first year followed by an annual molt of the females and a spring and fall molt of the males in succeeding years (van Deventer, 1937). The transition from darkness or very short daily exposures to light to day-lengths of twelve hours or more when the animals emerge from their burrows in the spring would provide a reasonable stimulus for the spring molt in both sexes. It is tempting to refer to the well-known retiring behavior of berried females as a possible factor in the delayed spring molt of this group which typically occurs two to three weeks later than the male spring molt. It would be possible to construct hypotheses to attempt to explain the rest of the molt cycle on the basis of variations in susceptibility to light as a stimulus or quiescent periods but there is not sufficient information available to limit the number of such schemes. Furthermore, the possibility that this response is characteristic only of immature animals cannot be discarded at present.

It should finally be pointed out that the results presented show a clear influence of day-length on the viability of these animals. There is a significant increase in the mortality of those animals which did not show gastroliths at the time of death as well as a clear increase in total mortality with increasing day-length. The fact that this increase occurs in animals which do not show gastroliths might suggest that the increase in mortality is not causally related to the increased tendency to molt. However, such a conclusion would rest on the statement that no animal which fails to exhibit gastroliths has entered the physiological pre-molt stage; this does not seem justified. However, it is of interest to compare this increase in mortality with the results of destalking reported by Brown (1938) which led him to postulate a hormonal effect on viability in crayfishes.

Summary

1. During the winter months, crayfishes maintained at moderate water temperatures (approximately 15° C.) respond to daily illumination by an increased tendency to molt.

2. The strength of this tendency to molt increases with increasing length of the daily periods of illumination.

3. If there is a threshold below which this response does not occur, it lies below a daily period of illumination of approximately ten and one quarter hours per day.

4. There is an increase in mortality in groups exposed to various daily photoperiods with the highest mortality occurring in animals exposed to the longest light period. This is true of animals which fail to show any sign of the initiation of molt at the time of death.

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DESOXYRIBOSE NUCLEIC ACID CONTENT IN THE NUCLEI OF SALAMANDER SOMATIC TISSUES

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Irregularities of ploidy in amphibians have been extensively investigated by Parmenter (1933, 1940), Fankhauser and his associates (*cf.* reviews by Fankhauser, 1945, 1952), and Costello and Henley (1949, 1950; Henley and Costello, 1951). These studies have dealt principally with conditions in early embryos and in the epithelium of larval tail-tips. Recently increasing attention has been given to the incidence of endopolyploidy in the adult somatic tissues of vertebrates, of which the mammalian liver and orbital glands have become classic examples (Sulkin, 1943; Biesele, 1944; Swift, 1950; Teir, 1944). The extent of such nuclear deviations in the normal tissues of adult amphibians has received only slight study. Mancini (1945) found a very rare occurrence of large nuclei in the liver of old salamanders. In the renal tubule cells of the Australian desert frog, *Cyclorana,* Dawson (1948) found striking variations in numbers and sizes of nuclei; a similar condition was reported by Schreiber and Melucci (1949) in the South American frog, *Leptodactylus.* In the integumental granular glands of *Triturus viridescens* Dawson (1937) described great volume changes of nuclei associated with secretory activity. Though changes in nuclear size often reflect corresponding changes in chromatin content, this relationship does not invariably hold. Chromosome counts are not feasible in adult tissues showing little or no mitotic activity. Cytospectrophotometry, however, affords a means for measuring the relative content of desoxyribose nucleic acid (DNA) in individual interphasic nuclei and thus provides a valuable technique for the determination of polyploidy and related conditions (*cf.* review by Swift, 1953). The present investigation was undertaken to provide such photometric data for adult tissues of the salamander *Taricha* (*Triturus*) *granulosa granulosa* Skilton. Parenchymal cells of the liver, pancreas, renal tubules, and granular glands of the skin were selected for study.

Methods

Pieces of liver, pancreas, kidney and skin from freshly collected water-inhabiting salamanders were fixed in 30 per cent neutral formalin. Paraffin sections were cut at thicknesses exceeding by two or three micra the average diameters of the constituent parenchymal nuclei. Sections of liver tissue from the same block were placed on all slides as a standard of photometric comparison. Staining with the Feulgen reagent followed the procedure described by Stowell (1945). The time

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of hydrolysis in 1 N HCl at 60° C. was 20 minutes, though a difference of five minutes did not produce an appreciable change in the maximal intensity of the color produced by the subsequent treatment with the sulphurous fuchsin reagent for one hour. Unhydrolyzed sections were also stained for controls. All the slides measured were treated with the same stock Feulgen reagent kept completely colorless by storage in tightly closed containers at 5° C.

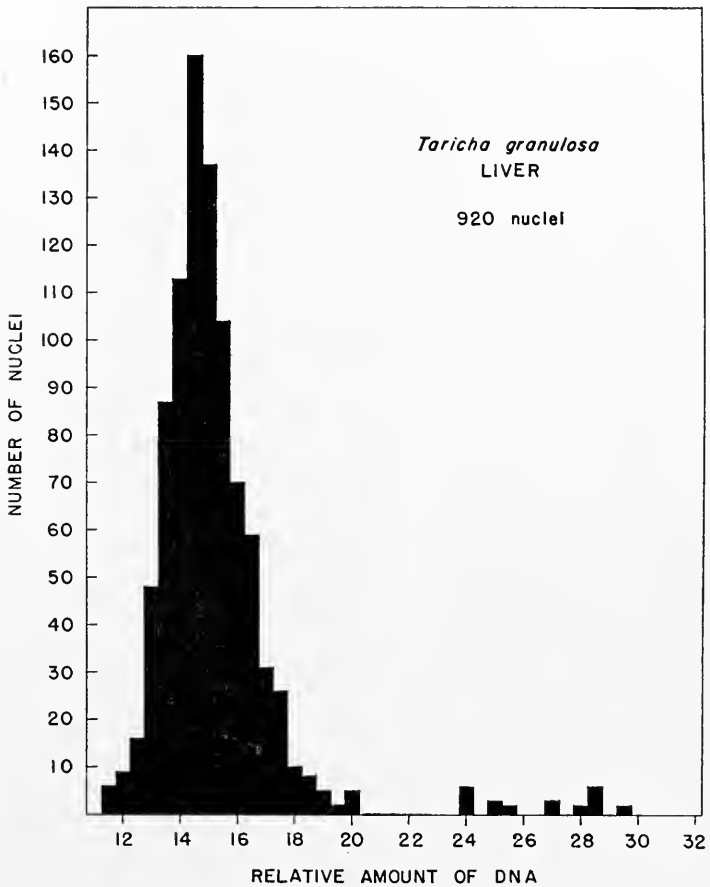

FIGURE 1. Distribution of DNA in liver nuclei of the salamander, *Taricha granulosa*.

Photometric measurements were made with an apparatus similar to that described by Swift (1950). Light of 550 mμ was isolated from a tungsten filament by a Bausch and Lomb interference filter. Optics on centerable mountings were carried on a Leitz microscope stand provided with a side-tube, and consisted of a B. & L. 4 mm. apochromatic objective (N.A. 0.95), a Leitz aplanatic, achromatic condenser (N.A. 1.40) stopped to 5 mm. diaphragm aperture, and 6 × or 8 × Leitz periplan oculars. Transmissions were recorded with a Farrand electron multiplier photometer, employing an RCA 1P21 phototube and a Rubicon galvanometer. The

phototube housing was mounted on a Leitz Aristophot camera. A calibrated iris diaphragm directly under the phototube permitted restriction of the field to any desired area. Measurements of nuclear diameters at predetermined magnifications were conveniently made on the reflex screen of the Aristophot camera.

Care was taken to measure only entire nuclei as determined by critical focusing. Since most nuclei in the present material were approximately spherical, the "plug method" for cytophotometry proved adequate and was used throughout; the largest nuclei of the granular glands, however, were rather irregular in shape, and hence were not included. Plug measurements were based on 60 per cent of the average nuclear diameter. Relative amount of the absorbing substance (DNA) in the whole nucleus was calculated from the formula used by Alfert (1950), *viz.:*

$$A = \frac{E \times r^2}{0.49} \times M^2$$

where A is the amount of DNA in arbitrary units, E is the extinction, r is the plug radius at the phototube level, and M is a magnification factor ($M = \mu$/mm. at the phototube level).

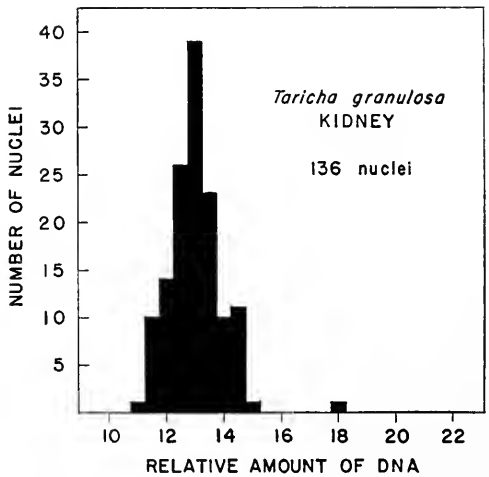

FIGURE 2. Distribution of DNA in kidney nuclei of the salamander, *Taricha granulosa.*

Theoretical aspects of the cytospectrophotometric method and sources of error have been fully discussed by Swift (1950) and Alfert (1950).

In order to obtain a reasonably representative number of determinations for the nuclei of the four tissues, all of the whole nuclei included in any one section were measured, except where deviations of shape did not allow their inclusion, as in the granular glands.

RESULTS

Liver

Interphasic nuclei of liver parenchyma were measured in two animals. The distribution of the relative amounts of DNA was similar in both, so that only one is illustrated (Fig. 1). In this specimen 920 nuclei were measured, in the other

644. Within the ranges 11.5 to 20 and 12 to 20, respectively, the great majority of DNA values formed in both cases a high, rather sharply delimited and fairly symmetrical unimodal curve. A few DNA values, 2.6 per cent of the total number of determinations in the animal figured and 2.2 per cent in the other, were scattered from 24 to 29.5 and from 22 to 33, respectively. The DNA values of liver nuclei were thus grouped into two classes. The peak value for class I nuclei was 14.5 in both specimens; respective means and standard deviations were 14.9 (1.4) and 14.8 (0.9). Class II nuclei, which included DNA values double the class I peak, showed means and standard deviations of 26.5 (2.0) and 25.3 (2.6).

Kidney

The number of kidney tubule nuclei measured was 136 in one animal (Fig. 2) and 129 in another. Unimodal curves similar to those of liver were obtained.

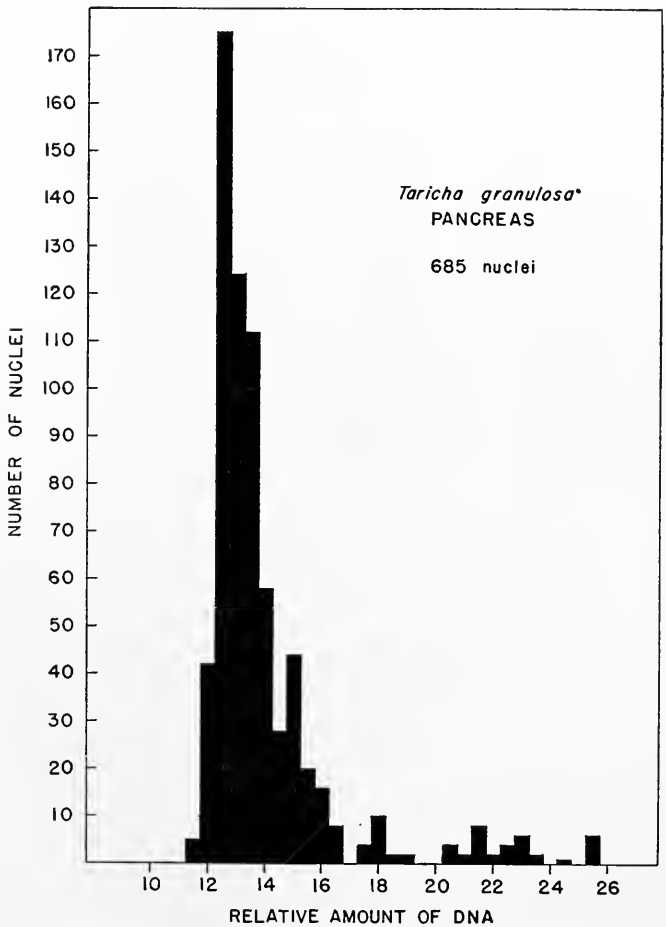

FIGURE 3. Distribution of DNA in pancreatic nuclei of the salamander, *Taricha granulosa*.

In both animals all DNA values ranging from 11 to 18 and from 11.5 to 19.5, respectively, with a peak value of 13.0 in both cases, could be placed into class I; means and standard deviations were 13.0 (0.9) and 13.3 (1.5). In the second specimen two DNA values fell at 21, but none twice the peak amount of class I were found. Careful scanning of several sections did not show any large and darkly stained nuclei which might give class II values. The average DNA content of 13.5 for 10 nuclei of the standard liver section which was mounted together with the kidney sections for comparison, seemed to indicate a slightly lower intensity of stain in the kidney slides. The comparison, however, also showed that class I nuclei of kidney cells were in close agreement with class I nuclei of liver cells with respect to DNA content.

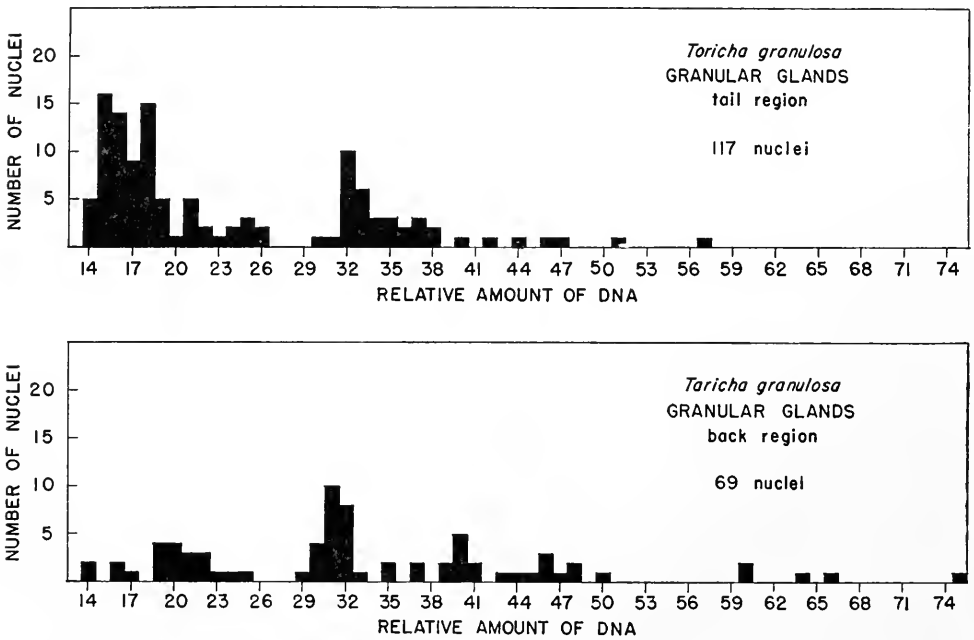

FIGURE 4. Distribution of DNA in the granular gland nuclei of the tail and back regions of the salamander, *Taricha granulosa*.

Pancreas

The distribution of DNA values was determined for 685 nuclei in one animal (Fig. 3) and for 944 in another. The results obtained were essentially similar to those found in liver nuclei. Within the ranges 11 to 19 and 11.5 to 19, respectively, the DNA values formed high unimodal curves (class I nuclei). Only a few values, 5.2 per cent and 1.6 per cent, respectively, of the totals were found in the ranges 20.5 to 25.5 and 20 to 24. The class I peak values occurred at 12.5 in the first animal (mean 13.5, st. dev. 1.3) and at 13.5 (mean 13.7, st. dev. 1.3) in the second. DNA content in the standard liver nuclei averaged 14.7 and 15.0, respectively. The slightly lower mean values of pancreatic nuclei can be accounted

for by their less homogeneous chromatin distribution. Class II means and standard deviations were, respectively, 22.6 (1.6) and 22.9 (1.3).

Granular glands

Skin sections were taken from the dorsal tail region in one animal and from the back region in another. In the first case most of the granular glands were at earlier developmental stages than in the second. In the younger glands the cell membranes were well defined, the cytoplasm was evenly filled with secretion granules, small nuclei were common, and most of the larger nuclei occupied a central position in the cell. A larger number of mature glands were found in the second location. In these the cell boundaries were lost (apocrine secretion), the cytoplasm was vacuolated, and peripherally located giant nuclei were numerous. These very large nuclei were usually flattened and could not be measured with the present photometric method. Because of this limitation only a small number of measurements were possible, 117 in the first animal and 69 in the second. Therefore the results, graphically shown in Figure 4, include only a partial representation of the granular gland nuclei.

A wide spectrum of DNA values was obtained in both specimens. In the tail region, where the smaller nuclei were most numerous, low DNA values were in the majority. In the back region, larger nuclei with high DNA values were relatively more abundant. Delimitation into nuclear classes is difficult. A possible grouping would include in class I the values 14 to 26, in class II 29 to 51, and in class III 57 to 75. Such a break-down would fit both graphs and would roughly yield successive doubling of mean values (I = 17.7 and 19.7; II = 35.5 and 36.5; III = 57 and 65). However, in comparison with the control liver sections, which showed means of 14.3 and 14.2, respectively, the above class I means are rather high. In part, this may be accounted for by the very homogeneous distribution of chromatin in granular gland nuclei, resulting in higher extinction values. If class I is taken to include only values 14 to 18, the means fall around 16 (in better agreement with the liver values), with doubling at 32 (at which peak groups occur in both graphs) and quadrupling at 64 (in harmony with the highest plots). This would leave considerable numbers of nuclei with intermediate values, notably the groups between 20 and 26 and between 39 and 51. Further consideration of this situation is deferred to the discussion.

Discussion

The nuclear DNA classes indicated by the distribution curves suggest a progressive doubling of chromatin. Class II nuclei might be interpreted as interphasic doubling prior to mitotic separation of diploid complements were it not for the fact that no mitoses were observed in the tissues examined. Hence it is more likely that these nuclei are definitively tetraploid or in an equivalent polytene condition. This conclusion gains strength in the case of the granular glands where class III (octoploid) values occur. The general picture is in agreement with similar phenomena in mammalian and other tissues (see Swift, 1953).

Variation within classes may be due in part to over-all error in the photometric technique, the exact value of which cannot be assessed but probably does

not exceed 15 per cent (Swift and Kleinfeld, 1953). Some of it might be accounted for by incomplete synthesis of DNA during endomitotic activity. The possibility of aneuploidy also cannot be disregarded, since its occurrence in the somatic tissues of mammals has been reported by Therman and Timonen (1951) and by Hsu and Pomerat (1953); Walker and Boothroyd (1954), however, believe that its incidence has been overestimated. In the absence of actual chromosome counts, the presence of aneuploid nuclei cannot be established.

The large number of individual nuclei measured in the several tissues of the present study permits estimation of polyploid frequencies. Unlike certain mammalian tissues, where polyploidy has been found to be common in beef liver and may even reach 50 per cent in old mouse liver, as reported by Swift (1950), salamander hepatic and pancreatic nuclei show very low polyploid frequency. Only about 2.5 per cent tetraploidy (class II nuclei) was found in the liver, 5.2 per cent in the pancreas, and no octoploid amounts in either. Kidney nuclei were entirely of the diploid class. The polyploid frequency, however, might vary with age of the animal, as is known to happen in mammals. Seasonal variation might also occur.

The granular glands of the integument present a case of special interest. The conspicuous nuclear changes in size, position and shape accompanying the various phases of secretory activity have been fully described by Dawson (1937) for the salamander *Triturus viridescens*. He thought, though the evidence was not conclusive, that these changes might suggest nuclear participation in the glandular activity. In the mature glands he noted nuclei of gigantic size, alveolar patterns of chromatin distribution, vacuolizations, and indications of possible passage of nuclear material into the cytoplasm.

In the present study, since a great part of the nuclear population could not be measured because of various shape irregularities, the account of the DNA distribution is not complete. There is, however, sufficient evidence that high polyploid (and possibly aneuploid) frequency exists, and an indication that it increases in degree as well as frequency during the development and maturation of the glands. This is exemplified by the greater prevalence of high DNA values in the older glands of the back region. Though only a few determinations were technically possible for nuclei in the octoploid range, large darkly staining and irregular or flattened nuclei, which might give similar or higher values, were frequently encountered. Thus it was not possible to establish the full extent of polyploidy in the mature glands. Giant nuclei which stained lightly were also observed, but their irregular shapes likewise precluded photometric determination by the present method.

In view of the considerable number of nuclei with apparent intermediate DNA values, it may be that chromatin multiplication is not wholly euploid. As already pointed out, mitoses are absent, so that direct evidence through chromosome counts could not be obtained. If polyteny is involved, it is conceivable that chromonemal duplication may be uneven. Or it may simply be that the intermediate values reflect a high rate of endomitotic activity, resulting in a picture similar to that obtained for tissues with high mitotic rates.

A point of additional interest attaches to the relation between volume and DNA content in the granular gland nuclei. This is graphically shown in Figure 5 which includes the data from both tail and back regions. Each plot shows the average

DNA content for all nuclei of the same size. It is seen that nuclear volumes increase regularly with DNA content. It may be observed, incidentally, that DNA values intermediate between class peaks are associated with intermediate nuclear volumes, which suggests that the error in photometry cannot be great.

Leuchtenberger and Schrader (1952) found that in the salivary gland of the snail *Helix* DNA content likewise varies directly with nuclear size, though both decrease with the production of cytoplasmic secretion. No loss of DNA accompanying secretory activity has been found in the granular glands. In this respect the granular glands resemble the pharyngeal and thoracic glands of honeybees, in

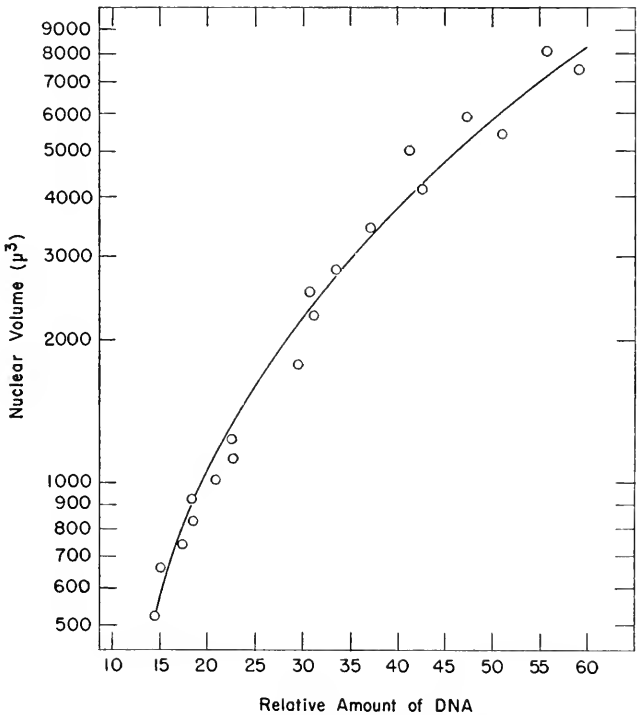

FIGURE 5. Relation between volume and DNA content in the granular gland nuclei of the salamander, *Taricha granulosa*.

which Merriam and Ris (1954) noted a positive correlation between degree of ploidy and secretory activity.

SUMMARY

1. Relative DNA content was determined photometrically for individual Feulgen-stained parenchymal nuclei of the liver, kidney, pancreas and granular glands of the salamander *Taricha* (*Triturus*) *granulosa granulosa* Skilton. Graphs of these values indicated the presence of nuclear classes falling into polyploid ratios.

2. A total of 920 liver nuclei were measured in one animal and 644 in another. A vast majority of the DNA values fell into the first class, representing the

diploid condition. Only 2.6 per cent and 2.2 per cent, respectively, of the total number of nuclei constituted class II, which included values roughly double those of class I. No higher values were found.

3. In the kidney tubules, 136 and 129 nuclei were measured. All could be included in class I.

4. In the pancreas 685 nuclei were measured in one animal, 944 in another. Only 5.2 per cent and 1.6 per cent, respectively, of the total number of nuclei fell into the range of tetraploid values. No higher values occurred.

5. Degree and frequency of polyploidy was marked in the granular glands of the integument. Diploid, tetraploid, and octoploid values were obtained, and presumably higher values occurred in very large nuclei too irregular in shape for photometric measurement by the method employed. Polyploid nuclei increased in frequency with maturation of the glands and exceeded diploid types in number. A considerable number of nuclei with intermediate values suggested the occurrence of aneuploidy or of incomplete DNA replication incident to high endomitotic activity. A regular increase in nuclear volume accompanied rise in DNA content.

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THE INFLUENCE OF BODY WEIGHT, TEMPERATURE AND SEASON UPON THE RATE OF OXYGEN CONSUMPTION OF THE TERRESTRIAL AMPHIPOD, TALITRUS SYLVATICUS (HASWELL)

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Talitrus sylvaticus, a soil-inhabiting amphipod, (Family Talitridae), is widely distributed in the coastal region of eastern Australia and is particularly abundant in the litter layer of sub-tropical rain forests in this region. The experiments described here were designed to obtain part of the data necessary to estimate the quantity of energy which would have been used during a year by a population of *Talitrus sylvaticus* in a tract of rain forest at Bulli Pass, N.S.W. A preliminary study was made of the relationship between the rate of oxygen consumption of *T. sylvaticus* and body weight. This was followed by a study of the effect of temperature on the rate of oxygen uptake of *Talitrus* in both summer and winter.

Methods

Large numbers of *T. sylvaticus* were brought from the rain forest at Bulli Pass at regular intervals and kept in the laboratory in jars of leaf litter and soil at room temperature. Animals which had been in the laboratory longer than a month were not used in the experiments.

Rates of oxygen consumption were measured in a Warburg respirometer, the temperature of the water bath being controlled to within ± .01 of a degree. The volumes of the reaction vessels ranged from 3 to 4 ml.

The *Talitrus* used in any vessel were weighed *en masse,* then dropped into the vessel where they remained quietly on, or close to, two strips of moistened filter paper of known area which had been placed in the bottom of the vessel. In all experiments the vessels contained between 80 and 120 milligrams live weight of *Talitrus* and 0.8 ml. of 10 per cent KOH as an absorbent for CO_2. After each experimental "run" the animals were removed from the vessels and discarded.

Experimental Results

The effect of body weight at 25° C.

Figure 1 shows the relationship between the rate of oxygen uptake of *T. sylvaticus* and body weight at 25° C. The curve, fitted to the scatter of points by

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use of a double logarithmic regression, has the formula:

$$y = 533\, x^{-0.164},$$

where y is the rate of oxygen uptake in $mm.^3$ O_2 per gm. per hour, and x is the body weight in milligrams. The rate of oxygen uptake, expressed as microliters of oxygen used per animal per hour by multiplying the right hand side of the above equation by x/1000, gave the formula:

$$y = 0.533\, x^{0.836}.$$

The effect of temperature

The relationship between the rate of oxygen uptake of *Talitrus* and temperature is illustrated by Figure 2. Figure 2 also shows the effect of the body weight

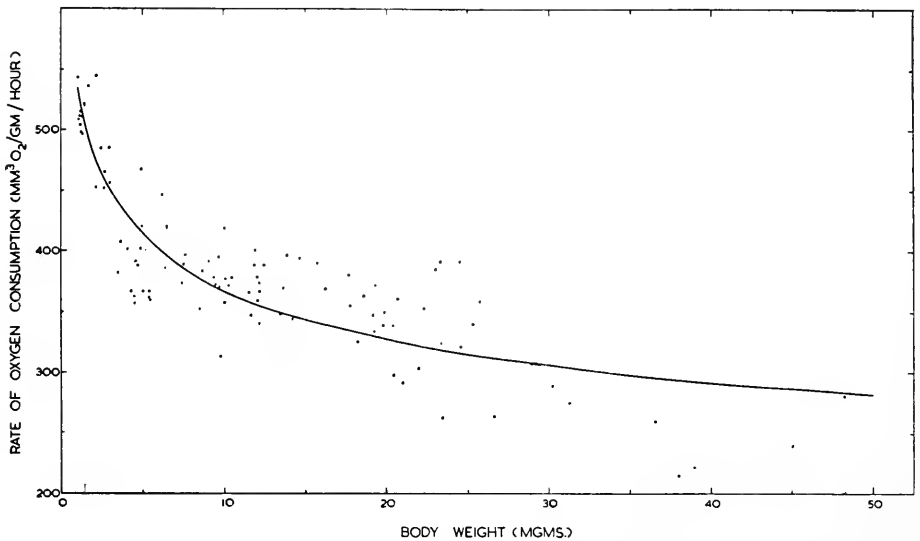

FIGURE 1. The relationship between the rate of oxygen consumption of *Talitrus sylvaticus* and body weight at 25° C.

of the animals (graphs A, B and C), and the effect of season on the rate of respiration of *Talitrus* over a range of temperatures.

A comparison of the graphs in rectangles A, B and C of Figure 2 shows that the rate of oxygen uptake of small *Talitrus* (mean body weight 1.5 mg.), was higher than that of medium and large animals (mean body weights 8.5 mg. and 21 mg., respectively), at temperatures above 15° C. The increase in the respiratory rate of the smallest animals, with increasing temperature, was more rapid than the corresponding increase in that of the largest animals. For example, between 20° C. and 30° C. the Q_{10} of *Talitrus* of mean body weight 1.5 mg. was 2.33 in winter while the Q_{10} of *Talitrus* of 21 mg. mean body weight was 1.66 in winter.

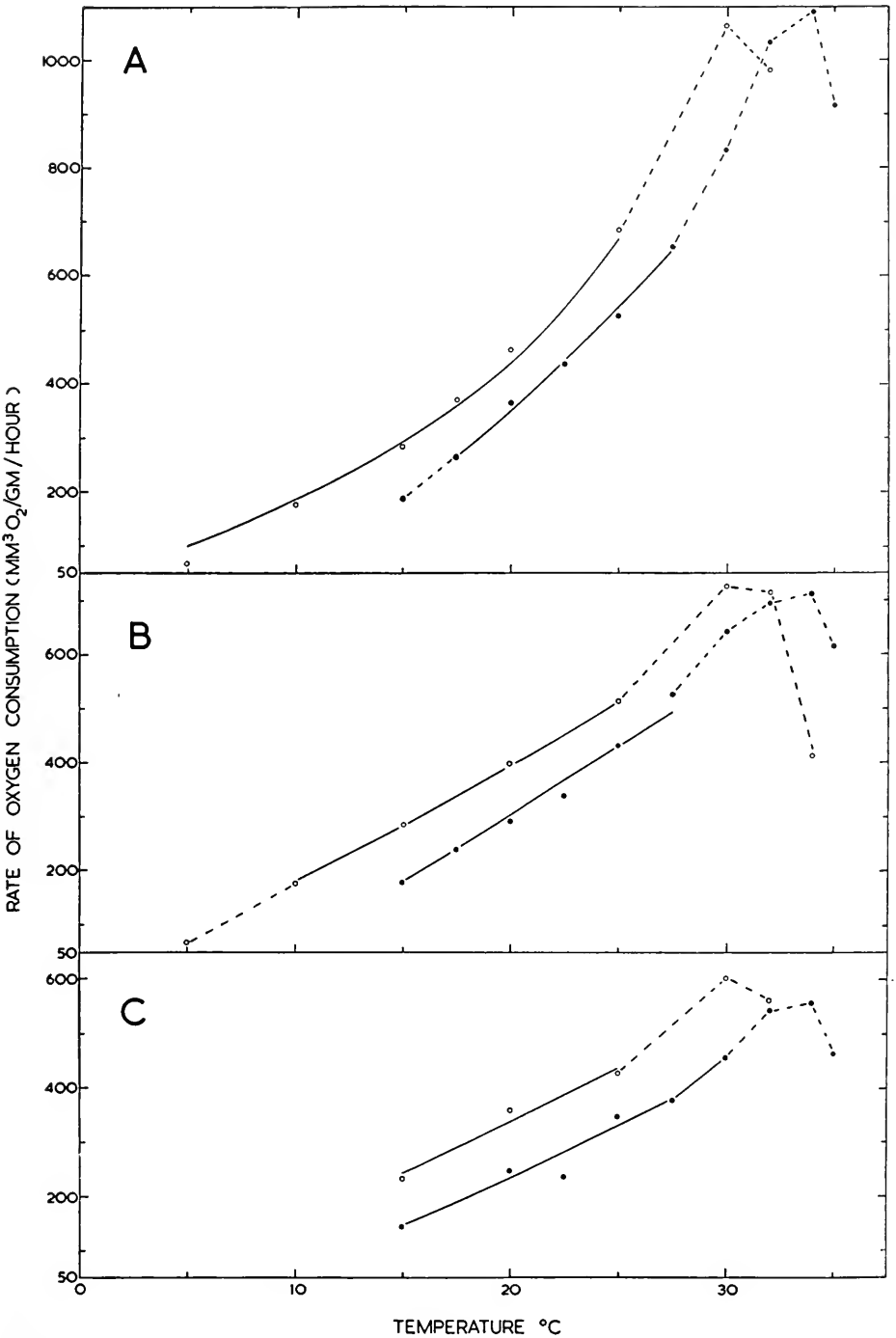

FIGURE 2. The relationship between the rate of oxygen consumption of *Talitrus sylvaticus* and temperature. Graphs A, B, C show curves for the rate of oxygen consumption of *Talitrus* weighing 1.5 mg., 8.5 mg. and 21 mg., respectively. Open circles, winter; black dots, summer. Broken lines are shown where the curves do not fit a logistic formula.

The effect of season

At any temperature the rate of oxygen consumption of *Talitrus,* which were taken from the field in winter, was approximately one and a half times higher than that of *Talitrus* of the same body weight which were taken from the field in summer. For example, the rate of oxygen uptake of *Talitrus* weighing 1.5 mg. was 187 mm.^{3} O_2 per gm. per hour at 15° C. in summer, and 272 mm.^{3} O_2 per gm. per hour at the same temperature in winter. Figure 2 shows that the rate of oxygen consumption of *T. sylvaticus* at any temperature in winter was the same as that at a temperature 2.5 centigrade degrees higher in summer.

An analysis of variance of these results showed that, of the factors influencing the rate of oxygen consumption of *T. sylvaticus,* significant first order interactions occurred between temperature and body weight, temperature and season, and season and body weight, P being less than .01 in each instance. The analysis also showed a significant second order interaction between temperature, body weight and season of the year upon the rate of oxygen uptake of *Talitrus,* ($P < .01$).

Discussion

The decrease in the rate of respiration of *Talitrus sylvaticus* relative to increase in its body weight at 25° C., as expressed by the exponent of 0.836, agrees well with previous results found on the respiration of crustaceans. Weymouth *et al.* (1944) found that the relationship between the rate of oxygen uptake and body weight of the kelp crab, *Pugettia producta,* was expressed by an exponent of 0.798 at 15° C. Scholander *et al.* (1953) found that an exponent of 0.85 could be applied to this relationship in many crustaceans ranging from the arctic fairy shrimp, *Branchinecta paludosa,* to the tropical giant land crab, *Sesarma ricordi.* Zeuthen (1953), in his review of the subject of body size and metabolic rate in organisms, stated that an exponent of 0.80 described this relationship for crustaceans containing more than 0.1 milligrams of body nitrogen.

The occurrence of a seasonal adaptation of the rate of oxygen uptake, as shown by *Talitrus sylvaticus,* has only been found infrequently in invertebrates (Scholander *et al.,* 1953). Edwards and Irving (1943a) found that the rate of oxygen uptake of the sand crab, *Emerita talpoidea,* from beaches at Woods Hole, Mass., was higher in winter than in summer at all temperatures below 20° C. In contrast, the amphipod, *Talorchestia megalophthalma* (family Talitridae), which also inhabits the beaches at Woods Hole, hibernates in winter and the rate of its respiration is the same in winter as in summer (Edwards and Irving, 1943b). Seasonal adaptation of metabolic rate to changing environmental temperatures has also been recorded in the gill tissues of the clam, *Venus mercenaria* (Hopkins, 1946).

The mean monthly temperature in the rain forest litter at Bulli Pass in 1951 ranged from 12.0° C. in July (mid-winter) to 21.0° C. in January (mid-summer) (Clark, 1954). The shift in the O_2 consumption-temperature curve of *Talitrus sylvaticus* to the left only covered a distance of 2.5 centigrade degrees along the abscissa (temperature axis in Fig. 2) and the R.Q. of *Talitrus* remained the same in summer and winter (Clark, 1954), so that its energy output was lower in winter than in summer.

The physiological mechanism which enables animals to alter their rate of oxygen consumption in response to season is unknown. Hopkins' (1946) demonstration of seasonal adaptation in the excised gill tissues of *Venus mercenaria* suggests that seasonal adaptation occurs within the tissues of the animal and hence may be effected by changes in the concentration of intracellular respiratory enzymes.

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Summary

1. The relationship between the rate of oxygen consumption and body weight of the terrestrial amphipod, *Talitrus sylvaticus,* was expressed by an exponent of 0.836 at 25° C.

2. The rate of oxygen consumption of *Talitrus sylvaticus* was significantly higher in winter than in summer at any one temperature.

3. Analysis of variance showed that, of the factors influencing the rate of oxygen consumption of *T. sylvaticus,* first order interactions occurred between temperature and body weight, temperature and season, and season and body weight. There was also a significant second order interaction between temperature, body weight and season on the rate of oxygen uptake of *T. sylvaticus.*

4. The adaptation of its rate of oxygen uptake by *Talitrus sylvaticus* to seasonal differences in temperature was not sufficient to enable it to maintain the same rate of output of energy in winter as in summer.

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PROTEASE IN AMMOCOETES ENDOSTYLE[1]

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It is now generally accepted that there is at least one proteinase associated with the breakdown of thyroglobulin in the thyroid gland. The action of such an enzyme is necessary to release thyroxine from protein, so that it may be able to diffuse through the follicular epithelium into the blood. The enzyme has been demonstrated in thyroids of rats and guinea pigs (Dziemian, 1943; De Robertis, 1941), man (De Robertis and Nowinski, 1946), and dog (Kamner *et al.*, 1950). From beef thyroid, Anson (1937) partially purified a proteolytic enzyme with properties of a catheptase; Weiss (1953) further characterized the beef protease biochemically. McQuillan and Trikojus (1953) were able to show the existence of a close thyroglobulin-enzyme association in hog thyroid and they devised a method for its purification.

Considerable interest attaches to the question of whether the larval thyroid gland of lampreys, the "endostyle," possesses a proteinase similar to that of thyroids of the higher vertebrates. The endostyle opens by means of a duct into the pharynx so that any thyroid-like secretion could be passed through it for digestion and absorption in more posterior parts of the gut. Thus, it would not appear to be essential for the endostyle to have an enzymatic thyroxine-freeing mechanism. On the other hand, when the lamprey larva undergoes metamorphosis, the duct to the pharynx is closed and the endostyle is in this way converted into a more or less typical follicular thyroid gland in the adult. Such closed follicles would require enzymatic hydrolysis of their contained thyroprotein. At what stage in these developments does the thyroidal proteinase appear? Does it precede the endostyle-thyroid transformation, or follow it? These are the questions that the present work has attempted to answer.

Materials and Methods

Because of the small size of the endostyle, it was necessary to employ a procedure especially adapted to the determination of the proteolytic enzyme on a micro level. The technic used for the measurement of enzyme activity was modified from the procedures of Anson (1937) and Kamner *et al.* (1950).[2] The animals used were the ammocoetes larvae of *Petromyzon marinus* collected in the Salmon River near Plattsburg, New York.

[1] We gratefully acknowledge the use of some facilities of the Brookhaven National Laboratory in the course of this work. This work was aided by Contract NR 163-208 between the Office of Naval Research, Department of the Navy, and Columbia University, and more recently by a grant from the National Science Foundation.

[2] We are grateful to Dr. L. C. Sze for his help in developing the microtechniques employed.

Larvae, ranging in size from 13 to 16 cm. in length, were used. The lower half of the pharynx was removed from unanesthetized animals and the endostyle was dissected out and weighed. Two endostyles at a time were homogenized in a glass microhomogenizer containing 50 microliters (μl) of 60% aqueous glycerol. Additional glycerol solution was added until the total tissue concentration was 0.1 mgm./μl. This homogenate was refrigerated for 15 minutes at 4° C., and then distilled water was added to give a final tissue concentration of 0.05 mgm./μl. After careful stirring with a fine, glass rod, the mixture was again refrigerated for one-half hour. The homogenate was then centrifuged, and the supernatant, used for analysis, was drawn off as needed. Endostyles of four or five ammocoetes were used in each separate test.

A 2.5% aqueous solution of hemoglobin,[3] was made up not more than three days before use as the stock substrate material. The final solution of buffered substrate, prepared just before use, contained 4 ml. of 2.5% hemoglobin solution and 1 ml. of a solution of acid (and/or salt) of desired molarity. The pH was determined on the Beckman pH meter.

TABLE I

The effect of hydrogen ion concentration upon endostylar proteolytic activity. Incubation time: 5 hours

25 μl of homogenate (1.25 mgm. tissue) +150 μl of buffered hemoglobin, pH:	Micrograms of tyrosine freed per 1.25 mgm. tissue
2.00	0
2.60	0
3.42	25.5
3.90	35.4
4.00	36.2
4.30	27.4
5.00	14.0

A series of conical two-ml. Pyrex centrifuge tubes, each containing 10 μl (or more) of the homogenate supernatant, was prepared in groups of three, two experimentals and one control tube. Into each was pipetted 150 μl of the buffered substrate of the desired pH. To the controls, 275 μl of 0.3 *M* trichloroacetic acid (TCA) also was added. This proved to be as satisfactory as heat inactivation suggested by Kamner *et al.* (1950). These tubes were then stoppered and placed in a water bath at 37.0° ± 0.02° C. After the desired interval, the reaction in the experimental tubes was stopped by the addition of 275 μl of 0.3 *M* TCA and thorough mixing. Since filtering of these small volumes was not practical, the tubes were centrifuged at 3000 rpm. and the clear supernatant was analyzed for liberated tyrosine as follows: 200 μl of the supernatant of each tube were quantitatively transferred into a correspondingly marked cuvette. To this, 400 μl of 0.5 N NaOH was added in similar fashion and the cuvettes were inverted three times to insure complete mixing. One part of Folin-Ciocalteu phenol reagent[4] was

[3] Powdered Bovine Hemoglobin Enzyme Substrate Powder, Pentex Incorp., Kankakee, Illinois.

[4] Eimer and Amend, New York.

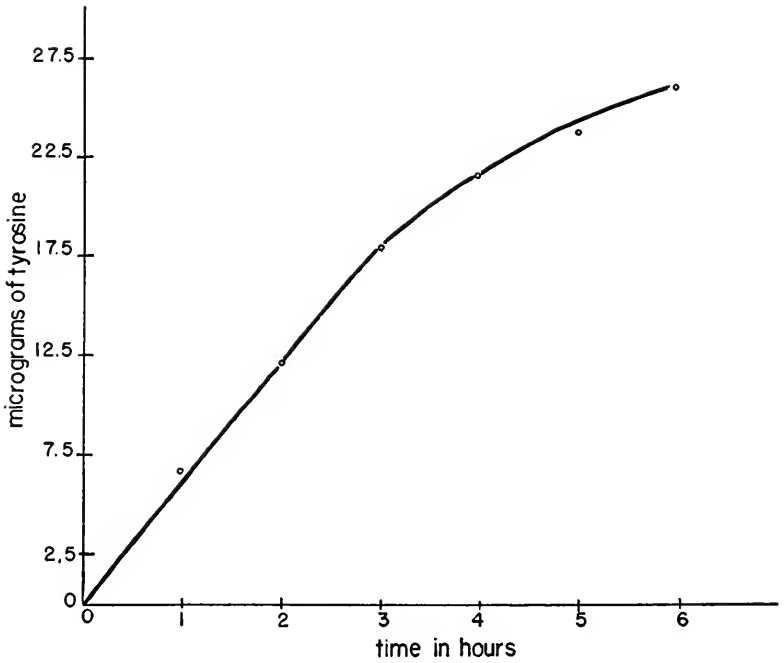

FIGURE 1. Proteolytic activity of endostylar enzyme as a function of time. Tissue concentration: 0.5 mgm./10 μl. of homogenate.

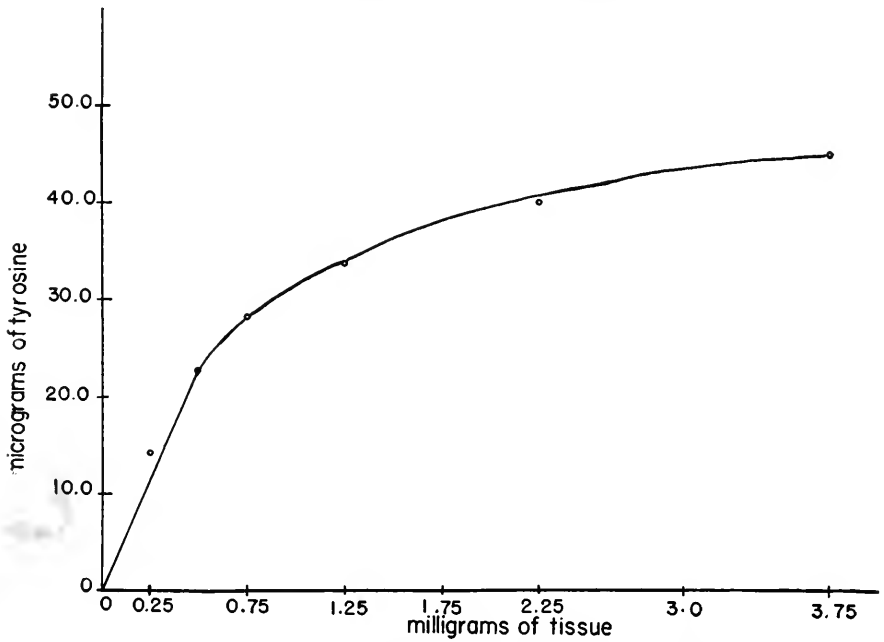

FIGURE 2. Proteolytic activity of the endostyle as a function of its concentration expressed as milligrams of tissue per unit volume. Incubation: 5 hours.

diluted with two parts of distilled water, and 150 μl of this dilution was added to each cuvette which was again inverted three times. The characteristic blue color produced in 15 minutes was measured in a Coleman, Jr. spectrophotometer at 700 λ and values were expressed as micrograms of tyrosine liberated by the homogenate after subtraction of the control values. The tyrosine standardizing solution was prepared from l-tyrosine, and the curve was made by reading spectrophotometric values corresponding to quantities ranging from 5 to 35 micrograms of tyrosine.

Results

The proteolytic activity of glycerol extracts of endostylar tissue first was measured as a function of the hydrogen ion concentration of the *in vitro* reacting system. Optimal activity for digestion of the hemoglobin substrate was found at pH 3.9–4.0, decreasing measurably on either side of this peak (Table I). It is interesting that this optimum is quite close to those found by others in studies of mammalian thyroidal protease.

To make possible further comparison of the ammocoetes endostylar protease with the thyroidal proteases of other animals, studies were made of the proteolytic activity of the enzyme as a function of time, and of its concentration. The data for both of these studies were confirmed by several repetitions, and two series of experiments are summarized in Figures 1 and 2. It may be seen that under the conditions employed, and within stated limits of time and enzyme concentration, proteolytic activity is a linear function of either of these two variables.

Discussion

Schneider (1879) first observed the histological changes during the transformation of the endostyle of metamorphosing larvae, and these observations led him to the conclusion that the endostyle is the forerunner of the well-defined thyroid gland of the adult. It was Dohrn (1875, 1886) and later, Leach (1939), who suggested that the endostyle might serve functionally in the production of a hormone or its precursor. They further believed that this secretion might pass through the pharynx to the alimentary canal where it could be absorbed. Recently, Sterba (1953) in an extensive study determined that the Type III secretory cells of the endostyle of *Petromyzon planeri* contributed to a large extent to the formation of the follicles of the adult thyroid. This supplied the basis for the correlation of morphology and function, for it was the Type III cells of *Entosphenus lamottenii* that accumulated radioiodine in radioautographic tests (Gorbman and Creaser, 1942). This ability of the endostyle to metabolize and store iodine demonstrates its thyroid-like function, even during the prolonged larval period. Further confirmation has been provided by Leloup and Berg (1954) who showed by means of radiochromatography that the endostyles of the larvae of *Petromyzon marinus* accumulate iodide, and form moniodotyrosine, and diiodotyrosine when maintained at 15° C. and kept at 2° C., they also form thyroxine. Evidently, the same cells that participate in follicular formation of the adult gland are able, long before, to elaborate the precursors of thyroxine as well as thyroxine itself.

Following Dohrn's suggestion (1886) that the endostyle is an organ of digestive secretion, Alcock, as early as 1899, tested the endostyle for ability to digest

fibrin. Although she found other parts of the digestive tract active in this respect, the endostyle did not, in her tests, digest fibrin. She concluded that the endostyle did not function as a source of digestive ferments. Since this negative report there has been no work produced to either confirm or contradict this conclusion. The data reported here seem to establish the presence of an active protease of the catheptase type in the endostyle of ammocoetes. Further, this enzyme is present in a concentration comparable to the protease in the thyroids of mice and adult lampreys (unpublished data).

The usefulness of the endostylar protease is difficult to assess in terms of thyroid function. Whether the endostylar secretion, particularly that of the iodine-metabolizing Type III cells, passes into the lumen by holocrine (Gorbman and Creaser, 1942) or merocrine (Sterba, 1953) means, it seems to move rapidly into the pharynx (Sterba, 1953). This would not seem to provide sufficient opportunity for enzymatic hydrolysis of the secreted iodoprotein. If not digested in the endostyle it appears more likely that digestion of the iodoproteins would be continued and completed in the intestine, and it would be followed by absorption of the hormone.

At any rate, it is reasonable to conclude that the thyroidal protease of lampreys is formed in the larval endostyle, and therefore it precedes follicular differentiation, when its presence would be obligatory for proper thyroidal function.

Summary

1. A proteolytic enzyme was demonstrated, by use of a new microanalytical technique, to be present in the endostyle of the ammocoetes larva of *Petromyzon marinus*.

2. The protease has an optimum pH of 4.0. The hydrolysis of hemoglobin (expressed as micrograms of tyrosine released) under the conditions employed, and within a period of three hours, is a straight line function of time. The hydrolytic activity of the endostylar enzyme is also a straight line function of enzymatic concentration, but within restricted and defined limits.

3. The probable site of action of the hydrolysis of the endostylar thyroprotein is discussed in regard to the usefulness of the investigated endostylar protease.

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INITIAL FLIGHT DIRECTIONS OF HOMING BIRDS

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Our understanding of the orientation of birds has been significantly increased by recent experiments in which Kramer (1952, 1953) and Matthews (1953a, 1953b) have demonstrated that homing pigeons, and probably Manx shearwaters as well, can choose approximately the correct homeward direction during the first few minutes after release in unfamiliar territory. Yet such a high order of homing ability is not invariably displayed, nor is a nearly correct heading always followed by a rapid return to the nest. For when certain birds are released in unfamiliar territory they tend to fly in a particular direction even though this is not the homeward direction (with pigeons it may be the direction of previous training flights). Still other species, such as gannets, do not seem able to choose any consistent direction at all (Griffin and Hock, 1949). For convenience these three kinds of orientation have been called types III, II, and I, respectively: type I being reliance on landmarks with wandering or exploration in unfamiliar territory, type II the tendency to fly in a particular direction which may differ from the actual homeward direction, and type III the ability to fly in roughly the homeward direction over unfamiliar territory, regardless of what that direction may be (Griffin, 1952). Type II and III homings seem to occur only under clear skies, so that the sun is probably one important factor enabling the birds to choose a particular direction.

In seeking a general understanding of the orientation of birds it is of obvious interest to inquire which species show each of these three types of homing, and under what conditions. Chiefly in the hope of finding a species which would consistently display type III homing, the initial flight directions of common terns (*Sterna hirundo*) and Leach's petrels (*Oceanodroma leucorhoa*) were studied by methods designed to obtain accurate information about each bird's direction of flight away from the release point as long as it could be seen through binoculars. The results are of interest, even though type III homing was not demonstrated, because the common terns showed a consistent tendency to fly in a southeasterly direction when released inland, even when home lay in a different direction.

We wish to express our indebtedness to many friends who assisted in catching or transporting the birds, in watching for returns at the nests, or during the actual observation of initial flight directions. We are also grateful to the Office of Naval Research which provided financial support through research contracts with Cornell and Harvard Universities, and to the Marine Biological Laboratory at Woods Hole, Mass. which provided facilities and boat transportation during 1952 and 1953.

Methods

Incubating birds were captured at their nests and set free at inland release points chosen to facilitate observation of the initial flight direction. Since earlier homing experiments had shown that the recorded homing times were far too long to establish direct homeward flights (Griffin, 1940, 1943), in only a few of the present experiments was an observer stationed at the nests to record the time of each bird's return. The methods of capture and observation at the nest were the same as in previous homing experiments with these two species. All birds were transported in boxes through which they could see nothing of their surroundings, and experience showed that the use of large boxes holding several birds enabled them to reach the release point in better condition than smaller individual compartments where the feathers were disarranged by rubbing against the walls. Water was provided every few hours, especially in hot weather, and most of the terns seemed to take advantage of it both for drinking and bathing. The birds were released with a minimum of handling, usually being allowed to take wing spontaneously. Each bird was released alone and not until some minutes after the previous one had been lost to view through the binoculars. Except for occasional instances noted below, each bird's flight thus represented an independent choice of direction.

During 1952 and 1953 most of the birds were observed through hand-held binoculars which allowed us to determine only the final bearing of the bird from the release point, the direction which Matthews calls the "vanishing point." Since in many cases the bird's bearing shifted steadily during the last minute or two it was clear that its actual direction of flight was different from the final bearing from the release point. In 1954 binoculars were therefore mounted on tripods equipped with alidades so that we could note the actual bearing throughout the observations. In all but the first experiment in 1954 two such alidades were stationed ¼ to ½ mile apart, one with 7 × 50, the other with 15 × 60 mm. binoculars. An assistant noted the bearings every 15 seconds, giving warning to the observer before each reading so that he could center the bird in the field of the binoculars. This procedure gave simultaneous cross bearings accurate to about one degree, so that the bird's actual flight path could be plotted for several minutes.

The flight paths plotted in Figure 2 are typical of the results obtained under clear skies, and in many cases the actual flight directions plotted in this fashion differed significantly from the bird's final bearing as seen from the release point. Even after one observer had lost sight of a bird the successive bearings from the other alidade allowed us to extrapolate the probable flight path as the bird receded into the distance. This was done by assuming that the actual distance flown during each 15 seconds remained constant; or, if the bird could be seen to be circling, it was assumed to cover less distance. Such extrapolated flight paths based on a single set of bearings are shown in Figures 2 and 3 by open circles or triangles connected by broken lines, while solid circles and triangles connected by continuous lines represent positions determined by cross bearings.

The selection of release points was of crucial importance. Not only was it essential to have a clear view in all directions, but it proved advisable to avoid the proximity of lakes or streams. Especially in cloudy weather the terns tended to alight on small ponds or streams within a mile or so of the release point. None

of the release points was closer than 15 miles to the ocean, nor closer than ten miles to large lakes or rivers. These requirements severely limited the choice of release points in New England, but two nearly ideal sites were found at Storrs, Connecticut, and Houlton, Maine.

Initial Headings of Common Terns

The results of fifteen releases involving a total of 145 terns are summarized in Table I, while Figure 1 shows the locations of the release points and nesting colonies (Penikese Island, Mass., and North Sugarloaf Island, Maine). Wind direction is shown in Table I as downwind, *i.e.*, the direction to be expected if a bird merely

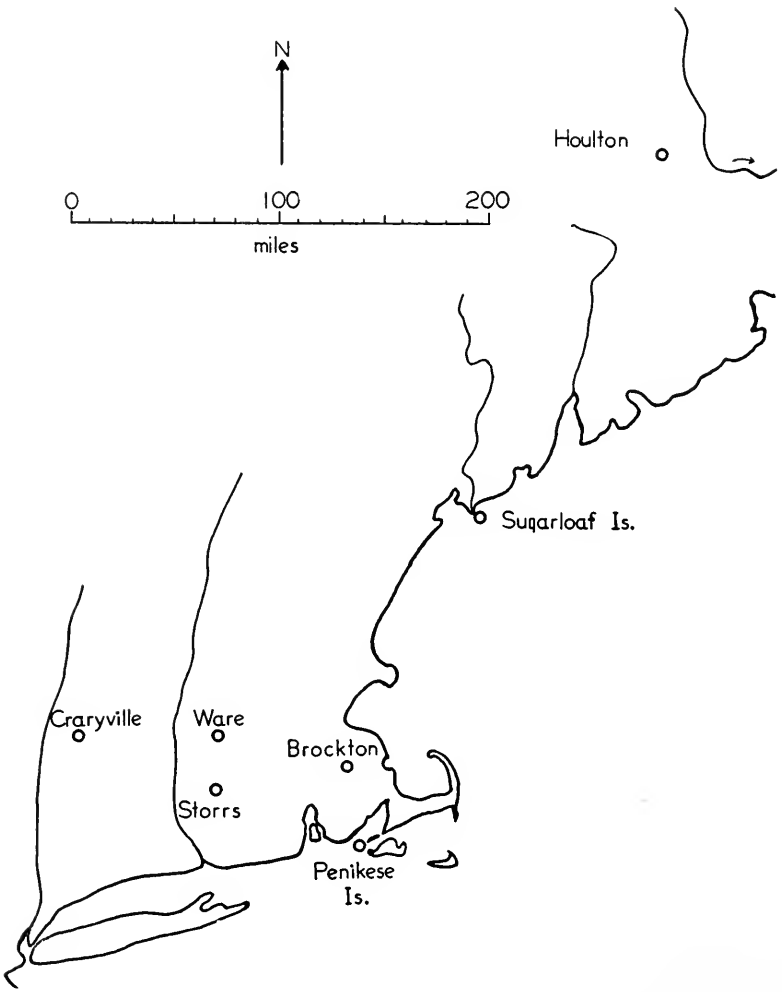

Figure 1. Map showing nesting colonies and release points (small circles). Birds from Sugarloaf Island are called "Me. birds" in Figures 2 and 4; birds from Penikese Island "Mass. birds."

TABLE I

Initial flight directions of common terns released inland. All birds from Penikese Island, Mass., except those noted as "Me. terns" which had been caught on Sugarloaf Is. (see Fig. 1). Brackets in right hand column indicate birds under shifted light schedules (see text)

Date	Release pt.	Down wind direction, velocity	Sky cond. and sun visibility	Home		Observed flight headings	
				Dist. (miles)	Bearing (degr.)	Mean	Individual terns
29–5 1954	Storrs, Conn.	N–NNE, 10–20	Br. clds., sun vis.	75	115	140	131, 185, 123, 105, 126, 142, 131, 135, 180
8–7 1954	Storrs	E, light	Br. clds., sun vis.	75	115	142	161, 146, 138, 140, 130, 154, 143, 127
8–7 1954	Storrs (Me. terns)	E, light	Br. clds., sun vis.	179	44	142	161, 151, 135, 127, 137, 144, 165, 90, 145, 180, 124
19–7 1954	Houlton, Me. (Me. terns)	N–NW, light	Overcast, some rain	190	211	—	6, 278, 42, 71, c110, 172, 105, c45, 171
28–7 1954	Houlton (Me. terns)	NE–SE, light	Clear	190	211	149	147, 187, 153, 133, 175, 133, 167, 144, 150, 129, 127, 154, 120, 160, 154
29–7 1952	Brockton, Mass.	W to NW, light	Sun visible	41	172	200	SSW, SSE, SSW, NNW, SW, SSE, SSE, SSW, SSE
6–8 1952	Brockton	S, light	Overcast, light fog sun not vis.	41	172	—	(E), (SE), (ENE), (S), (SW), (NE), (NE), (S), E, ENE, NE, W, SSW
6–7 1953	Brockton	E, 5–12 m.p.h.	Clear	41	172	151	E, SSW, S, S, S, ESE, SSE, ESE, SSE, SE, SSW
20–7 1953	Brockton	NW, light	Hazy, sun visible	41	172	158	SSW, E, S
22–7 1953	Brockton	S–SSW, 5–8 m.p.h.	Fog, sun not vis.	41	172	210	S, SW, SW
9–7 1953	Ware, Mass.	SSE light to mod.	Clear	87	131	116	E ESE, (E), (SE), E, (SE), (SSE), (S), (E), E, SE, ESE, E
23–7 1953	Craryville, N. Y.	Calm	High solid overcast	146	111	87	SSE, SE, NE, SE, (E), (NE, NE, NE) (see text)
9–7 1954	Craryville	N, very light	Clear	146	111	159	136, 160, 214, 145, 146, 155, 149, 170
9–7 1954	Craryville (Me. terns)	N, very light	Clear	222	60	131	120, 168, 119, 170, 151, 155
21–7 1954	Craryville	S–E, light	Br. clds., sun vis.	146	111	175 (most flew to lake)	185, 182, 175, 217, 189, 163, 181, 179, 178, 177, 161, 164, 184, 170, 149, 176, 173, 159, 154

drifted with the wind. Our best estimate of the general trend of each bird's flight after it had begun to head across country is listed in the right hand column of the table, in compass directions when hand-held binoculars were used, in degrees when alidades were employed. The actual precision of these estimates varied

widely, depending on the straightness of the bird's flight path, but when two alidades were used, almost all the estimates were accurate within ± 5°. Every bird for which any sort of meaningful initial homing direction was obtained is included either in Table I or in the section entitled "Miscellaneous Release Points." The only birds omitted from Table I were 20 lost to view (behind trees, against the sun, etc.) while still close to the observer, and 26 birds seen to land on ponds or other local bodies of water. Even these birds showed a southeasterly trend, so that their omission has not removed data inconsistent with the conclusions reached below.

The general behavior of the terns immediately after release was to circle or fly in irregular patterns with frequent changes of direction for about one to three minutes. Then, in good weather, they would generally begin flying an essentially straight course which soon carried them out of sight, although circling might continue at intervals. Examples of such reasonably straight flights are shown in Figures 2 and 3, with the initial circling omitted. When the individual points (one plotted for every 15 seconds) are close together the bird was circling. The time during which each bird was observed varied from four to twelve minutes, and most of them were lost to view at one to two miles from the release point. On some days the terns climbed on updrafts and even soared briefly, so that altitudes of a few hundred feet were reached. Our vertical bearings were only accurate enough, however, to convince us that neither at Storrs, nor at the other release points except possibly Brockton and Chester, could any of the birds have seen the coast while under observation.

Storrs, Connecticut

Consistent and significant headings were observed in all experiments at this release point. In a preliminary test of five terns from Penikese Island, two could be seen to head south or southeast, the others being lost to view before they showed any definite headings. Penikese Island is 75 miles from Storrs at 115°.

On May 29, 1954 a group of 16 terns was brought from Penikese Island to Storrs in excellent condition and watched for periods ranging up to 12 minutes with a single pair of binoculars mounted on an alidade. Although the first six birds released were lost behind trees before they had shown definite headings, we were then able to shift the observers' positions so that nine of the remaining ten could be followed for considerable distances. Despite a brisk wind from the south these birds showed a striking tendency to head southeast, their final bearings varying from 117° to 180°, and when reasonable corrections were made in the cases of four birds for which the bearings were shifting significantly during the last minute or two of observation, the estimated headings became those listed in Table I, with an average of 140°.

To determine whether this was a true case of type III homing, two groups of terns were next brought to Storrs from the Maine and Massachusetts colonies shown in Figure 1. On July 8, 1954 these 23 birds were released singly and in an irregular sequence not known to the observers by Dr. J. Manter of the University of Connecticut. Two observers stationed 2500 feet apart watched through binoculars mounted on alidades, and all but two terns showed definite headings while observed for periods ranging from 4 to 7½ minutes. All of these bearings

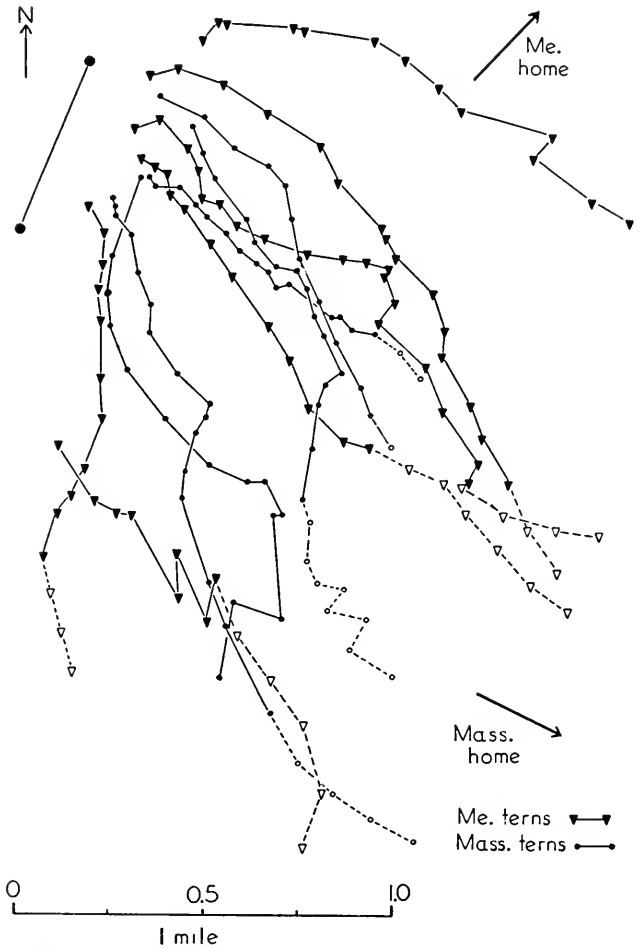

FIGURE 2. Flight paths observed after releases at Storrs, Conn., July 8, 1954. Solid triangles and circles stand for cross bearings, open triangles and circles for single bearings. The baseline connecting the observer's positions is shown in the upper left hand corner. Note that birds from the Maine and Massachusetts colonies show no significant difference in headings.

are included in Table I, and eleven of the longest series of observations are plotted in Figure 2. The flight paths omitted from Figure 2 were those which covered shorter distances, involved a small proportion of double bearings, or simply fell too close to one or more of the lines already drawn in this figure. These results demonstrate clearly that there was no difference in headings between the Maine and Massachusetts birds, both averaging 142° although the home directions were 44° and 115°, respectively.

Houlton, Maine

To analyze this southeasterly tendency further we next selected a release point at which the homeward direction was as different as practicable from those al-

ready tested. The airport at Houlton, Maine proved suitable since it provided excellent visibility in all directions and was in an area where the general drainage pattern was away from home; this is indicated in Figure 1 by the St. John River to the east which flows southward off the map. Terns from Sugarloaf Island were used, this island being 190 miles from Houlton at 211°. For the release on July 28 the weather was clear and mild, and the observation times ranged from 4 to 9 minutes. As shown in Figure 3 the headings were very similar to those observed at Storrs, with an average of 149° in comparison with 142° at the Connecticut release point. On the other hand the home direction for the Sugarloaf Island birds was 211° at Houlton and 44° at Storrs.

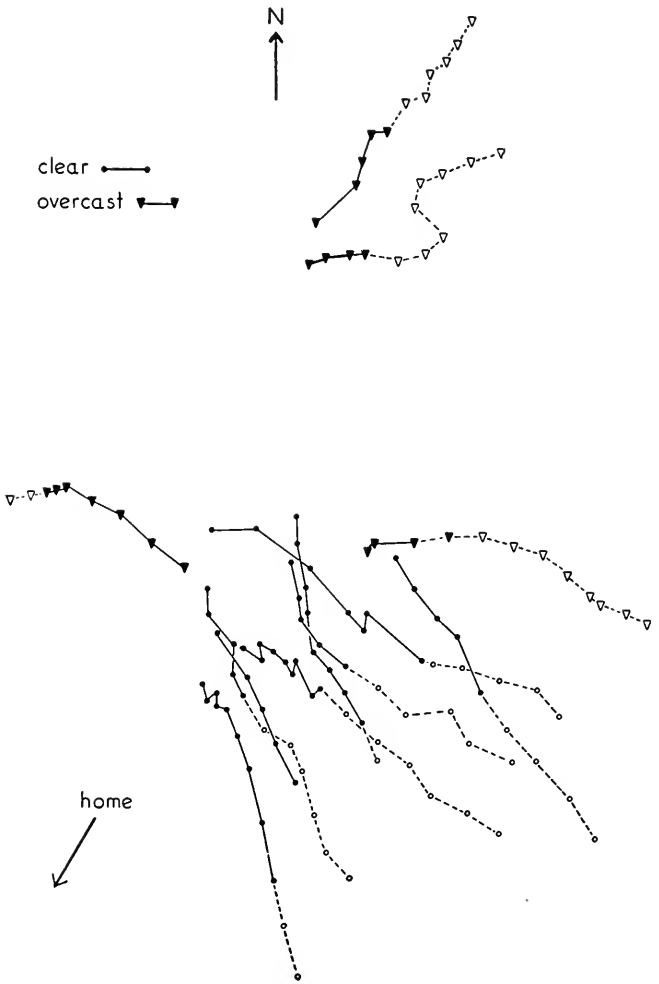

FIGURE 3. Flight paths observed after releases at Houlton, Maine on July 19, 1954 (heavy overcast and rain), and on July 28 (clear). Observers' positions not shown because they differed on the two days, being 3300 feet apart on July 19 and 2000 feet apart July 28. Scale and designation of cross and single bearings same as for Figure 2.

When terns from the same colony were released at Houlton under a heavy overcast on July 19 they obviously did not show the same tendency to fly southeast. Just as other terns had done at Craryville, Brockton, and elsewhere on overcast days, they showed much more hesitation and continued the circling phase of their flight for much longer than when the sun was visible. Observation times ranged from 4 to 10 minutes, but the birds did not fly as far from the release point as on the 28th. Eight landed on a small pond to the north of the airport, and these are not included in Table I. Indeed only 4 out of 20 showed a consistent enough heading to justify plotting their flight paths in Figure 3. The birds included in Table I and Figure 4, but not in Figure 3, were either followed by only one observer or yielded too few bearings to indicate a definite trend; their final bearings from the release point are the values listed in Table I.

Miscellaneous release points

Several other releases led to results consistent with those described above, and they are included in Table I or listed below even though they involved less satisfactory conditions. Those birds indicated in Table I by bearings in brackets were subjected to shifted schedules of light and darkness during two to four days in captivity in an attempt to shift their activity rhythms. No effect of this treatment could be discerned in their headings, but it is doubtful whether the treatment was long enough in duration to actually alter the activity rhythms.

Several groups of terns were released at the small airport south of Brockton, Mass., although the view is restricted by buildings and trees and Brockton is too close to the ocean to be certainly in unfamiliar territory. The time of day varied widely in the various releases, as did the observation time, but neither could be correlated with the initial headings. The results at Brockton were merely suggestive of a southerly (and approximately homeward) heading under clear skies, with a much wider scatter under a heavy overcast. A hilltop near Ware, Mass., was used only once because trees restricted the view and because a large pond was visible to the east, towards which several of the birds headed.

Eight terns were released at Craryville, N. Y., on the afternoon of July 23, 1953 under heavily overcast skies with light intermittent rain. The three birds indicated as "(NE, NE, NE)" had first flown off to the west, two of them remaining out of sight for several minutes. Several minutes later these two returned to the vicinity of the release point, were joined by the third, and then all flew off northeast together. While driving west towards the Hudson River shortly after releasing these birds it became obvious that the overcast extended only about five miles to the west of the release point and that the first two terns might have seen the afternoon sun during their flight to the west. Two releases at Craryville in 1954 confirmed the observations at Storrs, as is shown in Table I. The observers' alidades were 1300 feet apart, and the observation times ranged from 3½ to 9 minutes on July 9 and from 3½ to 7½ minutes on July 21st. But the results are complicated by the presence of Copake Lake 1½ miles south of the release point subtending an angle of 34° (156°–190°). On July 9 only about half of the birds seemed to be heading towards this lake, but on the 21st almost all did so, and we are certain that several actually landed on it.

In 1953 three terns were released at Keene, N. H., and two south of Barn-

stable, Mass., in both cases under heavy clouds with rain. Two of the Keene birds and one of the Barnstable terns landed on a pond; the other Keene bird flew west; the second Barnstable bird south. A group of seven terns was released July 9, 1953 in clear weather at Brookfield, Mass.; one flew to the east, while the others landed on a creek near the release point. Four terns from a colony at Plymouth, Mass. were released in clear weather at Chester, N. H., their nests being 75 miles away at 155°. Two were lost to view behind trees before choosing a definite heading, but the others were observed with two alidades for about six minutes and headed at approximately 214° and 145°, respectively. Unfortunately, the topography of this area, together with the altitude reached by these birds during our observations, might have permitted them to see the coast.

Initial Headings of Leach's Petrels

One petrel taken from Penikese Island to Storrs was observed to fly for some distance in a generally correct homeward direction. Twelve others from Kent Island, near Grand Manan, New Brunswick were released at Brockton and they also headed towards home. But these birds were rather weak and the homeward direction was downwind. The sun was visible during both these observations. Encouraged by these preliminary results, on June 19, 1954 we carried 22 petrels from Little Duck Island, near McKinley, Maine, to Houlton, Maine. They were released in excellent condition, some having been in captivity only eight to ten hours and the rest about 24 hours. There was a light wind blowing from the south. Ten petrels were lost to view soon after release, but satisfactory observations of a reasonably straight flight away from the release point were obtained for the other twelve. During six of these observations the sun was visible, for the other six it was hidden by clouds. Our best estimates of the final flight directions of the birds which could see the sun were: 27°, 105°, 205°, 226°, 330°, and 340°, and the six which could not see the sun showed headings of 15°, 15°, 28°, 31°, 280°, and 316°. Since the home island was 141 miles away at 189° there was no apparent tendency to fly towards home or in any other single direction. The northerly tendency of the birds released under overcast skies was possibly due to the presence of a small pond in that direction. This lack of any consistent directional trend after the release at Houlton, together with the greater difficulty of observing petrels at a distance, discouraged us from further attempts to study their initial headings.

Homing Performance

Earlier homing experiments with terns and petrels had shown that homing was relatively slow; hence in only a few of the present experiments was an observer stationed at the nesting colony to record the time of each bird's return. The following results of these experiments confirmed the previous conclusion that the homing times were far too long to establish whether or not direct homeward flight had occurred. After the release of terns at Brockton from 9:20 a.m. to 12:30 p.m. on July 6, 1953 a careful watch at ten of the nests showed eight returns by the end of the day following release. The most rapid was a tern that showed an initial heading of SSE (home being 41 miles away at 172°), and reached its nest in 3 hours 21 minutes. This corresponds to a direct flight at

12.3 m.p.h. The other birds were not nearly so rapid, however, the return times and initial headings being: 7 hours (SE), 20 hrs. (S), 24 hrs. (S), 27½ hrs. (SSE), 30 hrs. (S), 30 hrs. (ESE), and 31 hrs. (SSW). A less thorough watch for returns was maintained at Penikese Island after the July 9 release at Ware, and three returns arrived 16, 18, and 18 hours after release. The earlier homing experiments with terns had included one group released inland at 95 miles northwest, and return times ranged from 10 to 80 hours.

Of the 22 petrels released June 19 and 20 at Houlton, five reached their nests 141 miles away during the period from June 21 to 24 when the burrows were checked daily. Three of these birds had been released at Houlton late on the afternoon of June 19; one returned during the night of June 21–22, the other two the following night. One of the latter was the only return for which a satisfactory observation of initial flight direction had been obtained—330° in contrast to the true homeward direction of 189°. In previous homing experiments with this species a similar proportion of returns was recorded within four or five days after releases 120–170 miles at sea or along the coast.

Discussion

It seems clear that when these terns were released inland under clear skies (and were not attracted to local bodies of water) they tended strongly to fly in a southeasterly direction. This orientation was not appreciably affected by a wide variety of wind directions, and it was independent of the time of day. For instance in the four most significant experiments the actual times of release (EST) were as follows: Storrs, May 29, 2:00–5:15 p.m.; Storrs, July 8, 10:48 a.m.–3:56 p.m.; Craryville, July 9, 7:46–10:28 a.m.; and Houlton, July 28, 7:23–11:53 a.m. Yet there was no significant difference in the headings on these four dates, nor between the earlier and later releases on a given day. The release points at Storrs, Houlton, and Craryville were almost certainly in unfamiliar territory. This is true in the sense that recognition of landmarks during the first few minutes after release seems highly improbable, even though many hours of exploration might eventually have brought the birds to familiar territory. Since the headings of the Maine terns were clearly different from the homeward direction, we are evidently dealing with an example of type II orientation—a tendency to head in a certain direction whether or not this carries the birds towards home.

These experiments also indicate that the southeasterly orientation requires a view of the sun. But our data supporting this conclusion are less extensive than those demonstrating the consistency of the headings when the sun is visible. In one exceptional case (Brockton, July 22, 1953) three terns were released in a fog when the sun was not discernible to a human observer, and they headed in the same general direction that other terns had chosen at this release point in clear weather. The most satisfactory experiments, with respect to numbers of birds and good conditions for observation, are summarized in Figure 4, where the nearly random scatter under a heavy overcast is compared with the southeasterly tendency in clear weather. It is of some interest to note that while Matthews reports that as little as 50% cloud cover caused marked deterioration in the homeward orientation of Manx shearwaters, these terns continued to show equally consistent headings even when the sun could be seen only occasionally between breaks in a 90–

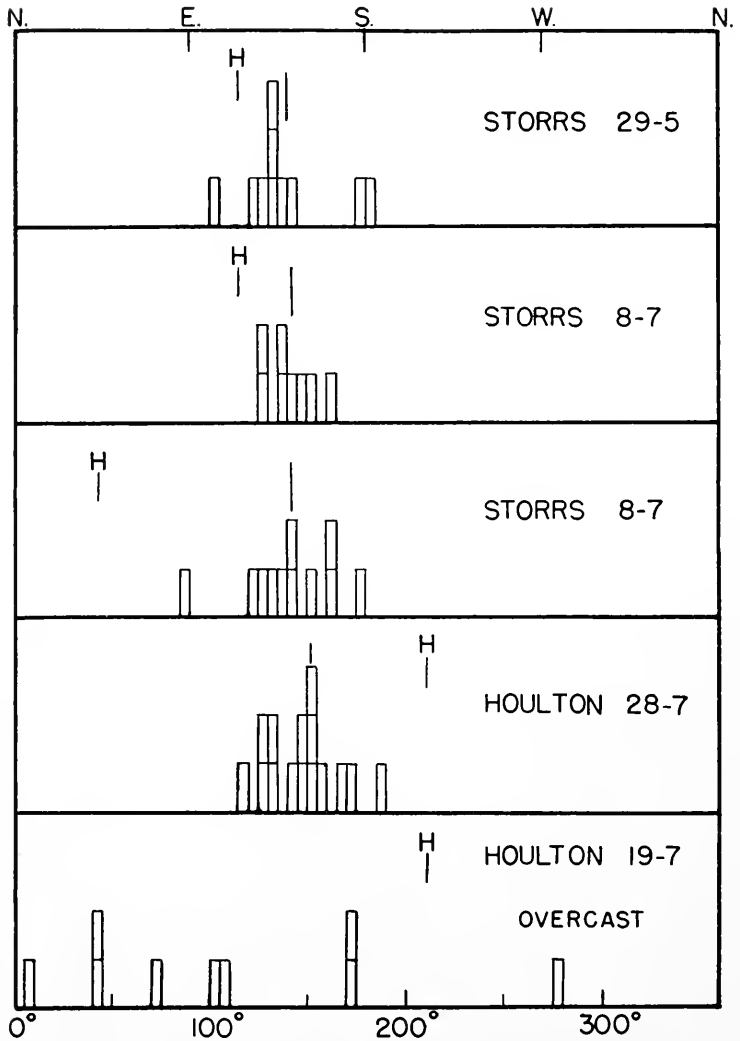

FIGURE 4. Graphic comparison of initial headings of 53 terns during the 1954 experiments at Storrs and Houlton. Homeward direction indicated by "H," mean heading of each group by vertical line. Sun visible to all birds except the last group labelled "overcast." Note the nearly identical average headings despite the 167° range of homeward directions.

95% cover of broken cumulus clouds. Only under a complete and thick overcast such as that prevailing at Houlton on July 19, 1954 did we observe a significant departure from the southeasterly headings.

Type II homing has been noted both in pigeons (Hitchcock, 1952; Matthews, 1951) and in certain wild birds (Rüppell, 1944; Rowan, 1946). Its appropriateness can be understood when the direction of flight corresponds to the previous training flights of pigeons or to the migration route which a given species can be ex-

pected to follow at the time of the experiment. But why do common terns tend so strongly to fly southeast? The migration route of this population of terns has been analyzed by Austin (1953) on the basis of a large number of recoveries of banded birds. This evidence could be interpreted as showing either that these terns first fly southwest along the coast and then move southeast to the general area of Jamaica, Trinidad, and Venezuela, or that they fly roughly south southeast across the ocean from their breeding grounds to their winter range. The initial headings we observed are thus similar to the direction of the fall migration; but our experiments took place during the nesting season when it is difficult to see why a tern should begin to migrate. It seems more likely that the observed headings represent a tendency to fly towards the coast. These terns do fly inland from time to time, and an ability to head towards the southeast would serve to guide them quickly back to the coast after any excursion inland from their normal summer range. This hypothetical explanation of the southeasterly headings also leads to the prediction that terns nesting in a different geographical area, such as the Great Lakes for example, would not show the same initial headings when released in unknown inland surroundings. We should thus like to suggest in closing that others interested in the phenomena of bird orientation attempt to repeat this type of observation with the same or similar species nesting in other regions, and with other species that have not yet been observed carefully as they begin homing flights over unfamiliar territory.

Summary

1. The initial flight directions of common terns and Leach's petrels were observed for as long as possible after release in unknown, inland areas. In some experiments two observers obtained cross bearings on each tern for as long as it could be seen through binoculars (maximum 12 minutes and two miles distance).

This procedure is strongly recommended whenever the initial headings of birds are of interest, since it reveals the actual direction of flight with greater accuracy than recording only the "vanishing point."

2. The petrels exhibited no consistent headings towards home or in any other direction, but one experiment resulted in returns from an inland release point similar in speed to those reported earlier from releases at sea or along the coast.

3. The terns showed a consistent tendency to head approximately southeast when the sun was visible. Average headings in the most satisfactory experiments were 140°, 142°, 142°, and 149°, while the total range of headings among 43 terns in these four experiments with the sun visible was 97°. The mean deviation from the average heading of 144° was only 16°. There was no significant difference between the headings when the direction of home was 44°, 115°, or 211°.

4. This southeasterly tendency disappeared almost entirely when the sun was hidden behind thick clouds, but it persisted under 90–95% cloud cover when the sun could be seen occasionally through breaks in the clouds.

5. The southeasterly tendency represents a special type of what has previously been called type II orientation, and it is probably based upon the sun. It may be a useful ability for terns that nest along the eastern coast of the United States, since whenever they find themselves inland, flight to the southeast will bring them quickly back to the coast.

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X-RAY EFFECTS ON ADULT ARTEMIA

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The available information concerning the lethal dosage of ionizing radiations in animals is limited to a certain few microorganisms, selected insects and laboratory types of vertebrates. Even more limited has been research on radiation-induced sterility in females. Because there are extreme differences between vertebrate results and those with holometabolous insects, it is of interest to fill in the gaps by extending research to a wide variety of representative animals. The present paper introduces a member of a primitive order of crustacea to this form of research. Except for attempts to induce visible mutations (Gajewskaja, 1923), we have been unable to find previous reports of a radiobiological nature on Artemia.

Materials and Methods

Commercial, dry eggs of the California strain (diploid amphigonic) of *Artemia salina* Leach, obtained from the M.B.L. Supply Department, were hatched in sea water and reared to maturity in large battery jars. Evaporation in our basement laboratory at Woods Hole was extremely slight; replacement was with sea water as is the standard procedure in bulk culturing. Cultures were fed daily with a few drops of either homogenized liver paste (commercial baby food) or yeast suspension unless the growth of natural microorganisms was obviously abundant. As soon as sex characteristics were distinguishable, individuals were segregated into male and female holding jars. When the accumulated total was adequate to provide five or more groups of at least five adult animals, experiments were set up. In this way animals were used within three days after reaching maturity.

The x-ray generator was that of the M.B.L., Woods Hole. This has two water-cooled G. E. (XTP) tubes in opposed position and operates at 30 ma and a 200 kv peak with an inherent filter equivalent to 0.2 mm. of copper. Air circulation in the room is assured by a fan.

Artemia were exposed 5 at a time in small plastic containers (4000 cu mm.). Exposures below 5000 r were made at an intensity of 2550 r per minute. Higher doses were delivered at 6120 r per minute. Calibration was achieved by M. Berman by inserting a rubber-covered ionization chamber through a hole bored in the side of one of the plastic containers which was then filled with sea water. Control samples of Artemia were held in exposure chambers in the anteroom for a period equalling that of the longest experimental treatment.

After irradiation, Artemia were maintained in a 30° C. incubator where evapo-

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ration was not a problem because the air was kept near the saturation point. Those used for life span records were segregated by sex and maintained 5 per wide mouth jar (50 by 80 mm.). For sterility studies, individual females were placed in stender dishes (60 by 35 mm.) and each provided with an untreated male. Regular mating associated with internal fertilization was observed periodically with all pairs. Feces, debris, and dead animals were removed daily by pipette and an equal amount of fresh sea water was added. If ova or nauplii had been deposited the pair of parents were transferred by glass tube to another stender dish. All incubator Artemia were fed daily by adding dilute yeast suspension in the amount of one drop per animal to the water of the container.

Results and Conclusions

Figure 1 presents survival of Artemia after exposures to various doses of x-rays. As shown, a sex difference was revealed at higher exposures by the tendency for females to die earlier than males in simultaneous experiments.

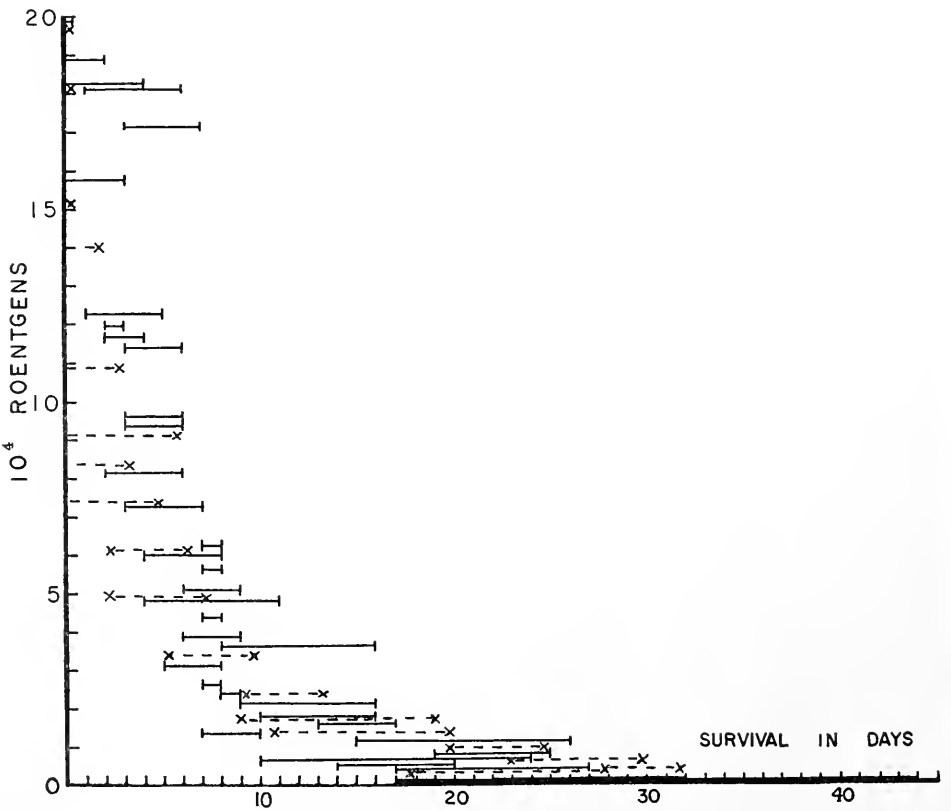

Figure 1. The range in time during which deaths in samples of five Artemia occur subsequent to x-ray exposure. Male records are designated by unbroken lines and short vertical marks; female records by broken lines ending in x marks. Control records are indicated by the thickening of the base line.

Furthermore, at 150,000 r and above, females failed to recover, although many males were able to survive for several days provided the exposure did not exceed 200,000 r. As a precaution, in the event that Artemia would be able to recove. from "knock down" doses as do adult insects, the following were delivered above those plotted: 224,910 r, 257,040 r, 289,170 r, 321,300 r, 353,430 r, and 358,785 r. No recovery occurred.

The minimal dosage at which all animals are killed seems to be the most feasible approach to lethal dose with materials of this type. As shown in Figure 1 for doses above 10,000 r, the variability in the time of death is not great when observations are taken in days for an organism whose life span is days. This is considerably different than the picture obtained with mammals whose life span is years. even decades, and the standard approach of 50% dead in 30 days provides a stable centering point for a high degree of variability. A 50% curve could be drawn for each sex through the ranges shown of Figure 1 and its shape would be

TABLE I

Summary of experiments on gamete deposit per individual female Artemia

X-ray dose in r	Highest no. of broods	Average no. of broods	Highest total no. of gametes	% Viviparous	Average no. of gametes	No. of females	Average adult life span in days
0	6	4.6	287	46.40	67.17	20	16.9
510	4	2.0	140	48.25	71.50	15	12.4
1,020	5	3.0	176	42.77	37.82	20	10.5
2,040	4	2.0	138	.04	57.46	15	9.2
3,060	2	0.9	130	.01	50.78	10	15.4
4,080	2	1.0	67	.14	29.40	10	10.7
5,160	2	0.7	55	0	18.29	15	5.9
11,527	1	0.7	79	0	31.40	5	3.4
15,900	1	0.5	23	0	6.60	5	9.8
21,200	1	0.4	51	0	12.60	5	6.6

the typical steep S. However no length of survival time suggests itself as particularly significant.

Table I presents a summary of observations on gamete production by females subsequent to their exposure. It is based upon the deposit of 46,966 ova and nauplii by 120 females. An initial experiment clearly demonstrated that no reproductive units except those pre-formed would be discharged after exposures above 5160 r and the groups of 5 experimental animals at each dose were not supplemented by replication. At 4080 r and 5160 r, exceptional individuals deposit a second meager brood but the great majority of females so treated die with empty uteri. At 3600 r second broods are adequate and death with a full uterus is not uncommon. Observations at the next lower dose, 2040 r, made it apparent that the sterility threshold had been bracketed. A comparison of the average life spans of Table I with the ranges at similar doses in Figure 1 demonstrates that deaths occurred relatively early in the sterility investigation. A number of cases of death of either or both parents following transfer lead us to suspect that brine shrimp are too delicate for repeated handling. Thus a number of females potentially able to give rise to additional broods of offspring may have been killed by

mechanical injury. Top individual performance as included in the table may be more valid as a basis for expectancy under normal conditions than are the averages obtained here. On either basis, the neighborhood of 2000 r is critical not only for numbers of broods produced but on the basis of inhibition of viviparity.

Normally, as occurred in controls, Artemia females void either live nauplii or brown heavy-shelled eggs (Lochhead and Lochhead, 1940). In addition to these, the experimentals produced several unusual types of gametes: partially formed nauplii, smooth shiny white eggs, dull white ragged eggs, orange eggs and tan eggs. Partially formed nauplii were able to complete development but none of the other types were seen to hatch. This may be due in part to their vulnerability through lack of a protective shell. Protozoa and copepods were seen actively attacking, presumably feeding on shell-less eggs.

Hatchability of brown shelled eggs will not be considered here. The data on various batches are not all identical because ways of handling this type of product were being explored.

Discussion

The lethal doses of ionizing radiation vary considerably among different types of animals. Best investigated from this standpoint have been the mammals where the total body dosage required to kill 50% of the adults within 30 days ranges from 200 to 800 r (Patt and Brues, 1954). To kill such types within hours after exposure requires higher doses, even as much as 25,000 r to 50,000 r for extreme cases (Hagen and Sacher, 1954; Patt and Brues, 1954). Massive as such doses may seem, they fall far below those producing immediate lethality for certain bacteria and protista on the one hand (Bonham *et al.*, 1947) and for adult insects on the other (Hassett and Jenkins, 1952; Sullivan and Grosch, 1953). The present results, 150,000 r for females and 200,000 r for males, place Artemia near but below holometabolous insects in radiation tolerance as adults. Demonstration of a sex difference was not unexpected. However females are usually found more resistant than males in the various animals studied.

Physiological factors undoubtedly are of importance in determining radiosensitivity and radiotolerance. One mammal, the bat, shows no effect on life span under 15,000 r just after hibernating and survives for several days after 60,000 r (Smith *et al.*, 1951). In work with insects the senior author has been impressed with the resistance of cell types specialized for physiological activity. Often this specialization is reflected cytologically in some degree of somatic polyploidy, a situation achieved through endomitosis and conceived to confer radioresistance (Grosch and Sullivan, 1954). Consistent with this view are the present findings in light of the fact that endomitosis accompanies differentiation to adult condition of a number of important Artemia tissues (Barigozzi, 1942). At earlier stages when mitosis is occurring and before endomitotic tissues have attained multiples of the basic chromosome number, Artemia should prove radiosensitive. Indeed, one of Gajewskaja's (1923) statements suggests this. Furthermore, even the most tolerant forms of holometabolous insects have radiosensitive developmental stages (for Habrobracon see Clark and Mitchell, 1952), while mammals on the other hand never achieve a radiotolerant stage. The latter depend upon mitotically active tissue to prevent loss of physiological fluids and for maintainance of the lines of bodily defence.

The most useful study found for comparison with Artemia results has been that of Henshaw (1939) on the cut branches of the colonial coelenterate, Obelia. Here 250,000 r were required to suppress hydranth activity and growth of the differentiated unit whereas only 6% of that large dose suppresses initiation and halts growth in the bud stage, a period characterized by cell proliferation. Henshaw used the same MBL generator employed for the present work.

Unlike the lethal dose response, the level of x-ray treatment causing Artemia sterilization is not appreciably above that required in some vertebrates for complete, irreversible destruction of ovaries. For example, although the 30-day lethal total-body dose in rats produces great degenerative changes in the ovaries, as much as 3000 r may be required in local exposure to insure permanent sterility (Lacassagne and Gricouroff, 1941). This may also be true for rabbits and guinea pigs. A similar dose, which as shown above affects Artemia gamete production profoundly, produces only brief temporary sterility in the wasp, Habrobracon (Grosch and Sullivan, 1954). To completely sterilize this wasp 5000 r is required. Glücksmann (1947) cites the same figure for Drosophila. Both of these holometabolous insects have a polytrophic type of ovariole in which large groups of polyploid nurse cells accompany each oocyte. Therefore on histological grounds the ovaries are not directly comparable to other forms. Evidence that insect ovaries of a simpler structure may be vulnerable at much lower doses is supplied with results with grasshopper nymphs (Tahmisian and Vogel, 1953) which have a panoistic type of ovariole. For this relatively simpler and more generally comparable tissue, 350 r is adequate to destroy all but the most advanced eggs and 800 r results in complete destruction of the ovaries. This is much like the traditional range for mammalian dosage, man and mouse, summarized by Glücksmann (1947). At present we can only speculate concerning the feature responsible for the dose requirement in Artemia sterilization. It seems likely that the interrelated shell gland may be involved. Further investigation along these lines is indicated since there is a notable lack of agreement in the literature on why reproduction is sometimes viviparous and at other times oviparous. In order to avoid mechanical damage to adults the more tedious transfer of gametes is suggested in future experiments.

Summary

As adults, Artemia males are killed by 200,000 r of x-radiation; females by 150,000 r. This approaches the doses required to kill adult holometabolous insects. Neither insects nor the brine shrimp depend upon mitotic tissues in adulthood. The sterility dose at 2000–3000 r for females is lower than in insects with polytrophic ovarioles and higher than in grasshoppers with simpler panoistic ovarioles. Further investigation is suggested for the interrelations of the Artemia shell gland and the reproductive cycle.

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LWOFFIA CILIFERA GEN. NOV., SP. NOV., A CILIATED MEMBER OF THE FAMILY SPHENOPHRYIDAE (HOLOTRICHA: THIGMOTRICHA)

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Up to the present time, three genera have been referred to the family Sphenophryidae. Two of these, *Pelecyophrya* and *Gargarius,* are monotypic. The third genus, *Sphenophrya,* includes several species, the most extensively studied one being *S. dosiniae.* An excellent summary of our knowledge of this remarkable group of ciliates has been prepared by Chatton and Lwoff (1949, 1950). Since the publication of their comprehensive monograph, one new species has been added to the genus *Sphenophrya* by Polianskiĭ (1951). A contribution of Raabe (1949), in which the development of *S. dosiniae* is compared with that of *S. sphaerii,* is apparently the only other publication dealing with this group which was not issued in time for consideration by Chatton and Lwoff.

The known genera of sphenophryids are parasitic on the gills of lamellibranch mollusks. As adults they are sessile and, with occasional exceptions in *Pelecyophrya tapetis,* are not ciliated. However, an infraciliature, disposed in two systems of a few rows each, persists in adult sphenophryids. In *P. tapetis* and the several species of *Sphenophrya,* the adults produce temporarily ciliated embryos which resemble in a general way some of the ciliates of the family Ancistrocomidae. In *Gargarius gargarius,* the embryo is also ciliated, but it is similar to the adult in all other respects by the time division has been completed. The embryonic ciliature of sphenophryids originates from the basal granules of those portions of the infraciliary rows which are conferred upon the embryo during division.

On the branchial filaments of *Brachidontes* (*Mytilus*) *recurvus* (Rafinesque) I have found a ciliate which is referable to the Sphenophryidae, but which differs from the other genera in having cilia throughout its life history. The morphology of this ciliate is most nearly like that of *Pelecyophrya,* but the process of division gives rise to two fully differentiated individuals, as is the case in *Gargarius.* The new genus is named for Dr. André Lwoff, of the Institut Pasteur, Paris, in recognition of his contributions to protozoology.

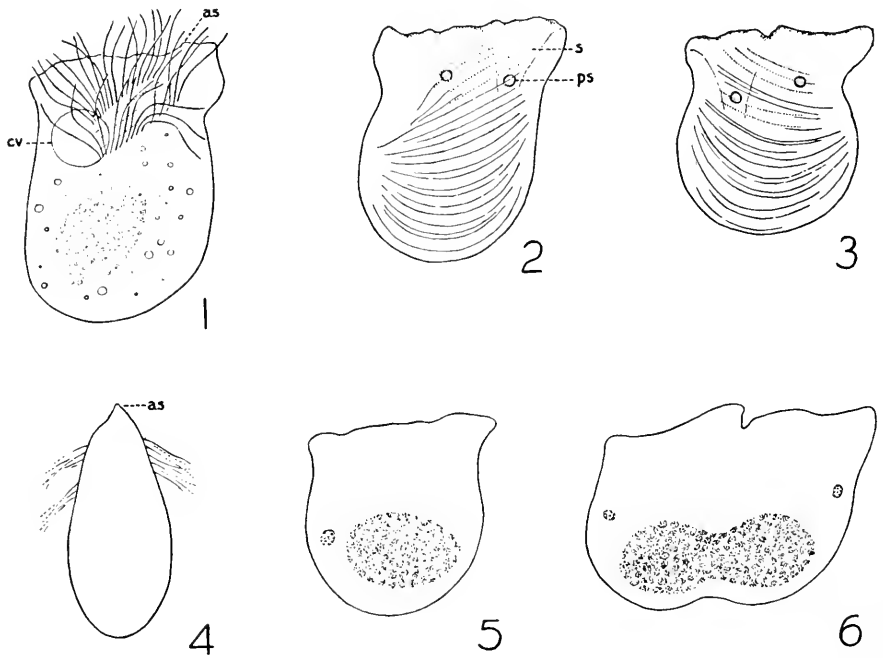
This study was carried out during the tenure of a Fellowship from the John Simon Guggenheim Memorial Foundation. It was begun while I was in residence at the Conservation Reserve of the University of Florida, and completed in the Department of Zoology, University of North Carolina.

Materials and Methods

Lwoffia cilifera was found in only one of ten specimens of *Mytilus recurvus* collected in a brackish lagoon confluent with the Caloosahatchee River approxi-

mately 6 miles southwest of Fort Myers, Florida. In the infected mussel, the ciliate occurred in enormous numbers. This permitted an extensive study of living material and the fixation of smears with hundreds of individuals.

For observation of living ciliates, small pieces of gill tissue were mounted in the fluid drained from the mantle cavity. Smears on coverglasses were fixed in Hollande's fluid with 3 per cent of acetic acid. These were stained with iron hematoxylin or the Feulgen nucleal reaction, or impregnated with activated silver albumose (Protargol) according to the method of Bodian. Impregnation with the sample of Protargol available to me was not satisfactory unless the smears were bleached, after fixation and washing, by Mallory's method, which consists of placing them in 0.5 per cent potassium permanganate for 5 minutes, washing them well, and then treating them with 5 per cent oxalic acid for 5 minutes.



Explanation of Figures 1–6

Lwoffia cilifera. All figures, except Figure 1, have been prepared with the aid of a camera lucida and are based on specimens fixed in Hollande's fluid and impregnated with Protargol (P) or stained by the Feulgen nucleal reaction (F); × 1640.

as, attachment surface; cv, contractile vacuole; ps, primordium of sucker; s, sucker.

Figure 1. From right side; after sketches from life.

Figure 2. From right side, showing rows of basal granules, pellicular fibril-like structures, sucker, and primordia of suckers; nuclei omitted; P.

Figure 3. From left side; P.

Figure 4. Optical section, posterior to middle; P.

Figure 5. Macronucleus and micronucleus; F.

Figure 6. Late stage of division; F.

Lwoffia cilifera gen. nov., sp. nov.

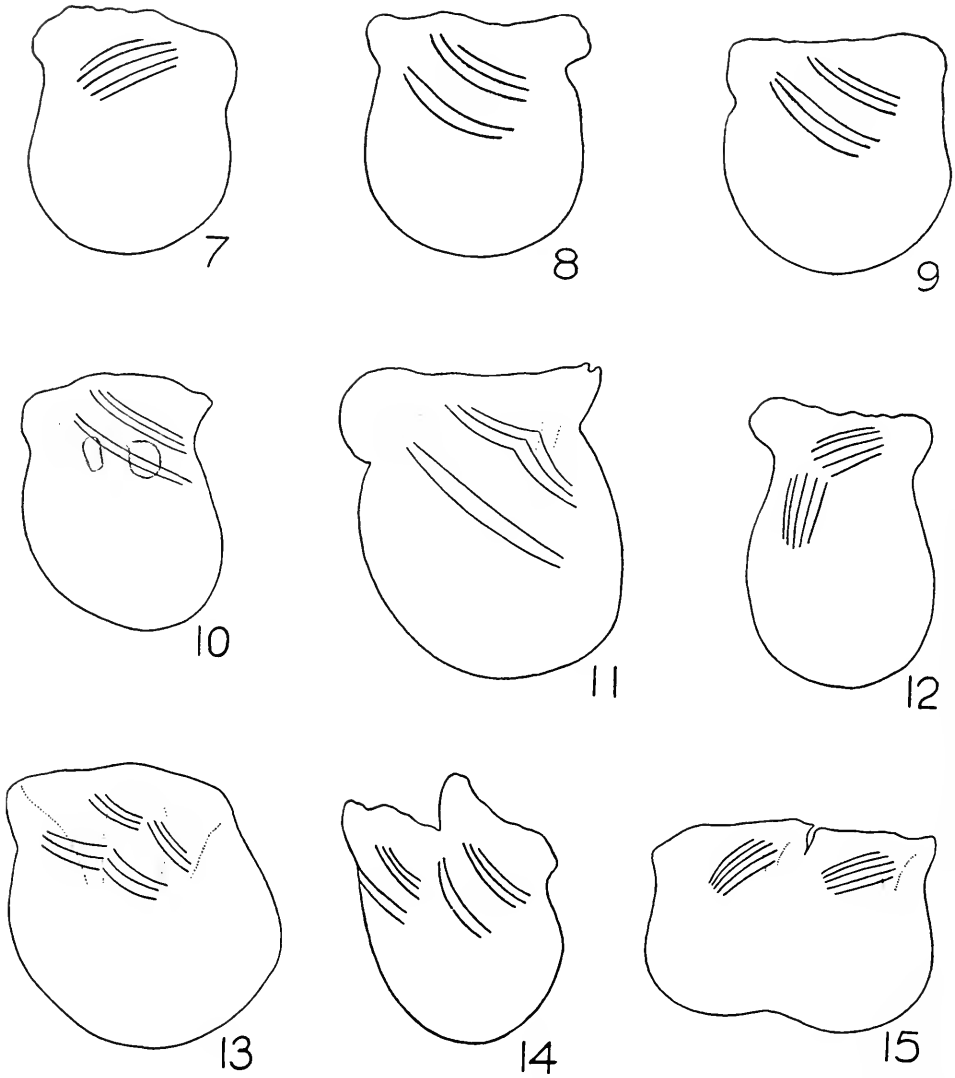
The body is compressed, having a thickness equal to about one-half the breadth of the ciliated surfaces (Figs. 1–4). The wide variation in the shape of the ciliates observed in fresh preparations as well as in fixed smears is due primarily to the plasticity of the ridge-like and somewhat corrugated attachment surface, which is in contact with the branchial tissue of the host.

The distance from the middle of the attachment surface to the opposite pole of the ciliate is usually approximately equal to, or slightly greater than, the greatest breadth below the attachment surface. Twenty living individuals in which these dimensions were measured ranged in size from about 16 μ by 14 μ to about 29 μ by 25 μ. However, as will be explained presently, these dimensions are not comparable to length and width of more conventional ciliates.

The orientation of *L. cilifera* may be defined satisfactorily only with reference to other thigmotrichs. Chatton and Lwoff, after considering the organization of a series of genera of Ancistrocomidae in relation to that of stomatous thigmotrichs such as *Ancistrum*, concluded that the ciliated surface of the more highly evolved ancistrocomids (*i.e.*, those in which the ciliature is reduced to a relatively few rows) is dorsal. In defining the orientation of the three genera of sphenophryids known to them, they regarded the rows of infraciliature in these forms to be homologous with the dorsal ciliary rows of ancistrocomids. In *Pelecyophrya tapetis*, therefore, the rows of infraciliature on either side of the body are collectively considered to correspond to the single system of dorsal ciliature in ancistrocomids. In the ciliated embryo of *P. tapetis*, before it becomes detached, the two ciliary fields destined to lie on opposite sides of the body in the adult converge rather closely upon one another toward the posterior end. The sucker occupies much of that part of the ciliate which is in contact with the gill tissue of its host, and is considered to be antero-ventral in position.

In species of *Sphenophrya*, in which the two systems of infraciliature evidently homologous with those of *Pelecyophrya* lie on the same side of the elongated body, the problem of orientation is more complicated. However, the ciliated embryo of *Sphenophrya* is much like that of *Pelecyophrya*, and during the changes involved in the development of the elongated adult form, the rows of infraciliature disposed on the right side of the embryo eventually come to lie on the same side as the rows which are on the left side of the embryo. The attachment surface was taken by Chatton and Lwoff to be dorsal, since it represents a differentiation and expansion of that portion of the embryo which separates the right and left ciliary systems.

The attachment surface of *Lwoffia* is more extensive than that of *Pelecyophrya*, and is disposed on those portions of the body which correspond to the anterior end and anterior part of the dorsal surface in *Pelecyophrya*. The sucker of *Lwoffia* resembles that of *Pelecyophrya* in having a broad funnel-shaped outline when viewed from the right or left side of the body (Figs. 2, 3). The margins of the sucker may be discerned in some specimens stained with iron hematoxylin, and individuals impregnated with Protargol show them quite regularly. The sucker becomes broader antero-dorsally, and its anterior margin may often be traced as far as the attachment surface. I have been unable to determine how much of the attachment surface is occupied by the origin of the sucker, and I have



EXPLANATION OF FIGURES 7–15

Lwoffia cilifera. All figures have been prepared with the aid of a camera lucida and are based on specimens fixed in Hollande's fluid and impregnated with Protargol; × 1640. The rows of cilia are rendered diagrammatically as continuous lines.

FIGURE 7. From right side.

FIGURES 8, 9. From left side.

FIGURE 10. Enlargement of primordia of suckers.

FIGURE 11. Elongation and early stages of division of ciliary rows.

FIGURES 12–14. Later stages in separation of ciliary systems of prospective daughter ciliates.

FIGURE 15. Attachment surfaces of prospective daughter ciliates distinct.

not observed an actual orifice. The sucker is evidently a protoplasmic channel. Although the cytoplasm of *L. cilifera* contains abundant lipoid granules, stainable with Sudan III in fresh preparations, and smaller, relatively non-refractile granules, cell fragments from host tissue cannot be identified. It appears probable that nutrition of the ciliate involves absorption of relatively fluid materials.

In impregnated specimens there is almost invariably observed within the sucker a small sphere whose periphery is argyrophilic (Figs. 2, 3). Another sphere of this type is characteristically located posterior to the region occupied by the sucker. These two spheres are primordia of new suckers formed during division of the ciliate, and may therefore be regarded as homologues of the sucker primordia observed by Chatton and Lwoff in about 30 per cent of the individuals of *P. tapetis* stained with iron hematoxylin. Apparently, however, neither of the two primordia in *P. tapetis* lies within the sucker of the parent ciliate.

The cilia are approximately 8 μ or 9 μ long. After being detached from the gill tissue of its host, *L. cilifera* is capable of swimming actively, although its movements are largely erratic rotations.

In proportion to the size of the body, the ciliary rows are much shorter than the rows of infraciliature in *P. tapetis*. In the latter species, some of the rows extend almost the full length of the ciliate. On the right side of the body of *L. cilifera* there are five rows arranged in one group (Figs. 1, 2, 7). On the left side of the body, there are two groups of rows (Figs. 3, 8, 9), as in *Pelecyophrya*. The group which is nearer to the attachment surface consists of three rows in all suitably oriented impregnated specimens which I have studied. The group which is farther from the attachment surface consists most frequently of two rows, but individuals with three rows in this group are common. In two specimens, out of hundreds I studied, there were four rows in this group, and in one specimen there was a single row.

In Protargol preparations, a number of delicate fibril-like structures, which appear to be pellicular, are observed on the right and left sides of the body, in both the ciliated and unciliated portions (Figs. 2, 3). Many of these, at least, traverse the entire broad surface, and at either end there is a convergence of the fibril-like structures from the right and left sides.[1]

The large macronucleus is located in the postero-ventral half of the body. It is elongated, and is usually about one and a half to two times as long as it is wide. In life, the macronucleus is coarsely granular (Fig. 1), with some of the granules being more or less rod-shaped. After fixation it is stained so heavily by the Feulgen nucleal reaction and by iron hematoxylin that its granular character is often obscured. The micronucleus generally lies close to the macronucleus. In the interphase it is typically spherical, with the chromatin dispersed in rather fine granules (Fig. 5). The contractile vacuole is located in the posterior part of the dorsal half of the body.

Stages of division are abundant in my stained and impregnated preparations of *L. cilifera*. In impregnated specimens, in which nuclear changes cannot be followed satisfactorily, the earlier stages of fission are evidenced by the division of each row of cilia into two segments. The two daughter segments are generally

[1] Comparable configurations are prominent in my Protargol preparations of *Gargarius gargarius* and *Sphenophrya dosiniae*.

approximately equal (Figs. 12–14), and it appears that the parent row elongates considerably before the break in the continuity of the row occurs (Fig. 11). As the furrow of cytoplasmic cleavage becomes more pronounced, the systems of ciliary rows formed by division of the parent system on either side of the body become more widely separated, and the attachment surfaces of the two prospective daughter ciliates become quite distinct (Figs. 14, 15).

During the early stages of division, the primordia of the new suckers to be conferred upon the daughter ciliates enlarge. Evidently this enlargement may be initiated before there is a break in the continuity of any of the ciliary rows (Fig. 10). Since one of the primordia lies within the sucker of the parent ciliate, the new sucker derived from this primordium essentially replaces the old sucker in the same position. As the development of new suckers progresses, the argyrophilic material of the primordia loses prominence.

Division of the micronucleus apparently begins after the primordia have begun to enlarge, and is completed before the attachment surfaces of the prospective daughter ciliates may be recognized as separate. Macronuclear division coincides with later stages of cytoplasmic division (Fig. 6).

Lwoffia gen. nov.

Diagnosis: Sphenophryid ciliates with cilia persisting throughout the life history. The body is laterally compressed, with an extensive ridge-like attachment surface which occupies the anterior end and anterior part of the dorsal surface of the body. The ciliature of the right side is composed of one group of rows; the ciliature of the left side is arranged in two groups of rows. The sucker is funnel-shaped in outline as viewed from the side, and is directed antero-dorsally; it is considered to be a protoplasmic channel. Primordia of new suckers to be formed during division are evident in the interphase, and one of the primordia lies within the sucker of the parent ciliate. Genotype: *Lwoffia cilifera.*

Lwoffia cilifera sp. nov.

Diagnosis: Size approximately 16 μ by 14 μ to 29 μ by 25 μ. The ciliary system of the right side of the body is composed of 5 rows. On the left side of the body, the ciliary rows in the group nearer the attachment surface are three in number, and the rows in the group farther from the attachment surface are commonly two or three in number, rarely one or four. On the branchial filaments of *Brachidontes recurvus* (Rafinesque), near Fort Myers, Florida. Syntype slides are in the collection of the author.

Summary

To the existing genera of sphenophryid ciliates (*Sphenophrya, Pelecyophrya,* and *Gargarius*) described by Chatton and Lwoff, a new genus, *Lwoffia,* is added. The genotype, *L. cilifera,* was found on the gills of *Brachidontes recurvus* near Fort Myers, Florida. Functional cilia persist throughout the life history of *L. cilifera,* and are disposed in two systems on either side of the body, in much the same way as the rows of infraciliature in *Pelecyophrya tapetis.* The attachment surface is proportionately more extensive than that of *P. tapetis,* and in form is much like that of species of *Sphenophrya.* Division produces equal and fully-

differentiated ciliates, rather than an ancistrocomid-like embryo from the parent ciliate, and in this respect is similar to division in *Gargarius gargarius*.

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THE SIZE, SHAPE AND HYDRATION OF LOBSTER HEMOCYANIN[1]

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Sedimentation (Eriksson-Quensel and Svedberg, 1936), diffusion (Polson, 1939a), partial specific volume (Svedberg, 1939), intrinsic viscosity (Polson, 1939b), and sedimentation equilibrium (Eriksson-Quensel and Svedberg, 1936) studies have been carried out on the hemocyanin of the lobster, *Homarus vulgaris*. While these studies serve in a general way to characterize the hemocyanin molecule, several important pieces of information are lacking. First, sedimentation coefficients were not extrapolated to zero concentration. Second, no information concerning hydration was provided. Third, no independent evidence of shape was presented. The purpose of the present study is to provide a more precise and more complete characterization of the lobster hemocyanin molecule.

A. Preparation of Hemocyanin

North American lobsters, *Homarus americanus,* obtained in Woods Hole, Massachusetts, were used in this study. The technical descriptions of the species appearing in Milne-Edwards (Milne-Edwards, 1837) permit the conclusion that *H. americanus* and *H. vulgaris* are very similar if not actually indistinguishable. The lobsters were bled, the blood was defibrinated by whipping, and the hemocyanin was purified by alternate centrifugation for ten minutes in a Servall angle centrifuge at 5000 rpm and for six hours in a type 40 rotor in a Spinco Model L centrifuge at 35,000 rpm. All centrifugations were carried out in the cold. Two low speed and one high speed runs were used to prepare the hemocyanin for diffusion and sedimentation experiments. Several additional cycles of low and high speed centrifugation were used in the preparation of material for partial specific volume, intrinsic viscosity, and specific refractive increment measurements.

B. Sedimentation Studies

1. Sedimentation as a function of concentration

Sedimentation studies on various concentrations of hemocyanin in $M/10$ phosphate buffer at pH 7 were carried out in a Spinco ultracentrifuge. Solution densities were measured with a pycnometer. Hemocyanin concentrations were determined by means of a refractometer, using 0.00193 as the specific refractive increment. Solution viscosities were measured at a temperature below and at a temperature above that of the sedimentation run, and the value at the tempera-

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TABLE I

Sedimentation of lobster hemocyanin at various concentrations

Hemocyanin concentration (g/100 ml.)	s Svedbergs	η Solution viscosity (cps)	ηs	ηs corrected	η_0 Solvent viscosity (cps)	$\eta_0 s$	$\eta_0 s$ corrected	Solution density
10.7	7.65	2.1454	16.4	20.1	1.0439	8.0	9.8	1.053
4.77	14.1	1.4008	19.8	23.0	1.0305	14.6	16.9	1.039
2.75	18.0	1.2179	21.9	24.5	1.0362	18.7	20.9	1.029
1.55	20.1	1.0982	22.0	24.1	1.0057	20.2	22.1	1.024
1.5	20.9	1.0156	21.2	23.2	0.9000	18.8	20.6	1.024
0.5	22.8	0.9859	22.5	24.3	0.9200	21.0	22.7	1.020
0.0				24.5			24.5	

ture of the sedimentation run was determined by interpolation. Solvent viscosities were determined in the same way. The product of sedimentation coefficient and solution viscosity and the product of sedimentation coefficient and solvent viscosity were then each corrected for solution density by multiplying by

$$(1 - V_h' \, d_w^{20}) \, / \, (1 - V_h' \, d),$$

where V_h' has the meaning defined in the next section, d_w^{20} is the density of water at 20°, and d is the density of the solution investigated in the ultracentrifuge. The figures thus obtained are identified by the symbols $\eta \, s$ (corr) and $\eta_0 s$ (corr), respectively. The data are listed in Table I.

It was found that viscosity-sedimentation products gave more nearly linear plots with concentration than did reciprocals of viscosity-sedimentation products. In the case of most virus characteristic particles, solution viscosity-sedimentation products are essentially invariant with concentration (Lauffer, 1944; Lauffer and Stanley, 1944; Taylor, Epstein and Lauffer, 1955). This was not found to be the case with lobster hemocyanin. Solution viscosity-sedimentation products, and solvent viscosity-sedimentation products were extrapolated to the same value at zero concentration. This value is listed in Table I. When one divides it by the viscosity of water at 20°, 0.01005, one obtains the sedimentation coefficient, corrected to water at 20°, at infinite dilution. This has a value of 24.5×10^{-13} cgs units. The previously reported value, 22.6×10^{-13} (Eriksson-Quensel and Sved-

TABLE II

Sedimentation of lobster hemocyanin in sucrose solutions

s Svedbergs	η Solution viscosity (cps)	ηs	d Solution density
18.85	1.158	21.8	1.0314
11.9	1.555	18.5	1.0587
7.0	2.196	15.4	1.1027
3.28	3.727	12.2	1.1445
1.05	8.287	8.7	1.1909
0.22	19.793	4.4	1.2420
0.06	25.463	1.4	1.2719

berg, 1936) is the mean of the values obtained from various dilutions of lobster blood in different electrolyte solutions; it does not represent the value at infinite dilution.

2. Sedimentation in sucrose solutions of various densities

Sedimentation studies on 0.9% hemocyanin in 0.1 *M* phosphate buffer at pH 7, to which various amounts of sucrose had been added, were carried out. Sedimentation coefficients, solution viscosities and solution densities were evaluated as described previously. The data are recorded in Table II. The product of solution viscosity and sedimentation coefficient, not corrected in any way, was plotted against solution density. The data fit the straight line, $\eta s \times 10^{15} = 104.7 - 80.9d$. The intercept at a density of 0.998, the density of water at 20°, is 24.0×10^{-15}. This is in good agreement with the solution viscosity-sedimentation coefficient product for 0.9% hemocyanin solution, 24.2×10^{-15}, as interpolated from the data of Table I. The intercept corresponding to zero sedimentation rate occurs at a

TABLE III

Viscosity at various concentrations

Concentration (g/ml.) C	Concentration (volume fraction) $\phi = CV$	Relative viscosity η_r	$\frac{\eta_r - 1}{\phi}$
0.107	0.0792	1.9881	12.5
0.0477	0.0353	1.3259	9.25
0.0275	0.0204	1.1448	7.1
0.0155	0.0116	1.0713	6.15
0.0083	0.0062	1.0300	4.85

density value of 1.294. The reciprocal of this value, 0.773, is taken to be the effective specific volume of the hydrated hemocyanin molecule, V'_h (Lauffer, Taylor and Wunder, 1952).

C. Diffusion Experiments

The diffusion coefficient of an 0.9% hemocyanin solution in 0.1 *M* phosphate buffer at pH 7 was determined at 20° in a Perkin-Elmer electrophoresis apparatus. A value not significantly different from that reported by Polson (Polson, 1939a) was obtained. Because Polson's data are more extensive, his value for D_w^{20} of 2.77×10^{-7} cm.²/sec. will be used in subsequent calculations.

D. Intrinsic Viscosity

Viscosities of solutions of various concentrations of hemocyanin in 0.1 *M* phosphate buffer at pH 7 were determined using a capillary viscometer in a water bath at 25°. Concentrations were measured by means of a refractometer using a value for specific refractive increment of 0.00193. The data are shown in Table III. Intrinsic viscosities or viscosity numbers were evaluated by plotting the quotient of relative viscosity increment divided by volume fraction against

volume fraction and extrapolating to zero. A value of 4.85 ml. per ml. was obtained. Polson (Polson, 1939b) reported a value of 6.4. The value obtained in the present study, namely 4.85, is used in subsequent calculations.

E. Partial Specific Volume

The apparent partial specific volume of lobster hemocyanin was calculated from the dry weight composition and the density of an aqueous solution of a highly purified preparation. A mean value not significantly different from that for the partial specific volume reported by Svedberg (Svedberg, 1939), namely 0.74, was obtained.

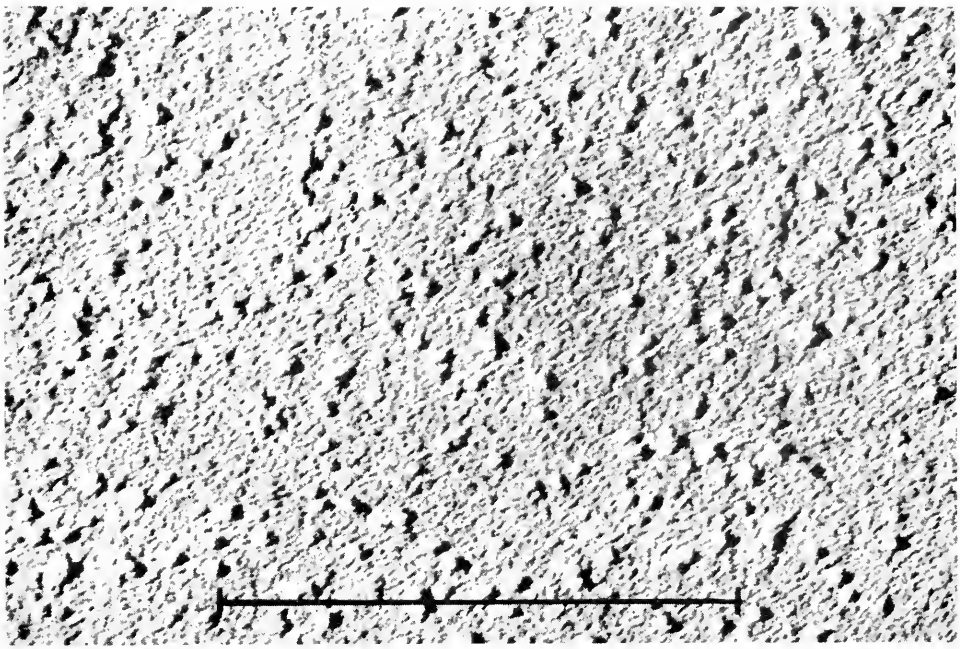

Figure 1. Electron micrograph of hemocyanin molecules. Uranium shadowed (× 69,000). Shadow angle slope — ¼. Line corresponds to one micron.

F. Specific Refractive Increment

The specific refractive increment was determined on aqueous solutions of highly purified hemocyanin by means of a Phoenix Precision differential refractometer. The concentration was determined by dry weight. The specific refractive increment obtained was 0.00193. Redfield had previously reported a value of 0.002 (Redfield, 1934).

G. Electron Microscopy

Electron micrographs of hemocyanin molecules shadowed with uranium were kindly made by Dr. Irwin Bendet of the Department of Biophysics at the Uni-

versity of Pittsburgh. A representative micrograph is shown in Figure 1. The electron micrograph shows clearly that the hemocyanin molecules are not rod-shaped. The shadow lengths are consistent with a height of 7.5 mμ and the modal image size corresponds to a particle with a diameter of 33 mμ. Thus, the micrograph is consistent with the assumption that the hemocyanin molecule is a flattened ellipsoid of revolution about 33 mμ in diameter and 7.5 mμ thick. Naturally, these dimensions refer to dried particles.

Discussion

The following parameters, determined in the present and in previous investigations, can be used to calculate the size, shape, and degree of hydration of lobster hemocyanin; sedimentation coefficient, $s_w^{20} = 24.5 \times 10^{-13}$; diffusion coefficient, $D_w^{20} = 2.77 \times 10^{-7}$; partial specific volume, $V = 0.74$; effective specific volume of the hydrated particle, $V_h' = 0.773$; intrinsic viscosity or viscosity number, $[\eta] = 4.85$. Details of these calculations have been reviewed recently (Lauffer and Bendet, 1954).

From the sedimentation coefficient, the diffusion coefficient, and the partial specific volume, the molecular weight and the friction ratio can be calculated to be 825,000 and 1.25, respectively. This molecular weight value supersedes the value of 752,000 reported by Eriksson-Quensel and Svedberg (Eriksson-Quensel and Svedberg, 1936) and compares with their sedimentation equilibrium value of 803,000. From the partial specific volume, V, and the effective specific volume of the hydrated particle, V_h', one can calculate that hemocyanin is hydrated to the extent of 0.15 ml. of water per g of anhydrous protein. The reservations which must attend the use of hydration values calculated in this manner have been discussed previously (Lauffer, Taylor and Wunder, 1952; Lauffer and Bendet, 1954).

If it is assumed that the molecule is a flattened ellipsoid of revolution and that its hydration is actually equal to the value calculated from sedimentation vs. medium density studies, namely, 0.15 ml. of water per g protein, then one can interpret the friction ratio, 1.25, to be consistent with a hydrated flattened ellipsoid of revolution with an axial ratio of 4.14, corresponding to a diameter of 32.2×10^{-7} cm. and a thickness of 7.8×10^{-7} cm. These values are consistent with those estimated from the electron micrograph. The axial ratio of the hydrated particle calculated on the basis of the same assumptions from the intrinsic viscosity was 4.05, in good agreement with the value obtained from sedimentation, diffusion and partial specific volume data.

Summary

1. The sedimentation coefficient of lobster hemocyanin corrected to water at 20° and extrapolated to zero concentration has been found to be 24.5×10^{-13} cgs units.

2. The intrinsic viscosity has been found to be 4.85 ml./ml.

3. From the variation of the sedimentation rate of hemocyanin in sucrose solutions of different densities and from the partial specific volume, the hydration of hemocyanin was calculated to be 0.15 ml. of water per gram of dry protein.

4. The specific refractive increment was determined to be 0.00193 ml. per 10^{-2} g.

5. From the above parameters and others taken from the literature, lobster hemocyanin molecules were calculated to have a molecular weight of 825,000, corresponding to oblate ellipsoids of revolution with hydrated diameter of 32.2 mμ and hydrated thickness of 7.8 mμ.

ERRATUM

AUTHORS' ERROR. Lauffer and Swaby, Vol. 108, pp. 294 and 295.

The dimensions of the lobster hemocyanin molecule, considered as a flattened ellipsoid of revolution hydrated to the extent of .15 ml. water per g. protein, were erroneously reported as 32.2×10^{-7} cm. for the diameter and 7.8×10^{-7} cm. for the thickness. The correct values are 21.4×10^{-7} and 5.2×10^{-7} cm. These are not very close to the electron microscope values. Therefore, the question of the interpretation of the hydrodynamic results is re-opened.

An additional possibility is that the hemocyanin molecules are spheres. If so, the friction ratio of 1.25 must be ascribed to hydration alone. By methods described by Lauffer and Bendet (1954), one can calculate a value of .70 ml. of water per g. of protein from this friction ratio. A sphere composed of protein with partial specific volume of .74, with an anhydrous molecular weight of 825,000 and with this degree of hydration, would have a diameter of 15.6×10^{-7} cm. Such a particle could collapse into a disk upon drying, but its total volume would be less than one-fourth that indicated by the electron micrographs. The new value for hydration is considerably higher than that calculated from the sedimentation vs. density experiments. As was pointed out in a reference cited in the discussion, however, the value calculated from sedimentation vs. density experiments represents only the water in excess of that associated with other constituents in the hypothetical hydrating medium and might underestimate the total.

If one takes the dimensions indicated by the electron micrograph at face value, the particles seen in the micrograph cannot be interpreted as single hemocyanin molecules, for their volumes are much too great. However, if the thickness of the uranium coat is taken into account, the resulting dimensions yield a volume which may not be inconsistent with the hydrodynamic data.

versity of Pittsburgh. A representative micrograph is shown in Figure 1. The electron micrograph shows clearly that the hemocyanin molecules are not rod-shaped. The [illegible] with a height of 7.5 mμ and the modal image [illegible] micrograph is [illegible] flattened ellip[illegible] rally, these d[illegible]

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3. F[illegible] tions of [illegible] hemocyanin was calculated to be 0.15 ml. of water per gram.

4. The specific refractive increment was determined to be 0.00193 ml. per 10^{-2} g.

5. From the above parameters and others taken from the literature, lobster hemocyanin molecules were calculated to have a molecular weight of 825,000, corresponding to oblate ellipsoids of revolution with hydrated diameter of 32.2 mμ and hydrated thickness of 7.8 mμ.

6. Electron micrographs are consistent with the assumption that the molecules are plate-like ellipsoids of revolution 33 mμ in diameter and 7.5 mμ in thickness in the dried state.

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STUDIES ON THE NEUROSECRETORY SYSTEM OF IPHITA LIMBATA STAL. I. DISTRIBUTION AND STRUCTURE OF THE NEUROSECRETORY CELLS OF THE NERVE RING

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Neurosecretory cells have been discovered by several authors in different groups of insects (see reviews: Scharrer and Scharrer, 1954a, 1954b). In *Iphita limbata* (Pyrrhocoridae: Hemiptera) the presence of these cells in the brain and the metathoracic ganglion was reported by Nayar (1953) who also described certain changes in the activity of these cells correlated with reproduction in the female. The functions of the neurosecretory cells of the brain have been worked out in certain insects, especially by Scharrer (1952) in *Leucophaea maderae,* by Thomsen (1952) in *Calliphora erythrocephala,* and by Williams (1952) in *Platysamia cecropia.* The present series deals with a more detailed study of the structure, functions and development of the neurosecretory cells of *Iphita limbata* Stal.

Materials and Methods

Adult insects were used for the study. They were frequently collected fresh from the field and were kept in insectary boxes where they were fed on cotton seeds.

When the dorsal wall of the cranium is removed and the head is stretched forwards by pulling the rostrum and fixing it with plasticine, the brain becomes exposed. The dissection of the brain was done under a stereoscopic binocular microscope (magnification $\times 40$). A longitudinal tracheal tube with a number of tracheoles traverses the middle of the cerebral ganglia. When that is removed, faintly whitish spots become visible underneath the firm and thin membrane investing the brain. When this membrane is teased with a fine needle, two groups of medial neurosecretory cells come into view as bluish-white masses on either side of the midline. Each contains about sixteen cells. The medial neurosecretory cells can be removed as a group from the pars intercerebralis of the brain with fine forceps (*cf.* Thomsen, 1952). When observed in insect Ringer, these remain without marked changes for about an hour.

For the study of topography and histological structure of the neurosecretory cells, the entire nerve ring was removed and fixed. The medial neurosecretory cells of the pars intercerebralis of the female were selected for the examination of finer cytological details; the corresponding male tissue shows no marked difference in cellular structure and distribution.

The following methods were used in this study:

1. For general histology: Bouin's, Helly's, Smith's, and Baker's formal-calcium were used as fixatives. Staining was done in Heidenhain's iron hematoxylin, Masson's trichrome (Foote, 1933), Gomori's chrome alum-hematoxylin-phloxin (Gomori, 1941), Gomori's aldehyde fuchsin (Pearse, 1953) and Heidenhain's Azan (Pantin, 1948).

2. For supravital observation: Phase-contrast and dark field microscopes. Light microscope for supravitally stained tissue (neutral red, methylene blue, and dahlia violet) in 0.001% stain for 10 to 15 minutes.
3. For the study of the granular system in the cytoplasm: Material fixed in Baker's ISO fixative (osmic acid in sucrose-iodate solution) stained in Altmann's acid fuchsin according to Metzner's method, and Helly-fixed material stained in Hirschler's hematoxylin (Baker, 1951).
4. For the study of the spheroidal system in the cytoplasm: Classical Golgi methods, such as fixation in Flemming-without-acetic and staining in iron hematoxylin; Weigl's Mann-Kopsch; Kolatchew's and Aoyama's methods (Baker, 1951); Thomas' (1948) method of study of gradual osmification in 2% osmium tetroxide; Baker's (1949) technique of sudan black staining; and Thomas' (1948) method of sudan black staining for paraffin sections.
5. For other structural details: Unna-Pappenheim's methyl green-pyronin method (Darlington and LaCour, 1947) after fixation in Heidenhain's saline-mercuric chloride, for nucleic acids; Baker's acid hematein test (1946) and pyridine extraction test for phospholipines; Nath's (1934) method of staining fats by Sudan III; Barnett and Bourne's (1942) method for ascorbic acid; treatment with Millon's reagent after Bouin-fixation, xanthoproteic reaction, Pollister's method after Bouin-fixation, Hartig-Zacharias' method after formal-calcium fixation, for proteins (Pearse, 1953); Best's carmine after Bouin-fixation for glycogen (Pearse, 1953); indole reaction, Vulpian reaction, Sevki's Giemsa-tannin method and Lison's chromaffin test (Pearse, 1953); and Schmorl's method for lipofuscins (Pearse, 1953).

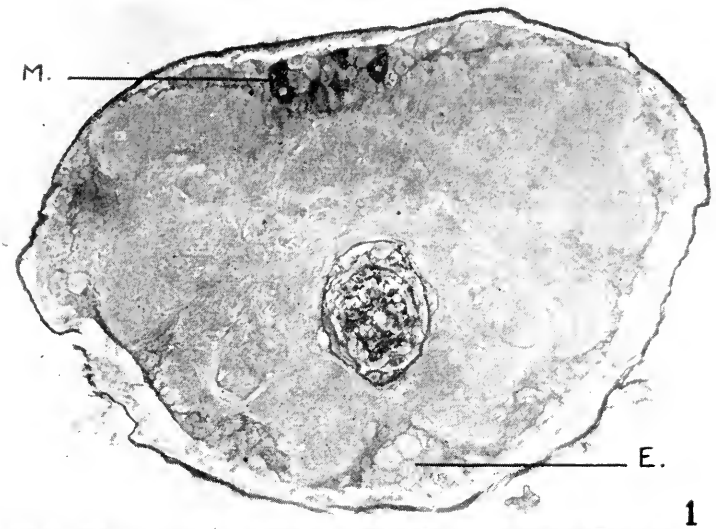
Fixed material was processed according to Peterfi's double embedding method with one-half to one per cent celloidin in methyl benzoate, and paraffin sections were cut at 5 μ for general staining. Some thick setions, 6 to 8 μ, were also cut for the study of the spheroidal constituents of the cells, while for mitochondria sections 2 to 3 μ were used. Frozen sections were cut at 10 and 15 μ after embedding in gelatine (25 per cent gelatine with trace of cresol).

Observations

For histological details fixation in Bouin's and Smith's fluids followed by Gomori's chrome alum-hematoxylin-phloxin gave the best results. The chrome hematoxylin selectively stains the neurosecretory cells a deep blue; sometimes in thick sections (6 or 8 μ) the cytoplasm appears blackish blue. Equally good results were obtained by using Azan stain where the cytoplasm is colored brilliant red by the azocarmine. The cytoplasm of these cells is fuchsinophilic in Masson's stain.

The neurosecretory cells are distributed in different parts of the nerve ring. In addition to the median neurosecretory cells of the brain (pars intercerebralis), there are the lateral groups of neurosecretory cells of the protocerebrum, numbering about three or four on each side. They are much smaller than the medial cells and rarely appear bluish in the fresh brain. The subesophageal ganglion contains scattered neurosecretory cells laterally and ventrally along the margin of the neuropile (Figs. 1, 5).

The neurosecretory cells of *Iphita* show two types of response to the staining procedures used. In one type, the cytoplasmic inclusions are stained deep blue in chrome hematoxylin-phloxin and dark red in Azan; these cells may be designated as "A cells." In the other type, the cytoplasmic contents stain red in chrome hematoxylin-phloxin and light blue in Azan; these may be designated as "B cells."



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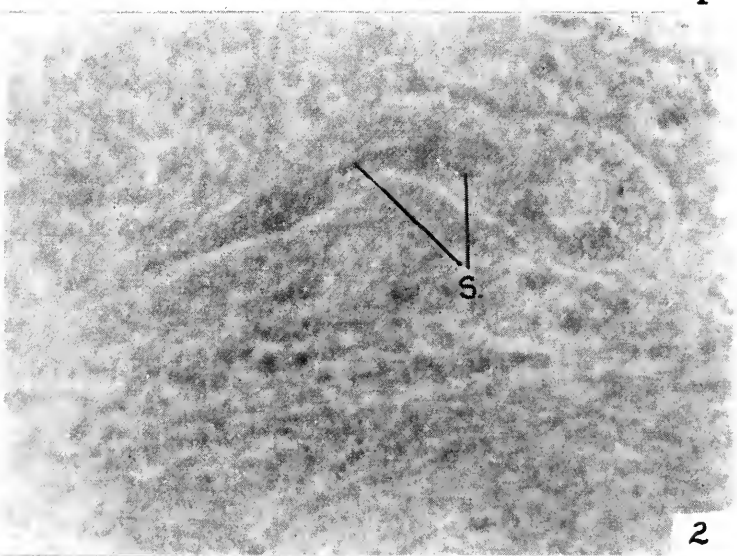

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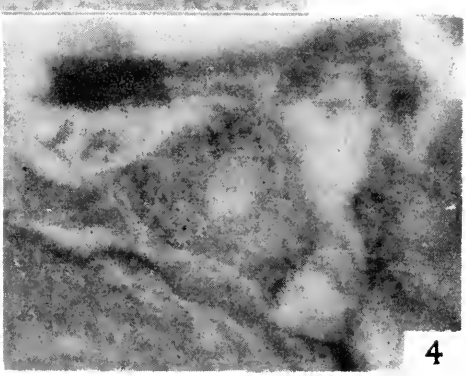

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FIGURES 1–4.

In Heidenhain's iron hematoxylin, the "A cells" are colored bright blue, and the "B cells" light blue. No selective staining of any kind was obtained by Gomori's aldehyde fuchsin.

The distribution of "A cells" and "B cells" is characteristic. In all preparations, the majority of the medial neurosecretory cells of the protocerebrum belong to the "A type" while a few (varying in number from two to six) are similar to "B type" cells. The latter show in their cytoplasm scattered granules. It is possible that these cells may be "A cells" deprived of the bulk of secretory material. Under certain experimental conditions where the insects have been fed on salt water or where salt water has been injected into the hemocoele, all the cells of the medial clusters are colored blue. The lateral neurosecretory cells and most of the subesophageal cells belong to the "B type" with phloxinophilic cytoplasm. A few cells of the subesophageal mass show a resemblance to the "A type." A map showing the distribution of these cells in the ring is given in Figure 6.

Lying on the two sides of, and closely apposed to, the anterior end of the aorta are the tiny corpora cardiaca with a slender bridge-like mass of cells in between which represents the hypocerebral ganglion. The cells of the hypocerebral ganglion are phloxinophilic, resembling the "B type" cells of the nerve ring. Laterally are the compact corpora cardiaca which show in their cytoplasm, in the vicinity of their nerves, granules colored blue in chrome hematoxylin. The cytoplasm here is heterogeneous, unlike that of the cells of the hypocerebral ganglion. The few cytoplasmic granules in the corpora cardiaca resemble those in the "A type" neurosecretory cells.

The medial cerebral neurosecretory cells have been used for a more detailed study. They measure about 52 to 97 μ in length. The cell tapers towards the axon and the apical part is swollen and carries the eccentrically located nucleus. The broadest part measures 32 to 39 μ. The round nucleus is 13 μ in diameter.

If pressure is exerted on a fresh preparation, within a few minutes globule-like droplets, measuring up to 13 μ, often separate off from the abaxonal part of the cell.

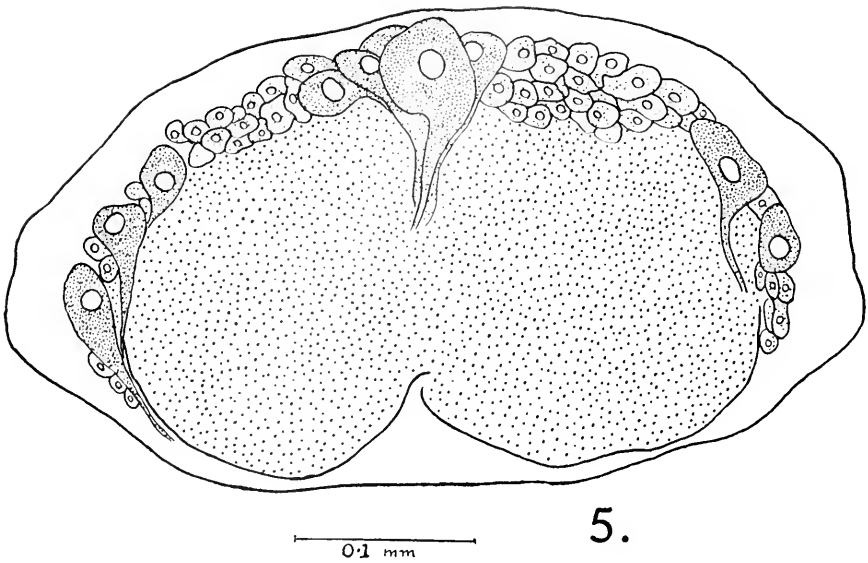
Under the phase contrast microscope, the cell is seen to contain a transparent and eccentric nucleus of low refractive index (Fig. 2). The chromocenters in the nucleus and the nucleolus appear dark, with a higher refractive index. The cytoplasm is filled with dark masses of granules which have generally a clumped appearance. Towards the broader edge of the cell are numerous tiny, dark granules which exhibit very active Brownian movement. The axons are traceable up to nearly three times the length of the cell. In the axons, as well as in the cytoplasm, are spheroids (see below) of variable size, with clear, dark rims and transparent

FIGURE 1. Transverse section of the nerve ring of *Iphita limbata* passing through the medial neurosecretory cells of the brain and the subesophageal ganglion. In the center is the esophagus. M = medial cells of the pars intercerebralis some of which are stained blue in Gomori's chrome-hematoxylin-phloxin. E = esophageal neurosecretory cells. Approx. × 95.

FIGURE 2. A medial neurosecretory cell of the pars intercerebralis under the phase contrast microscope. A few spheroids (S) are in focus. Approx. × 700.

FIGURE 3. Medial neurosecretory cells under the dark field microscope. They appear white; darker bodies in the cells are the nuclei. The white streaks are the tracheae. Approx. × 60.

FIGURE 4. The spheroids of the neurosecretory cell stained black in hematoxylin after fixation in Flemming without acetic acid. Approx. × 750.



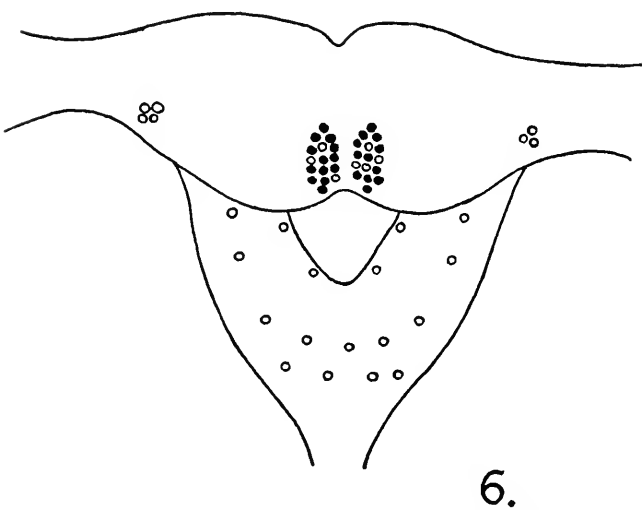


FIGURE 5. Camera lucida drawing (composite from a few adjacent sections) showing the marginal distribution of the subesophageal neurosecretory cells.

FIGURE 6. Diagram showing the distribution of the neurosecretory cells in the nerve ring of *Iphita limbata*. "A type cells" shown as black dots; "B type cells" shown as circles.

interior, appearing as extremely tiny droplets. Dark patches of a blotchy nature are seen on the nuclear membrane. The spheroids could be made out only with difficulty, but they could easily be distinguished when the distribution had been made out previously by vital staining methods.

In the dark field microscope the cytoplasmic content of the cell looks shiny and bluish-white, in the form of granules (Fig. 3). Besides granules there are larger bodies which are probably spheroids. The granules seem to flow along the axons.

Supravital staining in 0.001% neutral red gave good and uniform pictures of the cytoplasmic content. The cytoplasm shows red spheroids of variable size (Fig. 7). The neutral red spheroids measure from 0.71 μ to 2.86 μ in diameter. Somewhat similar results have been obtained by staining in 0.001% methylene blue. The spheroids here appear blue but the general staining effect is not quite as good as in neutral red.

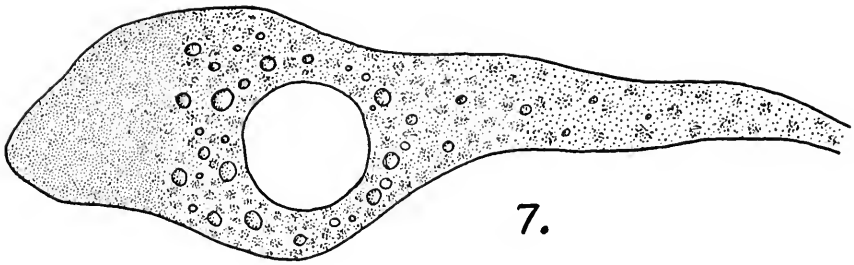
Supravital staining in 0.001% dahlia violet also gives a satisfactory picture of the cytoplasmic structures. The entire cell shows a very faint violet tint. The granulated mass stands out as dark greyish-violet bodies performing active movements. These granules show a blotchy appearance due to clumping. The spheroids do not always show up well; sometimes they appear as rounded bodies with dark violet rims and clear interior. Dahlia violet staining is not quite as good as neutral red; but in good preparations the granules and spheroids are traceable as conspicuously colored materials into the axons also.

The abaxonal broad part of the cell contains a vacuole-like structure which enlarges in due course into a conspicuous watery vacuole. Dense masses of granules fill this vacuole which show continual Brownian movement (Fig. 7). In the course of time these vacuoles part from the cells and appear as transparent drops filled with colored granules. These droplets sometimes take a faint reddish tint in neutral red. Such vacuoles have a low refractive index. The granules stain in the same way as mitochondria with Metzner's and Hirschler's methods. It is possible that these granules are derived from mitochondria, but this cannot be demonstrated conclusively with the methods used in this investigation.

Thus the study of living cells under phase-contrast and after supravital staining reveals that the cytoplasmic content of the medial neurosecretory cells of the brain is a complex of two substances: (1) a granular mass of small bodies, stainable dark greyish-violet by dahlia violet supravitally, contained in a fluid-filled vacuole; (2) a spheroidal system of tiny vacuole-like bodies, variable in size, stainable by neutral red, dahlia violet, and somewhat poorly by methylene blue.

An important structure in the cytoplasm of the neurosecretory cell is the spheroidal system. It consists of vacuole-like tiny spheres of variable size, supravitally stainable by neutral red, methylene blue, and dahlia violet.

The spheroids are demonstrable by the classical "Golgi" methods. When the cells are fixed in Flemming-without-acetic and stained in Heidenhain's iron hematoxylin, the spheroids appear as black bodies (Fig. 4). The spheroids are osmiophilic, and many of them appear as definite rings, while others look like large granules in Mann-Kopsch preparations after impregnation with osmic acid for two and a half days. These granules of different sizes could be seen on the nuclear membrane also. Similar bodies are discernible when cells are treated for about three days according to Kolatchew's method. The method of Aoyama is excellent to demonstrate the "Golgi system." The cytoplasm contains a system



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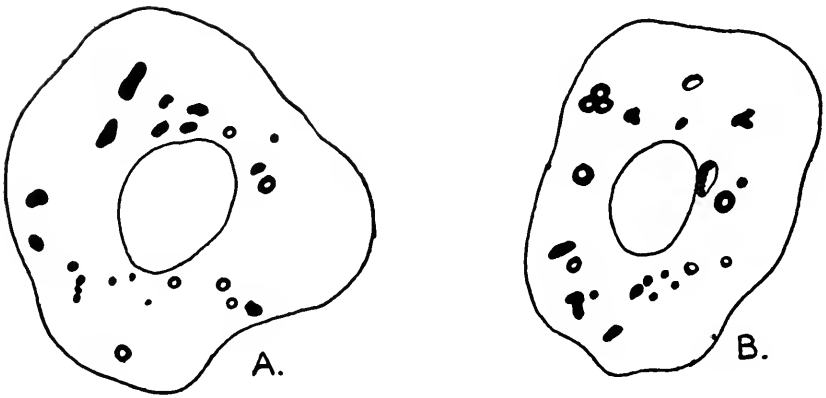
10μ.



10μ.

8.

FIGURE 7. Camera lucida drawing of a medial neurosecretory cell of the brain, stained in 0.001% neutral red. The neutral red vacuoles forming the spheroids are shown as circles. At the abaxonal part of the cell is a vacuole filled with mitochondria (fine stipples). The mitochondria of the rest of the cell appear in groups.

FIGURE 8. Camera lucida drawings of: A. A medial neurosecretory cell after treatment according to Aoyama's method, showing the "Golgi apparatus." The small spheroids which have coalesced appear as irregular masses, while the larger ones are ring-shaped. B. A neurosecretory cell after treatment according to Baker's sudan black method. Note similarity of the spheroids in both preparations.

of spheroids which show a deposit of silver around the periphery of the large spheroids, while the smaller vacuoles are more or less completely blackened. The picture in Aoyama preparations closely resembles the cells stained by neutral red; the neutral red spheroidal structures corresponding to the black ones in silver preparations (Fig. 8A).

Thomas' technique is very useful for the study of the development of the osmiophilic structures in the spheroids of the live cells. Freshly dissected neurosecretory cells were placed on a slide in a drop of 2% osmic acid. The coverslip was then sealed off. Within about five minutes the cells appear brownish. This becomes well marked in about thirty minutes; after about fifty minutes a few fine black granules and crescent-shaped black rims make their appearance. These are the developing osmiophilic elements. The subsequent deposition of osmium is comparatively slow. By about sixteen hours, the spheroids look like rings with the periphery almost completely blackened. In the deeper parts of the cell, such complete ring-like formation occurs in about a day. At room temperature (28–29° C.) the spheroids of all sizes become completely blackened after the fifth day. The cytoplasm as a whole then gets tinged with dark grey.

This method showed the gradual blackening of the margin of the spheroids which ultimately produced the configuration seen in the classical "Golgi preparations."

Baker's sudan black method shows that these spheroids are lipoidal in constitution. This is further supported by the acid hematein test which gives a positive result. Sudan black staining in both frozen and paraffin sections has similar results, and the preparations strikingly resemble those described above (Fig. 8B).

The spheroidal system of the neurosecretory cells could be reasonably described as lipochondria of variable size, characteristically osmiophilic, argentophilic, and sudanophilic.

In addition to the secretory granules and the spheroid system, other constituents of the neurosecretory cells were examined. In sections fixed in Heidenhain's mercuric-saline, and stained with methyl green-pyronin according to a modification of Unna-Pappenheim, the cytoplasm of the neurosecretory cells showed red or dark pink coloration indicating a concentration of ribose nucleic acid. The large nuclei of these cells are colored light pink, having in some cases a faint greenish tinge also, which indicates a comparatively low concentration of chromosome nucleic acid.

Baker's acid hematein test showed that the medial neurosecretory cells of the brain react strongly positively. The cytoplasmic products are colored a brilliant blue in both A and B types of cells. The mitochondrial and lipochondrial materials of the cytoplasm react like this: Pyridine extraction followed by acid hematein test shows no coloration at all. This is positive indication of the presence of phospholipines in the cytoplasm.

Though sudan black selectively stains the lipochondria found in the spheroids, simple staining by Sudan III according to Nath's method was unsuccessful. There was no indication of any coloring in these cases.

Barnett and Bourne's method for vitamin C revealed the presence of scattered black granules in the cytoplasm of the neurosecretory cells. They are more numerous in a perinuclear zone and close to the nuclear membrane.

inophilic and azocarminophilic. The cytoplasm contains granules and spheroids. The granules appear black in the phase contrast microscope, violet with dahlia and, like mitochondria, red with acid fuchsin; they exhibit continual Brownian movement in the living cells. The granules are associated with a fluid material. The spheroidal system which is osmiophilic, argentophilic, and sudanophilic, represents the lipochondria. Both the granular and spheroidal systems are revealed by supravital staining methods.

5. The cytoplasm of the neurosecretory cells contains a high concentration of ribose nucleic acid. The nuclear membrane and the cytoplasm show granular concretions of vitamin C. The secretory material contains proteins as indicated by various tests. The granular and spheroidal constituents are rich in phospholipines.

6. Tests for chromaffin substances and glycogen gave negative results. Staining methods for lipofuscins show that the product in the "A cells" probably contains these very complex substances.

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THE EFFECT OF X-RADIATION ON ENZYME SYSTEMS OF TETRAHYMENA PYRIFORMIS[1, 2]

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It had been shown previously by Eichel and Roth (1953b) that x-radiation at a level of 300,000 or 500,000 r depressed the respiration of *Tetrahymena pyriformis* W,[3] but did not appreciably affect the nuclease activity in homogenates prepared from irradiated whole cells. In irradiated homogenates, however, nuclease activity was reduced by approximately 50%. This paper presents some further observations of the effect of 300,000 to 600,000 r on respiration and several enzymes of *T. pyriformis* S. The S strain was chosen for two reasons; first, to obtain comparisons of the effect of x-radiation on respiration and deoxyribonuclease (DNase) activity in a strain different from that studied previously, and second, because the higher rate of succinate oxidation in the S strain (Eichel, 1954) would facilitate investigations of this specific enzyme system. The effects of x-radiation on catalase, and malic, glutamic, and succinic dehydrogenases, as well as some properties of succinic dehydrogenase and DNase, have also been observed. In addition, the effect of x-radiation on the oxidation of L-phenylalanine and acetate by the W strain has been studied.

Materials and Methods

T. pyriformis S cells were grown and harvested as previously described (Eichel and Roth, 1953b), and homogenates were prepared as described by Eichel (1954). Measurements of respiration and oxidative enzyme activities were by conventional Warburg techniques; the details of the procedures for x-irradiation and for the respiration and DNase studies were similar to those previously reported (Eichel and Roth, 1953b). Measurements of DNase activity were made at regular intervals for at least 100 to 136 minutes. The components of the test systems used in the study of oxidative enzyme activities are given in connection with the tables of results. All Warburg runs were made at 27° with air as the gas phase and a fluid volume of 3.0 ml. Catalase was determined by the method of Bonnichsen, Chance and Theorell (1947). Unless otherwise stated, 72 hour cultures of strain S were utilized for all experiments.

[1] Work performed under Contract No. AT(30-1)-1069 for the Atomic Energy Commission.

[2] A preliminary account of this project was presented at the General Scientific Meetings, Marine Biological Laboratory, Woods Hole, Mass., in August 1953 (Eichel and Roth, 1953a).

[3] *T. pyriformis* was formerly called *T. geleii.*

Experimental Results

The effect of x-radiation on respiration

Concentrated cell suspensions (approximately 2×10^6 cells/ml.), and cell suspensions diluted with an equal volume of glass-distilled water (referred to as diluted cell suspensions), were irradiated. The appearance of the irradiated cells immediately after exposure was similar to that of the x-irradiated W strain; many cells were non-motile, swollen, and highly vacuolated. The effect on respiration was comparable to that observed with the W strain (Eichel and Roth, 1953b) with the exception that the S organisms appeared more resistant at the 300,000 r level. It is evident from the data in Table I that 600,000 r was considerably more damaging than 300,000 r, and that at the higher level there was little difference in

Table I

Effect of x-irradiation with 300,000 and 600,000 r on respiration of T. pyriformis *S*
The figures give the average and range

Conditions	Per cent change in O_2 uptake from control cells			QO_2 of control cells[2] (μl O_2 per hr. per mg. of dry wt.)
	Minutes after irradiation[1]			
	30	80	140	
Concentrated cell suspension (7)[3]	+12	−3	−9[4]	32.3
300,000 r	(+3 to +17)	(0.0 to −7)	(−6 to −13)	(30.8 to 39.8)
Diluted cell suspension (6)	−4	−17	−23	29.9
300,000 r	(+40 to −43)	(0.0 to −47)	(−8 to −48)	(25.3 to 34.0)
Concentrated cell suspension (3)	−16	−40	−50	27.7
600,000 r	(+8 to −40)	(−22 to −52)	(−37 to −60)	(21.1 to 32.0)
Diluted cell suspension (6)	−5	−40	−54	27.8
600,000 r	(+35 to −38)	(−20 to −55)	(−40 to −70)	(26.7 to 29.5)
Part 2. Effect of 2,6-diaminopurine (AP)				
Diluted cell suspension+AP[5] (2)[6]	+13	−24	−41	
300,000 r	(0, +26)	(−17, −31)	(−36, −46)	
Diluted cell suspension (2)[7]	+31	−3	−9	
300,000 r	(+22, +40)	(0, −5)	(−8, −10)	
Diluted cell suspension+AP[5] (5)[6]	+23	−25	−41	
600,000 r	(+60 to −4)	(−9 to −45)	(−26 to −60)	
Diluted cell suspension (4)[7]	−8	−41	−57	
600,000 r	(+35 to −22)	(−20 to −48)	(−37 to −63)	

[1] The elapsed time between the end of irradiation and closing of the stopcocks was 20 minutes. Therefore, the first 10 minute reading was actually made 30 minutes after irradiation. In general, readings were made at 10-minute intervals during the first hour and at the end of 2 hours. For the sake of brevity, portions of these data are presented here.

[2] Calculated for the first hour.

[3] Numbers in parentheses indicate the number of separate experiments in both Part 1 and Part 2.

[4] Six determinations; two at 110 minutes, four at 120 minutes.

[5] AP concentration was 3.3×10^{-2} *M* during irradiation and 6×10^{-3} *M* after addition of aliquots to vessels.

[6] Control was unirradiated diluted cell suspension+AP.

[7] Control was unirradiated diluted cell suspension.

effect using concentrated or diluted cell suspensions. At 300,000 r, however, the concentrated cell suspensions showed less depression of respiration than those that were diluted. The results at 30 minutes after exposure to the x-ray beam were extremely variable except with concentrated suspensions irradiated with 300,000 r. The initial increase in respiration under these conditions is of interest; it is probably somewhat greater than the data indicate since staining (dilute methyl green) of the irradiated cells showed that many of them (25–50%) were dead immediately after irradiation.[4] In general, there was a significant decrease in respiration of the x-irradiated cells with time; this may be due to a continuing mortality of damaged organisms. The QO_2 values of control cells are presented in Table I to indicate the level of activity in these experiments.

A general interest in the purine antagonist, 2,6-diaminopurine (AP), led us to study the effect on respiration of the presence of this compound during irradiation of whole cells. Controls consisted of unirradiated cells in the presence of similar concentrations of the antagonist. The results obtained with AP are given in Table I, part 2. It can be seen that this compound afforded appreciable protection at the higher level of exposure, particularly in the early post-irradiation period. On the other hand, at 300,000 r AP had a significant deleterious effect. The concentration of AP used (0.006 *M*) was slightly inhibitory to the respiration of control cells. At the present time it is not possible for us to offer an explanation of the effects observed. With regard to the protective action, several amino compounds have been shown to protect other x-irradiated animals (Bacq and Herve, 1951; Bacq *et al.*, 1953), and it is possible that AP is another member of this class.

The effect of x-radiation on the oxidation of L-phenylalanine and acetate

Of the common amino acids, *L*-phenylalanine is most rapidly oxidized by *T. pyriformis* W (Roth, Eichel and Ginter, 1954). The rate of oxidation of acetate is also high in this organism (Kidder and Dewey, 1949). In view of the stimulatory effect of these compounds on the respiration of whole cells, it was deemed of interest to determine what effect they would have when added to x-irradiated cells. Strain W was used since it was investigated previously with respect to the oxidation of these metabolites. Diluted cell suspensions were prepared and then irradiated with 300,000 r. Immediately afterward, aliquots of the suspensions were added to Warburg vessels whose sidearms contained either neutral *L*-phenylalanine or acetate solution. Oxygen consumption was measured after tipping the sidearms and was compared to unirradiated controls to which the same additions had been made, as well as to controls, both irradiated and non-irradiated, to which only distilled water had been added from the sidearms. Experimental details and results are given in Table II. It can be seen that the oxygen consumption in the presence of *L*-phenylalanine was inhibited by 43% (average of 4 experiments) 30 minutes after irradiation. This inhibition was observed to increase at a uniform rate until, at 140 minutes post-irradiation, the value was 58%. The decrease in oxygen uptake of irradiated cells to which phenylalanine had been added was somewhat greater than that observed for the irradiated cells without phenylalanine, studied at the same time. The conclusion is that x-irradiation impairs the ability

[4] Living cells, even those visibly severely damaged, do not take up the stain; dead cells stain a light green with a darker green nucleus.

TABLE II

Effect of x-irradiation with 300,000 r on oxidation of L-phenylalanine and acetate by T. pyriformis. *Each vessel contained the following: body, 1.0 ml. of either control or irradiated cells and 1.0 ml. of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.3; sidearm, substrate as indicated below or water; center well, 0.2 ml. of 20% KOH. The vessels were allowed to equilibrate 5 minutes before tipping in substrate. After an additional 5 minutes, the stopcocks were closed and readings were taken at 10-minute intervals during the first hour and at the end of 2 hours*

Additions from sidearm	Conditions and strain	O_2 uptake in μl.					
		Minutes after closing stopcocks[1]					
		10	% change	60	% change	120	% change
L-phenylalanine[2]	(unirradiated) W(5)[3]	31		202		430	
L-phenylalanine	(irradiated)	24	−23	146	−28	249	−42
Acetate[4]	(unirradiated)	43		270			
Acetate	(irradiated)	51	+19	250	−7		
Water	(unirradiated)	30		182		354	
Water	(irradiated)	26	−13	143	−21	251	−29
L-phenylalanine	(unirradiated) S(3)	29		179		336	
L-phenylalanine	(irradiated)	15	−48	86	−52	142	−58
Acetate	(unirradiated)	31		177		337	
Acetate	(irradiated)	36	+16	167	−6	260	−23
Water	(unirradiated)	32		157		275	
Water	(irradiated)	18	−43	84	−47	131	−48
L-phenylalanine	(unirradiated) W(2)	17		148		301	
L-phenylalanine	(irradiated)	9	−47	64	−57	101	−66
Acetate	(unirradiated)	25		158		283	
Acetate	(irradiated)	20	−20	125	−21	170	−40
Water	(unirradiated)	14		104		203	
Water	(irradiated)	6	−57	47	−55	81	−60
L-phenylalanine	(unirradiated) W(2)	29		188		356	
L-phenylalanine	(irradiated)	14	−52	80	−57	129	−64
Acetate	(unirradiated)	35		184		341	
Acetate	(irradiated)	33	−6	153	−17	227	−33
Water	(unirradiated)	21		125		240	
Water	(irradiated)	16	−24	89	−29	154	−36
Averages							
L-phenylalanine			−43		−49		−58
Acetate			+ 2		−13		−32
Water			−35		−38		−43

[1] See note 1, Table I.
[2] Final concentration = 3×10^{-3} *M*.
[3] Numbers in parentheses indicate age of culture in days.
[4] Final concentration = 5×10^{-2} *M*.

of the cells to oxidize *L*-phenylalanine. In several cases acetate stimulated oxygen consumption by irradiated cells at 30 minutes after exposure; at 80 minutes after exposure, oxygen uptake was inhibited by only 13% as compared to the 38% inhibition of endogenous respiration. Although the inhibition of acetate oxidation increased considerably between 30 and 140 minutes after irradiation, the final level attained was less than that of either the endogenous respiration or phenylalanine oxidation.

The marked ability of acetate to stimulate the oxygen uptake of irradiated cells is of considerable interest. For example, in the third experiment at 80 minutes after exposure, irradiated cells to which acetate had been added showed an increase of 170% in oxygen uptake compared to irradiated cells without acetate. In the same experiment, acetate increased the oxygen consumption of control cells

TABLE III

Succinic dehydrogenase activity of x-irradiated homogenates and of homogenates prepared from x-irradiated cells of T. pyriformis *S. Each vessel contained the following: body, 1.0 ml. of either control or irradiated homogenate, 1.0 ml. of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.3, 0.3 ml. of 0.004 M $CaCl_2$ + $AlCl_3$, and 0.2 ml. of distilled water; sidearm, 0.3 ml. of 0.5 M sodium succinate adjusted to pH 7.4; center well, 0.2 ml. of 20% KOH. Since it has been shown that under the conditions employed here added cytochrome c has little effect on the activity of this system (Eichel, 1954), the compound was omitted. Conditions of equilibration and reading the same as those described in Table II. The figures give the average and range*

Conditions	Dose	Per cent change in O_2 uptake from control homogenates		
		Minutes after irradiation		
	(r)	30	80	140
Irradiated homogenates	300,000 (4)*	−28 (+5 to −45)	−34 (−14 to −48)	−27 (−4 to −50)
	600,000 (4)		−41 (−30 to −53)	−38 (−24 to −49)
Homogenates of irradiated cells	300,000 (4)	−15 (0 to −25)	−10 (−7 to −15)	−6 (+4 to −12)

* Numbers in parentheses indicate the number of separate experiments.

by only 52%. The net effect of the increased oxidation of acetate by irradiated cells is to give the appearance of a protective action by acetate, but in this case the acetate has been added after irradiation and the only protection would be that related to delayed effects of radiation. While certain unknown and probably complex factors will protect animals when given after irradiation (Kelly and Jones, 1953; Cole and Ellis, 1954), no simple substances have yet been shown to have this effect, and it may be worthwhile to test acetate for this action in higher animals.

The effect of x-radiation on respiratory enzymes

Succinic dehydrogenase

Homogenates were irradiated; also, whole cells were irradiated and then homogenized. In all cases suitable unexposed controls were prepared and as-

sayed simultaneously with the irradiated samples. The contents of the Warburg vessels and other experimental details are presented in Table III which gives the results obtained. Irradiation of homogenates at 300,000 or 600,000 r resulted in the loss of a considerable fraction of the activity. However, the activity of homogenates prepared from cells irradiated at 300,000 r declined only slightly, the decrease being just on the borderline of significance.[5]

Seaman (1952), using cystine as an inhibitor, concluded that *Tetrahymena* succinic dehydrogenase is a sulfhydryl-dependent enzyme. In Table IV are listed some effects of SH-reagents on the succinic dehydrogenase activity of normal homogenates. The strong inhibition of the system by *p*-chloromercuribenzoate (CMB) and *o*-iodosobenzoate (IOB) suggests further that it is SH-dependent, although the activity was not restored by the addition of glutathione at concentrations 10–20 times those of the inhibitors. The glutathione was either present in the main body of the vessels at the start of the experiment or tipped in from the sidearm midway through the run. The very slight reduction in succinic dehydro-

TABLE IV

Effect of p-chloromercuribenzoate (CMB) and o-iodosobenzoate (IOB) on succinic dehydrogenase activity of homogenates of T. pyriformis *S. Assays were performed as described in Table III except that in one experiment succinate was added to the body instead of the sidearm together with the sulfhydryl reagents. Concentrations given for the latter are final. The figures represent the average of 2 experiments*

Addition	Per cent inhibition
0.9×10^{-3} *M* CMB	−98
0.1×10^{-3} *M* CMB	−84
0.1×10^{-2} *M* IOB	−94
0.5×10^{-3} *M* IOB	−83

genase activity resulting from the irradiation of whole cells is of interest in view of the studies of Barron (1946) which indicated that inactivation of SH-enzymes is an important aspect of radiation damage in animal tissues. Previously, it had been shown that the activity of the SH-dependent ribonuclease of *T. pyriformis* W was relatively unchanged after exposing cells to very high doses of x-rays (Eichel and Roth, 1953b). These observations with protozoan enzymes are consistent with the recent reports that the activities of SH-dependent enzymes are frequently not altered in the tissues of mammals exposed to high levels of x- or γ-radiation (LeMay, 1951; Roth *et al.,* 1952; Thomson *et al.,* 1952; Ashwell and Hickman, 1952; Roth *et al.,* 1953).

Glutamic and malic dehydrogenases

The conditions and results are given in Table V. The activities of both enzymes were significantly reduced at 80 minutes; after an additional hour, the inhibition of glutamic dehydrogenase was apparently decreased while there was little further change in malic dehydrogenase.

[5] Changes of less than 10% are not considered significant.

TABLE V

Glutamic and malic dehydrogenase activities of T. pyriformis *S homogenates x-irradiated with 600,000 r. Both enzymes were assayed by the method of Seaman (1951) except that each vessel contained 0.5 ml. of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.3. Conditions of equilibration and reading the same as those described in Table II. The figures give the average and range*

Dehydrogenase	Per cent change in O_2 uptake from control homogenates	
	Minutes after irradiation	
	80	140
Glutamic (3)*	−28 (−14 to −40)	−10 (−9 to −10)
Malic (4)	−40 (−9 to −65)	−41 (−21 to −60)

* Numbers in parentheses indicate the number of separate experiments.

Catalase

T. pyriformis S contains an active and, under the conditions employed here, relatively unstable catalase which has not been previously described. No loss of enzyme activity was observed in homogenates kept at 0° up to 2½ hours. However, after 24 hours at this temperature, the activity decreased by 50%. In preliminary experiments, the activity was found to be proportional to the quantity of homogenate used. Catalase was significantly inhibited in x-rayed homogenates tested 5 minutes after irradiation (Table VI), but the activity was rapidly recovered when these homogenates were allowed to stand at 0° in the absence of substrate. In two experiments in which homogenates were irradiated at 600,000 r (not tabulated), and in which catalase assays were performed at 5, 35, 65, 95 and 155 minutes after irradiation, the average changes from control values were −36, −9, −10, −1 and +2%, respectively. After 24 hours, the irradiated homogenates were 29% more active than the controls. A similar recovery of activity was observed in homogenates prepared from irradiated cells (not tabulated). In one experiment with the latter, the catalase activity was 29% less than the control 10 minutes after irradiation, while 120 minutes later, it was 10% more active than the unchanged control.

TABLE VI

Catalase activity of x-irradiated homogenates of T. pyriformis *S. Each determination performed with 1.0 ml. of homogenate. Measurements made within 5 minutes after completion of irradiation*

Dose (r)	Specific activity × 1000		Change (%)
	Control (per sec. per mg. dry wt.)	X-ray (per sec. per mg. dry wt.)	
300,000 (2)*	2.14 (1.99, 2.29)	1.85 (1.99, 1.72)	−13 (0, −25)
600,000 (5)	2.36 (2.10 to 2.77)	1.67 (1.12 to 2.27)	−29 (−11 to −50)

* Numbers in parentheses indicate the number of separate experiments.

TABLE VII

Effect of x-irradiation with 500,000 r, and effect of sulfhydryl reagents, on DNase activity of homogenates of T. pyriformis *S. In part 1, experiments were performed as previously described for ribonuclease (Eichel and Roth, pp. 355–56, 1953b). Homogenates were irradiated in the presence of p-chloromercuribenzoate and aliquots were used for DNase assays. Before assaying, p-chloromercuribenzoate, in the concentration listed below, was in contact with the homogenate for 100 minutes, the time required for the irradiation procedure. The addition of aliquots of these samples to the assay mixtures diluted the p-chloromercuribenzoate to 3.5 × 10^{-4} M. The cysteine was added after irradiation in a final concentration of 3.3 × 10^{-3} M. In parts 2 and 3, the final concentrations are listed. The figures give the average and range of three experiments in part 1 and two experiments each in parts 2 and 3*

Part	Experimental conditions	Specific activity × 100 (units[1] per mg. dry wt.)	Change (%)
1	Control	1.38 (1.22 to 1.60)	
	Irradiated	0.66 (0.46 to 0.90)	−57 (−44 to −72)
	Control + 2.8 × 10^{-3} *M* CMB	1.20 (0.86 to 1.47)	−15 (+11 to −30)
	Irradiated + 2.8 × 10^{-3} *M* CMB	0.19 (0.13 to 0.29)	−86 (−78 to −94)
	Irradiated + 2.8 × 10^{-3} *M* CMB + 3.3 × 10^{-3} *M* *L*-cysteine	0.27 (0.07 to 0.55)	−82 (−58 to −99)
2	Control	1.23 (1.09, 1.36)	
	Control + 2.2 × 10^{-4} *M* CMB	1.09 (1.02, 1.16)	−8 (−6, −10)
	Control + 1.7 × 10^{-3} *M* *L*-cysteine	0.84 (0.79, 0.89)	−30 (−28, −31)
	Control + 2.2 × 10^{-4} *M* CMB + 1.7 × 10^{-3} *M* *L*-cysteine	1.04 (0.98, 1.11)	−11 (−8, −14)
	Control + 1.1 × 10^{-3} *M* IOB[2]	1.28 (1.13, 1.44)	+8 (+4, +11)
3	Control	0.76 (0.80, 0.72)	
	Control + 5.6 × 10^{-5} *M* CMB	0.87 (1.00, 0.74)	+19 (+26, +3)
	Control + 2.8 × 10^{-4} *M* CMB	0.73 (0.84, 0.63)	−4 (+6, −13)
	Control + 5.6 × 10^{-4} *M* CMB	0.72 (0.72, 0.72)	−5 (−10, 0)
	Control + 1.1 × 10^{-3} *M* CMB	0.34 (0.32, 0.35)	−55 (−60, −51)

[1] One unit of activity is defined as that amount of enzyme which, in 25 ml. of test solution, causes a decrease in optical density of 0.1 in 60 minutes.

[2] *o*-Iodosobenzoate.

The effect of x-radiation and sulfhydryl reagents on DNase activity

It has been reported previously that DNase activity decreased 50% in *T. pyriformis* W homogenates irradiated at 500,000 r (Eichel and Roth, 1953b). Similar results were obtained with the S strain and these are given in Table VII, part 1. The DNase activity of control homogenates was of the same magnitude as that found previously for the W strain. The effect of CMB, both alone and in com-

bination with x-radiation, was also investigated. The combined inhibitory effects of radiation and the presence of CMB were somewhat more than additive, while the addition of cysteine after irradiation had little effect on the inhibition. In a similar experiment with ribonuclease of the W strain, cysteine added to irradiated homogenates completely reversed the inhibition due to CMB (Eichel and Roth, 1953b). While cysteine (Table VII, part 2) inhibited DNase by 30% and CMB depressed the activity by 8%, together the two compounds produced only an 11% inhibition. CMB alone had a variable effect depending on its concentration (Table VII, part 3). At the highest concentration used, DNase activity was depressed strongly, while at the lowest concentration there was a slight acceleration. This variable response to CMB has also been reported with pancreatic ribonuclease (Ledoux, 1953). IOB accelerated DNase activity slightly. These facts suggest that in *T. pyriformis* S DNase is dependent for optimum activity on a certain ratio of -S-S- to -SH groups.

Discussion

From the results reported in this paper and the previous one (Eichel and Roth, 1953b) it seems clear that irradiation of *Tetrahymena* homogenates at 300,000 to 600,000 r results in appreciable destruction of enzyme activity. However, in the case of catalase, where irradiated homogenates were allowed to stand at 0° and assayed at varying intervals, all of the activity was recovered within 1–2 hours after x-raying. On the other hand, the irradiation of whole cell suspensions, while causing considerable decrease in respiration, does not markedly affect the activity of several enzymes tested. It seems probable, therefore, that the explanation of the high cell mortality observed does not lie in alterations in enzyme activity due to the direct or indirect action of radiation. Other factors, such as denaturation of nucleoprotein resulting in lethal mutations, or formation of toxic products *in vivo,* may be involved. Further biochemical studies on radiation effects might be more profitably directed toward changes in nuclear activities, but before this can be done we must learn more about the fundamental properties of the nucleus.

Summary

1. The effect of 300,000 to 600,000 r on respiration and oxidative and other enzymes of *Tetrahymena pyriformis* S has been determined. Both homogenates and whole cells were irradiated. At 600,000 r, respiration was significantly decreased; the activities of the succinic, glutamic, and malic dehydrogenase systems, catalase, and DNase were depressed in homogenates irradiated at this level. Within a short time after irradiation, catalase activity was completely recovered in homogenates allowed to stand at 0°. Irradiated cells showed only minor changes in succinic dehydrogenase activity.

2. Whole cells irradiated with 600,000 r in the presence of 2,6-diaminopurine were considerably protected from the effects of radiation on respiration for a short time after exposure. Cells of the W strain, irradiated with 300,000 r and then placed in the presence of *L*-phenylalanine, showed a loss of ability to oxidize this compound compared to controls. Under the same conditions acetate stimulated the oxygen consumption of x-irradiated cells to a much greater degree than that of control cells when measured 30 minutes after irradiation. Some effects of

sulfhydryl reactants on succinic dehydrogenase and deoxyribonuclease activities of homogenates were studied, and the general implications of the results were discussed.

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EFFECTS OF VARIOUS LEVELS OF X-IRRADIATION ON THE GAMETES AND EARLY EMBRYOS OF FUNDULUS HETEROCLITUS [1]

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Fish gametes and embryos have not been studied very extensively in radiobiology. This may be due to the fact that these cold blooded forms appear to be rather radioresistant and experiments must therefore be conducted at high levels of exposure. Nevertheless, the abundance and availability of such marine material and the transparency of the egg and the developing embryo make the fish an attractive object for radiobiological research. In this study an attempt is made to determine (1) the levels of radiation sensitivity of the egg, sperm, and early cleavage stages and (2) the effect of x-irradiation upon early development of the embryo of *Fundulus heteroclitus*.

Materials and Method

Adult specimens of this fish were brought to the laboratory immediately after being caught, and the sexes were separated into different 15-gallon tanks through which ran fresh sea water. Since such fish in captivity generally release their gametes within a day or so, they were used immediately and discarded.

Eggs and sperm were obtained by the usual procedure of stripping. However, when eggs or sperm were to be x-irradiated, this was done prior to stripping by x-irradiating the entire fish. From 3 to 6 fish of the same sex were wrapped in cheese cloth, soaked in sea water, and were then placed in a covered plastic fly box measuring 7 cm. in diameter and 2 cm. in depth. The dish was always filled to capacity with fresh sea water during irradiation. Eggs and sperm were stripped from the fish immediately after their exposure.

Early cleavage stages and later embryos were always selected and were x-irradiated in 40 cc. of filtered sea water for each 25 eggs (or embryos) in the same plastic dish, described above. Immediately after irradiation, the exposed material was removed from the sea water and placed in fresh, filtered sea water in order to avoid any possible indirect effect of contamination from extraneous materials irradiated in the sea water. Post-irradiation eggs or embryos were kept 5 per finger bowl in 100 cc. of filtered sea water which was changed every 3–4 days. All material was kept at the laboratory temperature, which was generally about 25° C.

The x-ray facilities used were those available at the Marine Biological Laboratory, Woods Hole, Massachusetts, and consisted of two alternate parallel tubes

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[2] Working under contract AT-30-1-GEN-70 for the Atomic Energy Commission.

run at 182 KVP and 25 MA and having equivalent filtration of 0.2 mm. Cu. The maximum output of this machine at the closest position of the tubes (position "A") gave 6000 r/minute, air dose. The position "B" gave a lower rate of 600 r/minute. For long exposures at the higher rate an electric fan was directed to the container in order to dissipate any heat emanating from the tubes themselves.

Observations and Experimental Data

X-irradiation of the unfertilized egg

The unfertilized eggs of Fundulus were exposed within the body of the fish to various levels of radiation from 1000 r to 200,000 r. They were then stripped into a dry suspension of fresh, normal sperm. These eggs were flooded within 15 minutes with filtered sea water and were studied side-by-side with unirradiated control eggs which had been fertilized simultaneously by normal sperm from the same source.

X-irradiated eggs, whether exposed to 1000 r or to 200,000 r, showed separation of membranes to form a perivitelline space, generally considered as one of the criteria of successful fertilization. It is true that unfertilized eggs placed in sea water will often show this membrane separation after a period of time, but these irradiated eggs showed the effect almost immediately. This occurred even though cleavage did not follow. Eggs exposed to 100,000 r and placed in sea water without benefit of sperm tended to form blastodiscs but there was no real activation. The eggs appeared to be deformed.

Eggs exposed to 100,000 r were often fertilizable with normal sperm and some developed at least as far as stage 15 of Oppenheimer (1937) without any evidence of gross morphological defect (see Figs. 1, 2). These embryos developed optic vesicles, at least 4 pairs of somites, and pericardial vesicles. Some of these embryos reached stage 17 in 46 hours, but all exhibited lordosis and reduced development of the anterior neural structures (see Figs. 3, 7 and 10). No corpuscles appeared in the experimental embryos, while the simultaneous controls had reached stage 22 and had circulating corpuscles. Some such embryos from 100,000 r x-irradiated eggs lived fully two weeks, finally acquiring pulsating hearts but still no corpuscles. They had retinal pigment but were never able to hatch from their surrounding membranes (see Fig. 4). Since the controls (Fig. 5), under identical conditions of temperature, light and sea water, did develop into free-swimming fish fry, we must admit to considerable retardation in development in consequence of x-irradiation of the egg alone. The failure to hatch may have been due to irradiation damage to the hatching enzymes.

Eggs exposed to 50,000 r showed less retardation of development and better morphological differentiation than those exposed to 100,000 r. However, some of these were also without anterior neural differentiation, even though the posterior end and tail development appeared to be quite normal.

X-irradiation of the sperm

X-irradiation of the sperm to very high doses did not have so devastating an effect upon the subsequent development. Sperm exposed to 200,000 r and used to fertilize normal eggs caused development to reach, by 13 days, a stage which compared favorably with the development of the controls at 12 days (compare

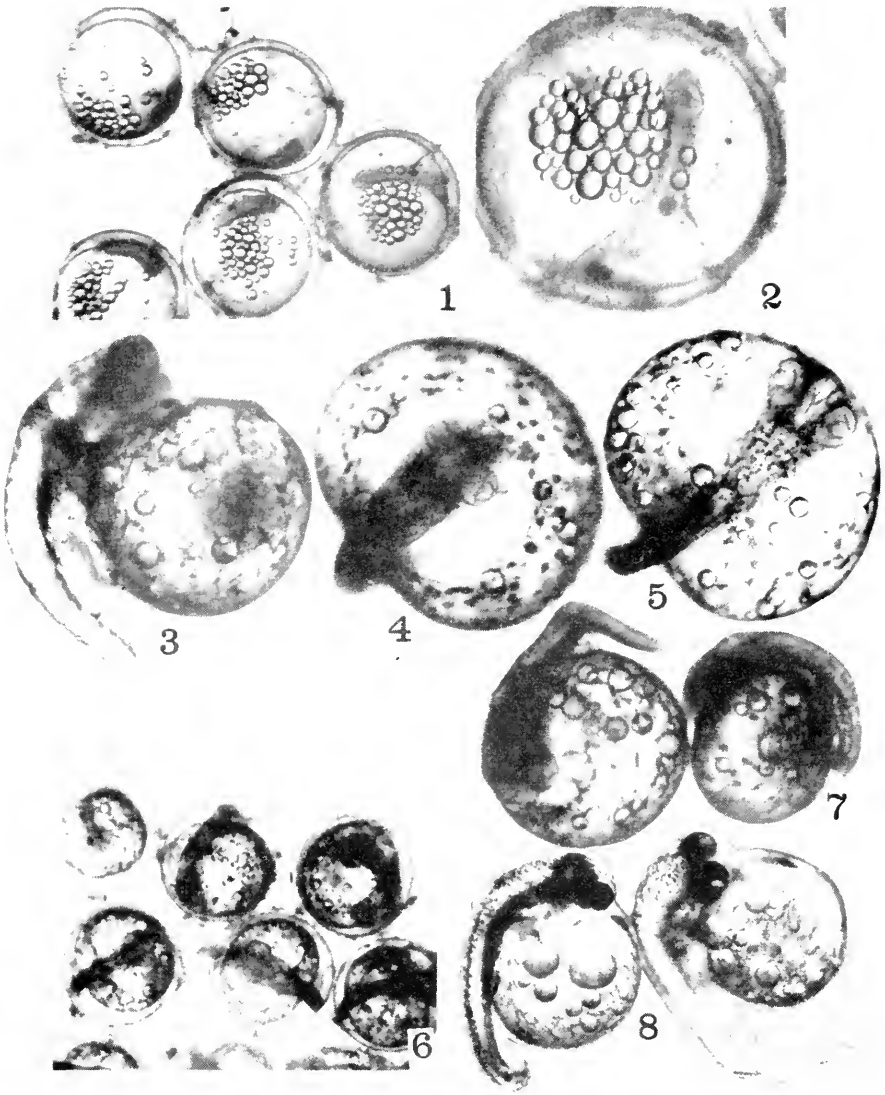

PLATE I. X-Irradiation of sperm and eggs of *Fundulus heteroclitus*.

FIGURE 1. Normal eggs of *Fundulus heteroclitus* fertilized by sperm after exposure to 100,000 r x-rays. Development comparable to stage 15.

FIGURE 2. Single specimen from previous group which developed to stage 17 in 46 hours, while controls developed to stage 22.

FIGURE 3. Development to 11 days of normal egg fertilized by sperm after exposure to 100,000 r. Note reduction in yolk mass, failure of anterior structures, and lordosis.

FIGURE 4. Sperm exposed to 200,000 r prior to use in fertilizing normal eggs of Fundulus. Note better development than in Figure 3, with distinct optic vesicles and chromatophores. Such embryos had pulsating hearts but the circulatory system was devoid of corpuscles. This specimen was 13 days of age, to be compared with control in Figure 5 at 12 days.

Figs. 4 and 5). Retinal pigmentation and pulsating hearts developed, but no blood corpuscles were formed, and none of these embryos ever hatched.

Following 100,000 r x-rays to the sperm, prior to their use in fertilizing normal eggs, some 33% of the eggs cleaved, and many of these developed to stage 12 within 34 hours. Their anterior neural structures developed but were twisted. There were no true somites. Some of these embryos eventually reached stage 30 and hatched, appearing to be quite normal. These may well have been haploids. A few lived for 19 days and all showed cyclopia without other neural differentiation.

X-irradiation of early cleavages and the embryo

The 1- to 2-cell stage is the most vulnerable to x-irradiation insult. Following as little as 300 or 400 r there was stunted but otherwise normal development (Fig. 9). Following 600 r some 25% of the embryos finally hatched, but all were very much stunted. Following 1000 r all were grossly abnormal and without any neural differentiation. The controls had reached stage 22 when the irradiated embryos had reached a stage best identified as stage 15.

The 2- to 8-cell stages were only slightly less vulnerable. The embryos exposed to 500 r exhibited quite normal development.

Stage 11, which is the expanding blastula stage, is slightly more radioresistant than are the early cleavage stages, so that it took 1,000 r to produce a slight quantitative effect on early development (Figs. 10–13). Only a very few of these hatched, however. This may indicate again that the enzymes necessary for hatching are even more sensitive than are some of the morphogenetic processes, for the embryos contained within the chorionic membranes were quite normal. Above 1000 r there appeared all kinds of teratologies, and rarely did development proceed beyond stage 21 (Figs. 14–17). The anterior neural structures were again the ones most seriously affected. However, it must be emphasized that within any group of similarly x-irradiated embryos there was a wide divergence of effect on development.

Point irradiation

By means of a silver point coated with polonium and mounted in a ball-point pen holder, it was possible to spot irradiate with alpha particles a single cell or a limited region of a developing embryo. While irradiation dosage could not be determined accurately, it was estimated that at point of contact the cell would be exposed to about 10,000 reps per minute. A non-radioactive but similar silver

FIGURE 5. Control at 12 days, normal sperm and egg of Fundulus, raised under identical conditions as above experimentals.

FIGURE 6. Eggs exposed to 50,000 r x-rays and then fertilized with normal sperm and allowed to develop for 5 days. Retarded from stage 29 of the controls to about stage 25, with poor development of the anterior structures.

FIGURE 7. Eggs exposed to 100,000 r x-rays and then fertilized by normal sperm and allowed to develop for 4 days. Should be stage 26 but gross abnormalities evident. Compare with Figures 3 and 4.

FIGURE 8. Same as Figure 7 but allowed to develop for 10 days. Should be stage 32, probably comparable to stage 26. Note rather normal development of the eyes, but abnormal head anterior to the eyes and curvature of the tails. Artificially removed from covering membranes.

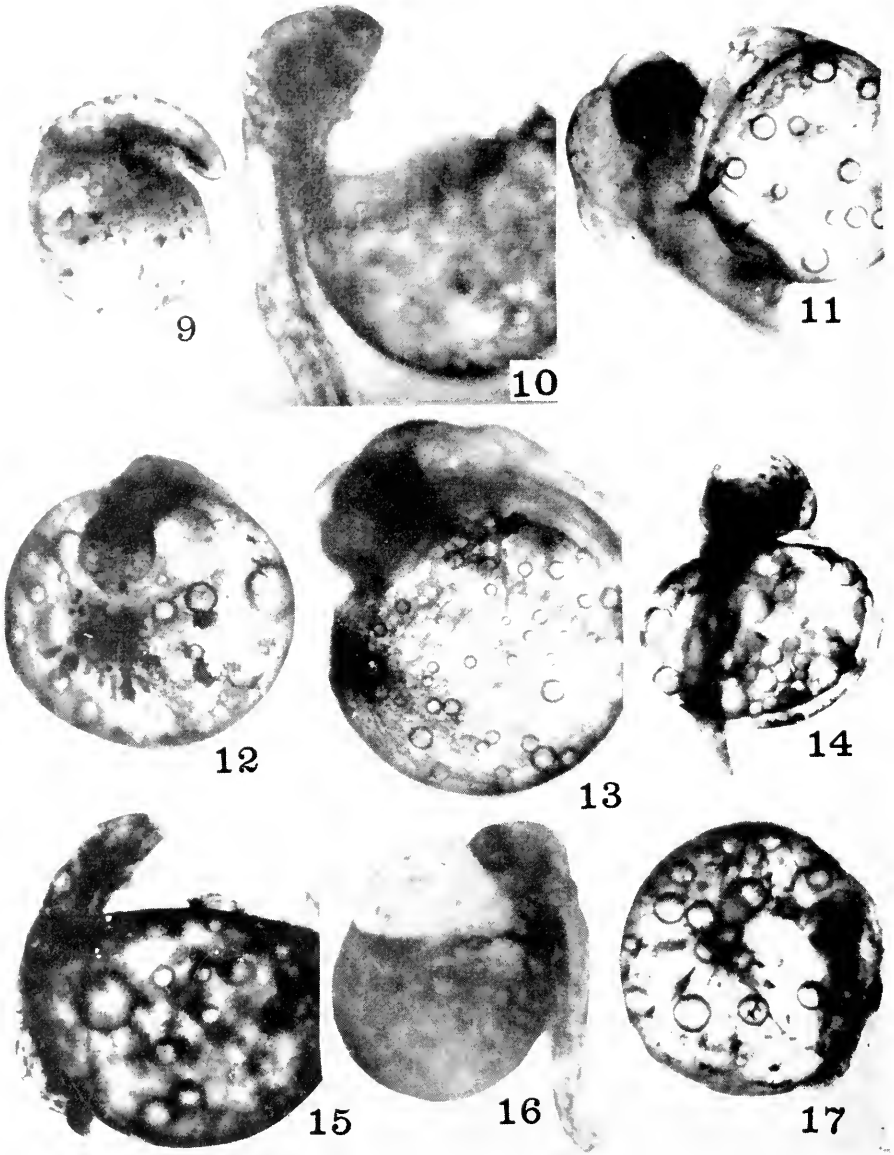

PLATE II. X-irradiation of the early embryo of *Fundulus heteroclitus*.

FIGURE 9. Fertilized egg x-irradiated to 400 r at the one- to two-cell stage and allowed to develop for 10 days. Should be in stage 31 but shows only faint evidence of structural development, poorer anteriorly than posteriorly.

FIGURE 10. Stage 11 from same batch as Figures 11–17 experimentals (expanding blastula at 14 hours) showing normal development at 8 days with head structures straightening out, and well developed and functioning circulatory system (note heart).

FIGURE 11. Stage 11 exposed to 500 r and allowed to develop for 8 days. Development slightly stunted but quite comparable to control in Figure 10.

point was used as control. This method of spot irradiation with polonium was probably first used by Lacassagne and Desclin in 1928.

Eggs were dechorionated [3] and placed in a shallow depression slide. The point of the polonium pencil was brought into direct contact with one of the cells of the two-cell stage and held there for two minutes. This would represent an exposure of approximately 20,000 reps, but the penetration was not more than 30 microns.

The cleavage pattern of the cells was not immediately altered by such spot irradiations. One, or more frequently two, apparently normal cleavages succeeded exposure. This would seem to indicate that there is a latent period of about an hour's duration before the early cleavages can be affected by this dosage of alpha irradiation. Subsequently, however, the progeny of the irradiated cell ceased to divide, although the unirradiated portion continued to cleave in the usual manner. These eggs usually developed into high blastulae which had the appearance of normal blastulae except that they covered less surface of the yolk and the area of contact with the yolk was oval in shape rather than round. By this time the irradiated cells seemed to be no longer a part of the blastoderm but had flattened out and fragmented. At the time of gastrulation no trace of the damaged cells could be found and the experimental eggs were indistinguishable from the controls.

Gastrulation usually began in the unirradiated eggs, and ordinarily no differences were observed until the blastoderm had reached the equator of the egg. After this point, however, there appeared constrictions and lines of tension in the unincorporated yolk of the irradiated eggs. As epiboly continued, the yolk was under such pressure from the tension of the inadequate blastoderm that there appeared bulges of yolk, which often ruptured. Though these ruptures were able to heal over in some cases, only one embryo was observed to complete gastrulation and finally develop into a normal but small fish fry.

When the polonium point was applied to the hindbrain of the dechorionated embryo at stage 25, the embryos failed to develop beyond stage 27, although similarly treated controls, using the dummy pencils, developed normally.

Discussion

As Solberg (1938b) pointed out, the embryos of Fundulus exhibit decreasing radiosensitivity with increasing age. Greatest sensitivity was shown by the fertilized egg and the early cleavage stages, which were retarded substantially by as small a dose as 300 r in our experiments. Later stages acquired increasing radio-

[3] With the much-appreciated technical assistance of Dr. J. P. Trinkaus of Yale University.

Figure 12. Stage 11 exposed to 1000 r x-rays and allowed to develop for 5 days. Note failure of development of blood vascular system.

Figure 13. Same as Figure 12, photographed at 8 days to show functioning blood vascular system but retardation of anterior structural development.

Figure 14. Stage 11 exposed to 1500 r and allowed to develop for 5 days. Note abnormal yolk, and edematous brain vesicle.

Figure 15. Stage 11 exposed to 2000 r x-rays and allowed to develop for 7 days. Extreme stunting but large pericardial vesicle, even though no circulating cells were formed.

Figure 16. Same as Figure 15, photographed the next day. Note rather normal eye development and heart, but no circulating blood cells.

Figure 17. Stage 11 exposed to 2500 r and allowed to develop for 5 days. Extreme stunting and failure of both neutral and circulatory development.

resistance, the blastula developing fairly normally after 1000 r and the 8-day embryo tolerating up to 3000 r without a marked increase in the percentage failing to develop normally.

Contrary to Solberg's results, however, were our observations after irradiation of the unfertilized eggs or the sperm. Using doses of 2000 r to the gametes, Solberg found abnormalities to be produced in 68% of the embryos when the eggs were irradiated and in 86% when only the sperm were treated. He found (1938a) that the testes of Oryzias were 3 or 4 times as radiosensitive as were the ovaries. In our laboratory, we employed much higher doses of irradiation, *i.e.*, 100,000 r and 200,000 r to the gametes of each sex. We found that the relative number of developing embryos resulting from the fertilization of normal eggs by irradiated sperm was greater than the number developing from irradiated eggs and normal sperm. Furthermore, some of the irradiated sperm produced fairly normal embryos, some of which eventually, in the case of those receiving 100,000 r, were able to hatch into viable fish fry. In no case were those embryos developing from eggs irradiated to these high doses able to develop circulating blood corpuscles or hatch.

The disparity of our results from those reported by Solberg may perhaps be explained on the basis of the doses of x-irradiation used. It is still conceivable, upon consideration of our data, that his assumption of greater sensitivity of the sperm is substantiated. Perhaps the x-ray doses which we gave were sufficiently high to destroy the genetic functions of the sperm so that parthenogenetic development followed. Nuclear constituents of cells are more radiosensitive than is the cytoplasm, and the sperm, while retaining their motility, could have been so damaged that the chromosomes were no longer functional. In this case, the irradiated sperm would have activated the eggs, initiating development, yet they would not have contributed genetically to the embryos. Hence the resulting offspring would have been haploid. In Solberg's work, abnormalities probably resulted from the attempt of damaged sperm chromosomes to achieve syngamy.

It is concluded from our observations following the alpha irradiation of cleavage stages in Fundulus that the mechanism of gastrulation was not impaired by the early destruction of one half the germinal material. The process began in the irradiated eggs as it did in the controls. Many investigators (Morgan, 1893, 1895; Lewis, 1912; Hoadley, 1928) have performed experiments in which one cell of the two-cell stage was surgically excised, and the resulting cell was able, if it survived the operation, to form a blastoderm which gastrulated normally and eventually hatched. Morgan presumed that he had not removed as much as half the embryonic material in his experiments. In such a case, any increase in the amount of cytoplasm would probably produce a corresponding increase in the embryo's chances for successfully completing the gastrulation process. It is thought that our failure to achieve normal closure of the blastopore (except in one instance) was due to the great quantitative reduction in blastodermal material. Normally the blastoderm encircles and covers the yolk (Trinkaus, 1951) but here its amount was reduced by half while the amount of yolk remained unchanged. If it were possible to remove some yolk and thereby restore the original ratio of blastoderm to yolk, it is conceivable that more of the irradiated eggs would have been able to complete gastrulation. Attempts to accomplish this were unsuccessful.

Summary and Conclusions

1. X-irradiation of unfertilized eggs of *Fundulus heteroclitus* often caused the separation of the outer membrane to form a perivitelline space, but none of these eggs proceeded to cleave.

2. X-irradiation of males up to 200,000 r did not destroy the motility or the fertilizing power of all the spermatozoa.

3. Eggs exposed to 100,000 r within the body of the female were sometimes fertilizable with normal sperm and many developed as far as stage 17 without gross teratologies. While lordosis was frequent, due to imperfect development of the central nervous system, the pulsating heart was generally found functioning without benefit of corpuscles.

4. Sperm x-irradiated to 200,000 r and used to fertilize normal eggs could often produce quite normal development to 12 days, when the embryos were comparable to the controls at 11 days. These may have been haploids, due to a genetically non-functional nucleus.

5. An exposure of the spermatozoa to 100,000 r frequently resulted in quite normal embryos that were able to hatch.

6. The first (or earliest) cleavage is the most vulnerable to x-irradiation insult, such embryos tolerating a maximum of 300 r without gross abnormalities. Following 1000 r the embryos were unable to achieve any neural differentiation.

7. A dose of 1000 r to stage 11 (expanding blastula) was qualitatively comparable to a dose of about 500 r to the first cleavage stage.

8. The anterior neural structures appear to suffer most following x-irradiation of any embryonic stage from the first cleavage through the expanding blastula. Pulsating hearts without corpuscles are often found.

9. Point x-irradiation of a single cell of the two-cell stage with polonium (20,000 reps) appears to damage the cell to such an extent that regulation and successful gastrulation cannot be achieved, except in rare cases. This may be due to mechanical factors, or to a quantitative alteration of the blastoderm-yolk relations.

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ON THE DISTRIBUTION OF NEREIS DIVERSICOLOR IN RELATION TO SALINITY IN THE VICINITY OF TVÄRMINNE, FINLAND, AND THE ISEFJORD, DENMARK

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In the literature on the salinity tolerance and osmotic behavior of the widespread estuarine polychaete, *Nereis diversicolor* O. F. Müller, there occur suggestions such as that of Ellis (1937) that physiologically distinct races exist in different regions. Ellis had noted that *N. diversicolor* from Roscoff, France, differed from worms of the same species from Plymouth and Bangor in Britain in rate of weight regulation in response to suddenly lowered salinity, and suggested that the physiological differences observed were of "racial rather than environmental" origin. Unfortunately, Ellis did not report the conditions of salinity under which his experimental animals had existed in nature nor even the salinities at the time and place of collection, so that one cannot decide whether the observed differences in response might reasonably be considered of adaptive significance. Similarly, certain of the findings of Beadle (1937) of differing responses in winter and summer cannot be evaluated because the pattern of salinity variation in the Blythe estuary from which he obtained his worms is not stated.

It is obvious that *N. diversicolor* as a species experiences, and is presumably adapted to withstand, markedly different salinity conditions in different parts of its geographical range. In Britain the species is most characteristic of estuaries, in which it may experience extreme tidal and seasonal variations in salinity, whereas in the tideless Baltic Sea it encounters over much of the year a low but relatively stable salinity. Since the larvae of *N. diversicolor* tend to be benthic (Dales, 1950), populations in British estuaries must be fairly well separated from each other and from those on the continental coasts, and have certainly been isolated for a long time from these of the inner Baltic. Because conditions of extreme salinity variation must present different selective pressures than do conditions of stable low salinity, *N. diversicolor* seems a favorable species in which to investigate the possible existence of physiological races, differing from each other in their tolerance of low salinities. In other words, the character of the distribution of this species is such as to favor the formation of populations genetically distinct in respect to physiological response to low salinity. But while thus suggesting that physiological races in *N. diversicolor* may be a reasonable possibility, we should bear in mind that sound evidence for such races is almost non-existent. In this discussion a physiological race is thought of as a population in which a characteristic and adaptive level of physiological performance exists in response to a given environmental factor, in this instance low salinity. It seems clear that before one can claim to have demonstrated a physiological race of this sort, one

must have demonstrated a clear difference in response to the variable factor under comparable conditions in the separate localities, and must also know the pattern of variation of this factor in the different portions of the range of the species. The problem thus divides itself naturally into laboratory studies to measure the response to the controlled variable, in this case low salinity, and field studies to determine the salinity conditions under which *N. diversicolor* lives in representative parts of its geographical range.

The writer has had the opportunity to carry out such studies upon the ecology of *N. diversicolor* and upon its chloride regulation at several selected localities in northwestern Europe, localities which represent three extremes of the salinity conditions within the range of the species.

1. A "marine-dominated" habitat has been studied at Millport, Scotland, and the results reported in a previous paper (Smith, 1955). In brief, the above study (which has been supported by observations in the Salcombe estuary in southern England) shows that *N. diversicolor* when in an essentially "marine" environment finds its optimum in local, relatively brackish, zones.

2. "Stable low salinity" represents perhaps the opposite extreme of the ecological range of *N. diversicolor*. Such conditions are best exemplified in the mesohaline [1] waters of the Baltic Sea, with which the present paper is largely concerned.

[1] Owing to the number of salinity classifications which have been proposed, it is necessary to state that in this paper reference is to the system of Redeke as amended by Välikangas (1933), expressed as salinity in parts per mille (‰). More recently Ekman (1953) has proposed a more complex terminology which embodies essentially the same divisions as the previous one, as seen in the following comparison:

Välikangas (1933)		Ekman (1953)	
Fresh water	< 0.5 ‰	Fresh water	< 0.5 ‰
Oligohaline	0.5–3.0	Oligohaline brackish	0.5–3.0
β (meio-) mesohaline	3.0–8.0	Mesohaline brackish	3.0–10.0
α (pleio-) mesohaline	8.0–16.5	Polyhaline brackish	10.0–17 (20?)
Polyhaline	16.5–30.0	Oligohaline sea water	17 (20?)–30
Ultrahaline (sea water)	> 30.0	Mesohaline sea water	30–34 (?)
		Polyhaline sea water	> 34 (?)

I have elected not to follow the Ekman system because it seems more likely than the older system to lead to confusion as a result of repetition of adjectives, by the use of generally accepted terms in new quantitative meanings, and by the restriction of the term "brackish" to a portion of the range of salinities which it has been taken to cover during long usage. There is much to be said for retaining the general terms oligo-, meso-, and polyhaline to describe waters of low, intermediate, and higher salinities, respectively, and keeping "seawater" for these salinities above 30‰ which characterize the open seas (here some modification of the Välikangas system may be needed). "Brackish" can be omitted as a classificatory term, and the boundaries of the oligo-, meso-, and polyhaline left somewhat flexible, to be adjusted by zoogeographers to fit local conditions (in which it is necessary to state specific salinities anyway). It seems possible that the upper limit of a mesohaline body of water, as judged on faunistic grounds, would be somewhat different in a warm brackish sea than it is in the Baltic, to which both of the above systems rather specifically apply.

It should be noted that the limits of the meio- ("less") and the pleio- ("more") mesohaline of Välikangas have been determined on the basis of Baltic faunas; these divisions thus represent

3. Another type of extreme is represented by "estuarine" habitats such as are common in Britain. Here salinity *variation* is the predominating characteristic, the salinity changing semi-diurnally with tides, often semi-monthly with the lunar cycle, seasonally or erratically with winds and rainfall, and topographically according to relative volumes of estuary bed and fresh-water inflow. Conditions of salinity in the estuarine habitat of *N. diversicolor* are difficult to characterize in any simple way (Bassindale, 1943a, gives one excellent account), and in connection with the present work will be discussed in a later paper.

The results of the comparative physiological work which has been carried out concurrently with observations upon the distribution of *N. diversicolor* in the several areas visited will be assembled in a single paper elsewhere, but it seems advisable to summarize the ecological studies on each type of habitat separately. The attempt to carry out both field and laboratory studies in several widely separated localities within the space of a year has necessarily resulted in a superficial picture. Nevertheless, these studies are probably the first attempt to see *N. diversicolor* with the same eyes over so wide an ecological range, and to support laboratory studies of its salinity tolerance by systematic field observations. It is hoped that our picture of this species may be somewhat clarified, if only by defining gaps in our knowledge. The present paper reports observations made on the south coast of Finland, not far from the eastern limit of *N. diversicolor* in the Baltic Sea, together with subsequent observations in a Danish fjord of comparably stable but not so low salinity. A summary of ecological conditions in each area has been included to complete the picture and as an aid to future visiting workers.

Part I. Observations at Tvärminne, Finland

General description of the area

The Baltic Sea is not only the largest and best-known brackish-water area of the world, but offers in its practically tideless condition a most illuminating contrast to estuarine waters with their tidal fluctuations in level and salinity. For the present study the Tvärminne Zoological Station of Helsinki University has proved most favorably situated. Located on the southeastern side of the peninsula of Hangö at the southern tip of Finland (Fig. 1), it lies near the eastern limit of the range of *N. diversicolor* (Välikangas, 1933), in the west-east gradient of decreasing salinity of the Gulf of Finland. This Gulf is essentially a continuation of the central Baltic, not being cut off (as is the Gulf of Bothnia) by a shallow sill or group of islands. The region about Tvärminne has been extensively studied, so that much information is available to support the studies of the short-term visitor. Thus the bottom fauna of the area has been studied by Segerstråle (1933a, 1933b, 1949, 1955), the larger aquatic vegetation by Luther (1951a, 1951b), the bottom micro-fauna by Purasjoki (1945), the hydrography and plankton by Halme (1944) and Halme and Kaartotie (1946), as well as by others, references to whom may be found in the papers cited. One of the regular water-sampling stations of The Institute of Marine Research, Helsinki, lies in Tvärminne Stor-

a regional adaptation of the more widely applicable but less precise term "mesohaline." Ekman's divisions seem to embody the same ideas, but have gone beyond the concept of a characteristic (or "true") brackish-water fauna to express the idea that the waters inhabited by this characteristic fauna are the "true" brackish waters, a somewhat doubtful logical step.

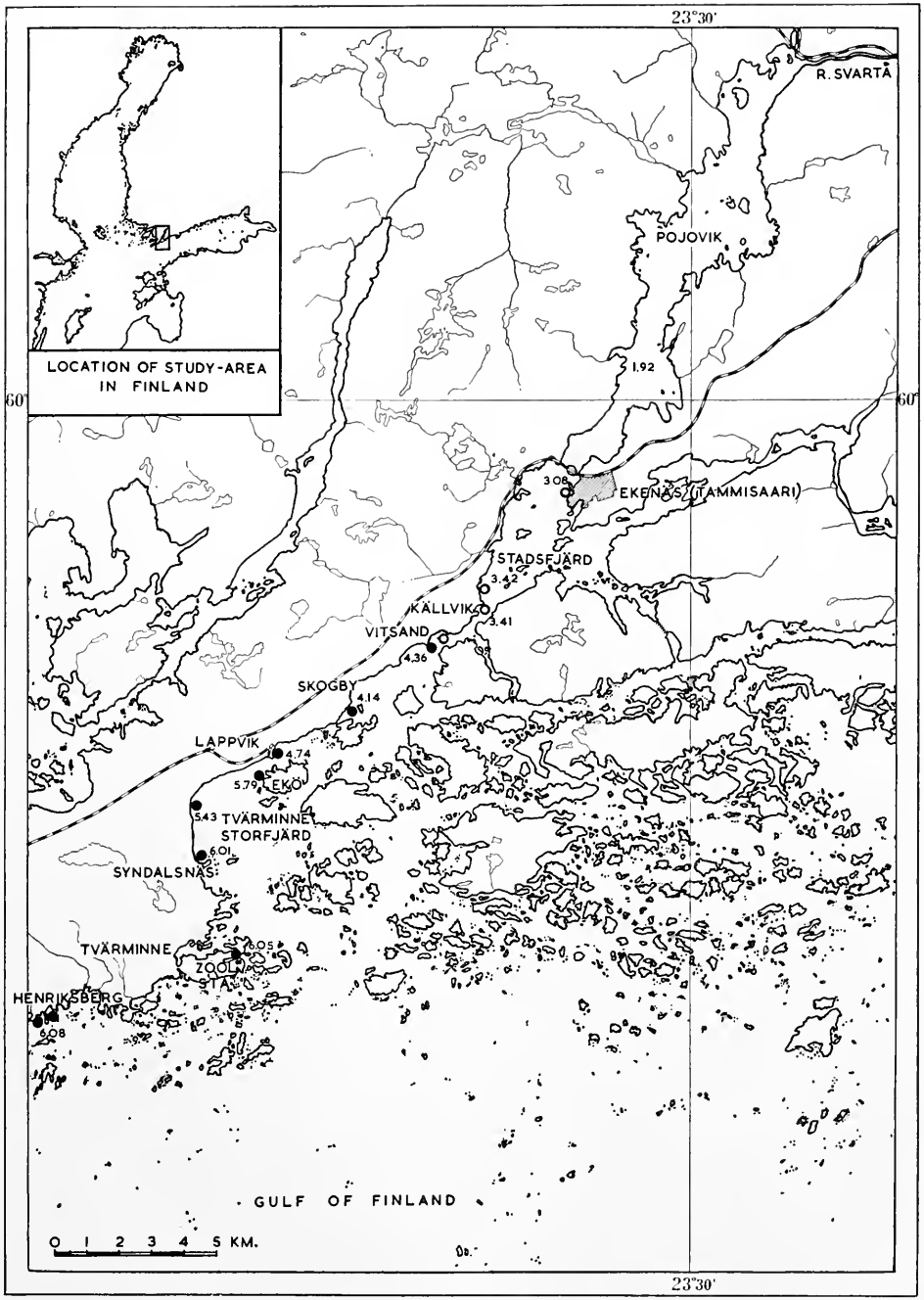

FIGURE 1. Map of the Tvärminne area, Finland, showing localities mentioned in text. Numerals indicate surface salinities observed in July–August, 1954. Black circles, *N. diversicolor* present; open circles *N. diversicolor* not found after careful search on suitable bottom. Detailed data in Table I.

fjärd near the Zoological Station; hence nearly complete series of salinity and temperature records are available for the past 25 years (*e.g.*, Granqvist, 1951). Summer salinities of the open coastal waters near Tvärminne have been about 6°/₀₀ at the surface and 7°/₀₀ at 30 meters (*loc. cit.*). In the past 15 years the region, in common with the rest of the Baltic, has experienced a noticeable rise in salinity of 0.4–0.7°/₀₀ (Granqvist, 1949, 1952). This is not known to have affected the distribution of *N. diversicolor*, but the breeding ranges of certain animals, *e.g.*, *Aurelia*, have been extended eastward (Segerstråle, 1951a), while the amphipod *Calliopius laeviusculus* has extended its range shoreward in the Tvärminne area (Segerstråle, 1953).

Salinity variations in the coastal water mass are quite marked at certain seasons and have been summarized by Segerstråle (1951b). Mainly as a result of the large fresh-water discharge of the River Neva, salinities along the south coast of Finland decrease to the east, and are subjected to further lowering at the time of melting snow. In addition to the main west-east coastwise gradient, there is a gradient of decreasing salinity as one passes northward (inshore) from the outer islands, this gradient being most marked where rivers from the Finnish mainland reach the Gulf. Pronounced salinity stratification may occur, especially when the ice cover prevents mixing by winds. Thus in Tvärminne Storfjärd the salinity of the deeper waters may be almost unaffected by a layer of fresh water from the River Svartå (Fig. 1) flowing beneath the ice (Granqvist, 1938). Its effect upon the bottom fauna of deeper waters must be negligible. Segerstråle (1951b) has shown that spring lowerings of salinity to less than 50% of the annual mean (taken at a depth of 5 meters to avoid the surface layer) occur only about once in 20 years in Tvärminne Storfjärd; the coastal waters are thus of relatively stable salinity. However, in shallow inshore bays the spring dilution may be a much more frequent and serious matter. Thus the Krogarvik, a little bay adjacent to the Zoological Station, whose depth is mostly under three meters and which receives fresh water drainage from a nearby pond and marsh as well as being subjected to the regional lowerings of salinity, may experience salinity reductions to less than 40% of the mean in three years out of four. Segerstråle (1933a) records salinities at the bottom of the Krogarvik as low as 2.02°/₀₀ in 1929 and 0.21°/₀₀ in 1932, although from 1945 to 1950 none lower than 3.77°/₀₀ were recorded (Segerstråle, 1951b). These critical periods of greatly lowered salinity occur in the spring (February–May) and may last as long as a month. The topography of Tvärminne Storfjärd and the Krogarvik are illustrated in Segerstråle's papers (1933a, Abb. 1; 1949, Fig. 4), where more complete descriptions of the region are available.

Tidal changes in water level are virtually absent. A tide of 4 cm. is statistically demonstrable at Hangö (Lisitzin, 1945), but variations caused by local winds and by general water levels in the Baltic are much greater. Thus, during the period of the present study (July 5 to August 9, 1954), the water level at the Zoological Station varied over a range of 40 cm. and averaged 10.3 cm. above mean water level. Luther (1951a) discusses water levels and related matters in some detail, and points out the serious effects which may be caused by low water coincident with the spring discharge of fresh water beneath the ice at shallow inshore localities.

Although to the zoogeographer the distribution of *N. diversicolor* in the west-

east salinity gradient of the south coast of Finland is of primary interest, the physiological ecologist with limited time at his disposal finds the local gradient of decreasing salinity from the open Baltic inshore towards the land more convenient for study. The region about Tvärminne receives no fresh water streams of any consequence except for the River Svartå, which discharges a large flow into the northern end of the long fjord-like Pojovik (Fig. 1). This fresh to oligohaline water passes out over deep-lying mesohaline water in the depths of the Pojovik, where the salinity at 30 meters approximates 4‰ (Halme and Kaartotie, 1946), and over a shallow (7 m.) sill at Ekenäs (Tammisaari) into the Stadsfjärd. The latter bay shows a distinct salinity stratification in summer. Halme (1944) describes a labile β-mesohaline wedge of deeper water entering the Stadsfjärd via the narrow but relatively deep (15 m.) strait at Källvik, sometimes extending inwards past the Ekenäs sill and at other times being displaced seaward by the massive outflow of oligohaline water. The Källvik strait thus represents a topographical interruption or discontinuity in the salinity gradient between Ekenäs and Tvärminne Storfjärd, a point to be amplified later.

Segerstråle (1933b) reported that *N. diversicolor* occurred regularly but sparsely on sandy bottoms down to depths of 6–8 meters and occasionally on soft bottoms. The maximum density (44 per m.^{2}) and weight (7.4 gm. per m.^{2}) were obtained on a muddy bottom. Although reported from a number of stations, the species does not appear from this report to have been as abundant at that time as it is at present. Since Segerstråle's stations were restricted to the area about Tvärminne and the southern part of the Storfjärd, the limits of the salinity range of *N. diversicolor* were not known, and a main objective of my field work was to establish the distribution of the species in relation to the local gradient of decreasing salinity inshore from Tvärminne.

Field observations

In the course of collecting worms for physiological work, and on a limited number of other days, water samples were taken at points indicated on Figure 1, and a careful search made for *N. diversicolor*. Quantitative sampling could not be used in the time available; rather, as careful a search as possible was made at each spot on the most suitable bottom, and a rough estimate of abundance based upon the time and effort used in securing specimens. Negative results, where indicated, are based upon at least two visits to the site on separate days under good collecting conditions, employing both visual search and digging in shallow water and dredging in deeper water. All collecting stations were at sites where the bottom appeared to be, on general grounds, suitable for *Nereis*. The most favorable substratum was clearly sandy, ranging in consistency from decidedly coarse on exposed beaches to fine, gray, and firm on protected shores. At deeper levels, worms could be found in mud or gyttja (a soft deposit rich in organic matter), usually with some admixture of sand. Contrary to Segerstråle's experience (1933b) I have obtained the largest specimens in firm sand rather than in mud and at depths of less than one meter. Worms were not taken on clay bottoms, such as were encountered along much of the shoreline of the Stadsfjärd.

Samples were preferably dug with a spade in water up to waist-deep, and washed through a sieve of about one-mm. mesh. On open sandy bottoms it was

TABLE I

Summary of field observations in Tvärminne region

Scale of exposure
E —Exposed
Pr—Protected
SP—Semi-protected

Scale of abundance of Nereis
R—Rare; occasional or single individuals
S—Scattered; consistently found by careful search; 5–10 worms per hour of digging or hand dredging
C—Common; 1 or 2 worms per spadeful or dredge haul
P—Plentiful; several worms per spadeful or dredge haul

Scale of size
L —Large
M —Medium
Sm—Small

Collecting site (see Fig. 1)	Surface salinity ‰	Exposure and substratum	*Nereis diversicolor*		Associated flora (conspicuous elements only)	Associated fauna (conspicuous elements only)
			Abundance	Size		
Henriksberg (outside point)	6.08 (5.95–6.21)	E; coarse sand	S	L	Flocculent or encrusting algae on sand; *Fucus vesiculosus* on rocks	*Macoma baltica* (abundant) *Mya arenaria* *Mysis vulgaris*, etc.
Henriksberg (inside point)		SP; medium sand	F	M and Sm		
The Krogarvik (Zool. Sta.) head and north shore	6.05 (5.99–6.10)	Pr; shallow; fine firm gray sand	S	L	*Fucus vesiculosus* (allochthonous) *Chorda filum* (autochthonous) *Potamogeton perfoliatus* *Ranunculus obtusiflorus* *Myriophyllum spicatum* *Chara tomentosa* *Phragmites communis*	*Cardium edule* (large), *Macoma* (all sizes) *Mytilus edulis* (small), *Mya* (small) *Lymnaea ovata*, *Theodoxus fluviatilis* *Corophium volutator*, *Pontoporeia affinis* *Mesidotea entomon*, *Gammarus* sp. Chironomid larvae, *Dendrocoelum*, etc.
Central parts		Pr; depth 2–3 meters; mud, gyttja, plant remains	C	M and Sm		
Syndalsnäs	6.01	SP; medium sand	P	M	*Phragmites* (otherwise as at Henriksberg	As at Henriksberg
Shore south of Lappvik	5.43	E; coarse sand	C	M	As at Henriksberg but more sparse	*Mya* (large), *Macoma* (small and scarce) *Bathyporeia pilosa*, etc.
Behind point at Lappvik	4.74	SP; medium sand, slightly muddy	C	M and L	Sparse algal mat *Potamogeton*, *Phragmites*, etc.	As at Henriksberg

TABLE I—*Continued*

Collecting site (see Fig. 1)	Surface salinity ‰	Exposure and substratum	*Nereis diversicolor*		Associated flora (conspicuous elements only)	Associated fauna (conspicuous elements only)
			Abundance	Size		
Small bay on Ekö	5.79	Pr; muddy sand below *Fucus*	S	M	Autochthonous *Fucus*, other algae *Phragmites*	*Macoma*, *Mesidotea*, *Lymnaea stagnalis*, etc.
Below Skogby	4.14 (4.09–4.18)	SP; fine muddy gray sand	S	M	Some autochthonous *Fucus* *Phragmites*, etc.	*Macoma* (large and abundant) *Mya* (medium), etc.
Vitsand	4.36 (4.00–4.72) (5 m) 4.81 (10 m) 6.08	SP; fine sand and clay in shallows; mud, coarse sand and debris at 3–5 meters	R; 1 at 4–5 meters	M	*Phragmites* Allochthonous *Fucus*	*Macoma*, and others as in Krogarvik, but restricted to depth below 3 meters
Källvik passage	3.41 (3.10–3.56)	SP; as at Vitsand	Absent		*Phragmites* Allochthonous *Fucus*	In channel, below 5 meters: *Mytilus* (large and abundant), *Mya* and *Cardium* (medium) *Membranipora crustulenta* and *Balanus improvisus* (large and abundant, on *Fucus* and *Mytilus*) Rest of "marine" fauna as in Krogarvik
Southern end of Stadsfjärd	3.42 (2.97–3.87) (5 m) 5.72	SP; as at Vitsand	Absent		*Phragmites*, *Potamogeton*, and other freshwater plants in shallows Allochthonous *Fucus* below 3 meters	In shallows: *Bithynia tentaculata*, *Theodoxus* *Asellus aquaticus*, chironomid larvae, etc. Below 3 meters: *Balanus* and *Membranipora* (common) *Cardium* (small, 1.2 cm.; free in coarse sand) *Macoma* (all sizes, abundant) *Pontoporeia affinis* (abundant), *Mesidotea* (large and breeding) (*Mya* and *Mytilus* absent)
Ekenäs (Tammisaari)	3.08 (2.66–3.49)	SP; fine gray sand, slightly muddy	Absent (no dredging at this station)		*Enteromorpha* Filamentous green algae	*Condylophora lacustris* (on pilings); *Macoma* at deeper levels (*Mesidotea* beyond this point at deeper levels as breeding resident of Pojovik)

often possible to locate worms by observing the burrow openings; digging at such spots commonly yielded worms where random digging and sifting had failed to reveal a single individual in an hour's time. Hence calm weather permitting a clear view of the bottom was considered a requisite of good collecting conditions on sandy shores fairly free of macroscopic algae, but was of less value in protected bays where algal growth often covered the bottom. Dredging was carried out from a skiff or motorboat, employing a light triangular ring-dredge whose line was weighted with chain to encourage the dredge to bite into the substratum. This dredge would secure a good sample of soft mud and/or coarse sand but proved useless on the type of fine, hard, sandy bottom (as at the head of the Krogarvik) where spading showed the presence of scattered to common very large worms, rather deeply buried.

Salinity determinations have been made as in the previous study (Smith, 1955) by determining chloride in grams per liter on duplicate one-ml. samples. Because all previous reports on the Tvärminne area have used salinity rather than chlorinity, my results in the present work have been expressed as approximate salinity in parts per mille, calculated by multiplying my chloride (essentially chlorosity) values by 1.81. The results are thus not exactly what would have been obtained by routine hydrographic methods, but are close enough for an ecological account.

The field observations are summarized in Table I and the distribution of *N. diversicolor* and of mean surface salinities (deep salinities in table) shown in Figure 1. It may be seen that in all suitable localities from Henriksberg on the outer shore to the vicinity of Lappvik, worms are fairly common in shallow water, but disappear from the shore somewhere above Skogby. At Vitsand *N. diversicolor* could not be found in shallow water despite the presence of apparently suitable bottom, but a single individual was taken by repeated dredging at a depth of 4–5 meters. The species thus reaches its limit outside the constricted Källvik strait, at a point which, incidentally, is also the limit of autochthonous (attached) *Fucus vesiculosus* (Luther, 1951a). No other reduction in the marine fauna has been noted in the deeper water at this point, and it is significant that at a depth of 10 meters the salinity is still as high as at the surface on the outer shore. In the narrow Källvik strait a vigorous marine fauna, except for *Nereis*, also exists. Within the Stadsfjärd we see a drastic reduction of this fauna, with the dropping out of *Mya* and *Mytilus* and the reduction of *Cardium*, as well as the appearance of a number of fresh water forms.

Discussion

A study of Table I reveals several facts, some of which when viewed in the light of other findings are not a little puzzling:

1. In typical tidal estuaries, marine animals generally tend to occupy an upper intertidal level as they extend upstream into less saline waters. While this may be attributable to differences in the position of suitable substratum (as pointed out by Spooner and Moore, 1940, for mud-dwellers in the River Tamar), it has the advantage that the higher salinities of the flood tide are received and the lower salinities of the ebb avoided. But here in the tideless Baltic, the distribution of the worms (which are of course always "subtidal") follows the zone of favorable salinity to a lower level as fresher waters are approached. The salinities actually

encountered by these worms in summer are thus not so low as the surface salinities over the range might imply.

2. It would appear that *N. diversicolor* in the Tvärminne area does not normally have to endure summer salinities below about 4°/₀₀. When we consider that worms of this species living in estuaries have a range extending from salinities of 22°/₀₀ or more down to salinities below 1°/₀₀ (Percival, 1929), and when we further consider the fact observed in the experimental part of this study that worms from any part of the Tvärminne area can at summer temperatures tolerate soft fresh water (chloride about 0.02°/₀₀), it is surprising indeed that *N. diversicolor* is limited in the field at a salinity as high as 4°/₀₀. The obvious conclusion is that the species is not being limited by the salinities prevailing in summer near Tvärminne, but if salinity is a limiting factor at all, it must be operating at some other time of year, most probably during the spring reduction of salinity associated with melting snow. Two possible ways in which the species might be affected are (1) by inability of the adults to perform the necessary osmotic regulation when temperatures are near 0° C. or (2) by a failure in some aspect of reproduction and/or dispersal. The first alternative would imply that *N. diversicolor* could not survive the winter or spring even if it had become established in the Stadsfjärd in the previous summer. We have no specific information on which to base further discussion of this possibility. The second alternative implies that *N. diversicolor,* even if capable of surviving in the Stadsfjärd as an individual, would fail to reproduce itself there, and that larvae produced elsewhere are unable to enter to replenish the population. The available information on the breeding habits of *N. diversicolor* (summarized by Dales, 1950) indicates that in most localities it is a winter or early spring spawner. Although the breeding season at Tvärminne is not yet known, it would appear to have been completed prior to July in 1954. In examining and taking coelomic fluid samples from over 100 worms in the laboratory during the present work, besides handling a much greater number in the field, not a single male has been identified. Female worms, greenish and with obviously over-ripe eggs, were found on several occasions, and worms with small undeveloped oöcytes were numerous, probably representing the next year's breeding stock. Small worms (under 15 mm. in length) were common in collections, and many more undoubtedly escaped through the sieves used. The smallest nereid seen (collected by Dr. T. G. Karling at Henriksberg on August 4) measured 2 mm. in length and had 15 setigerous somites. According to the rates of growth reported by Dales (1950) at unspecified temperatures, this worm was probably 6–8 weeks old. Spawning must then have been essentially completed before the summer months, but whether it fell in or after the spring period of low salinity could only have been determined by field studies at that time.

3. Although in British estuaries *N. diversicolor* penetrates further into waters of low salinity than any other "marine" types except for certain crustaceans which do not enter the present discussion (Percival, 1929; "Plymouth Marine Fauna," 1931; Spooner and Moore, 1940; Bassindale, 1943b), this species in the Tvärminne area is one of the first elements of the characteristic brackish-water faunal assemblage to drop out. To be sure, certain marine types such as the amphipods *Pontoporeia femorata, Calliopius laeviusculus,* and *Gammarus locusta,* as well as the isopod *Idothea baltica,* the priapulid *Halicryptus,* the polychaete *Harmothoë sarsi,* and others, seem to be restricted to the highest salinities found about Tvär-

minne. These have been poorly or not at all represented in my collections, and will not be included in the following discussion. Leaving them aside, we see from Table I that *N. diversicolor* does not penetrate as far into lower salinities as do the rest of the animals commonly associated with it. To an observer whose experience has been gained outside the Baltic this fact seems paradoxical, but it would appear that the situation in the shoreward gradient of decreasing salinity from Tvärminne to Ekenäs reflects a condition characteristic of the Baltic as a whole and not unusual in terms of this brackish sea. Thus the ranges shown by Välikangas (1933) indicate that in the gradients of decreasing salinity of both the Gulf of Finland and Gulf of Bothnia, *N. diversicolor* stops short of such forms as *Mytilus, Macoma, Membranipora, Corophium volutator,* etc. Other information indicates that *Mya* extends further into the Gulf of Bothnia than does *Nereis* (Segerstråle in litt.), and *Cardium* further east in the Gulf of Finland (Segerstråle, 1933b). One is thus encouraged to hope that an analysis of the factors limiting *N. diversicolor* in the local salinity gradient shoreward from Tvärminne may shed light upon the more general problem of its distribution in the Baltic Sea. And the closer we can come to understanding why the relative distribution of *Nereis* and its associates in the Baltic is so different from that seen in typical estuaries, the closer are we to understanding the effects of these different environments upon the animals which are able to inhabit them, and the nature of physiological adaptation to such environments.

4. With reference to Table I, the following facts and comments are offered: It may be significant that of the associates of *N. diversicolor* which do penetrate the Stadsfjärd, at least two, *Mesidotea entomon* and *Pontoporeia affinis,* are glacial relicts which are also found in fresh water lakes (Segerstråle, 1955). *Membranipora crustulenta* likewise extends into very low salinities in the heads of the Gulfs of Finland and Bothnia (Välikangas, 1933) and so would not be encountering severe conditions in the Stadsfjärd. Of the properly marine species, *Macoma baltica* is a summer breeder (Segerstråle, 1951b) and would be reproducing when salinity in the Stadsfjärd is maximal. A further point to notice is that *Cardium, Balanus improvisus,* and *Membranipora,* even if unable to reproduce within the Stadsfjärd (a point which has not been confirmed or denied), might have entered it as planktonic larvae at a time of saltwater ingression following the spring outflow; Levander (1915) has recorded larvae of the latter two forms within the Stadsfjärd in summer (at his station VIII). It is also possible that these three species could have been carried in as post-larval stages on drifting *Fucus. Membranipora* in the Stadsfjärd is generally on this plant; *Balanus* is commonly so found. One large individual of *B. improvisus* has been found on a clean pebble weighing 27 grams, suggesting settlement *in situ,* but the possibility that the stone was carried in by *Fucus* at some previous time cannot be entirely ruled out. Likewise, very small *Cardium* are abundant, byssally attached to *Fucus* outside the Stadsfjärd, and could readily drop off after drifting in. But in the case of *N. diversicolor,* the possible planktonic stage is at most a brief one (Dales, 1950), and attachment to allochthonous *Fucus* does not seem a probable means of transport. Although Thorson (1946) reports larvae presumed to be of *N. diversicolor* in the plankton of the Danish Øresund from late March to July–August (with maximum abundance in April and May), there are no records of nereid larvae in

the plankton about Tvärminne. In response to a question on this point addressed to Drs. Purasjoki and Segerstråle, the latter has replied (in litt.) "We are of the opinion that there is rather convincing evidence of the worm not having a planktonic stage in our waters. No such larva has been found in the numberless plankton samples taken since decades, including the cold season." We may further note that a nereid larva entirely lacks a protective shell, and may for this reason be less resistant than larvae of *Balanus* or bivalve molluscs. If conditions in and above the Källvik strait are such as to prevent the reproduction of *N. diversicolor* or the survival of its early larvae, and if the species with its brief and benthic larval life lacks the means of colonizing this region beyond its actual breeding range, then we may consider that the distribution of the species is limited not by one, but by the interaction of several factors. More specifically, the factors which limit the spread of *N. diversicolor* (while permitting the advance of its marine associates) are not restricted to the physiological tolerances of the worm and its larvae, but must include physical factors of hydrographic conditions which may not be favorable to dispersal at the time larvae are produced. The data of Halme and Kaartotie (1946) suggest that the narrow Källvik passage is subjected to greater spring disturbances of salinity at its deeper levels than is any other part of the area studied. If we consider the 5-meter depth as a level likely to be critical for *N. diversicolor* (which has not been reported deeper than 6–8 meters), the records of Halme and Kaartotie show that in 1936–37 the Tvärminne Storfjärd had an annual salinity variation of 1.25‰ (6.45–5.20), the Pojovik varied by 1.35‰ (2.30–0.95), while in the Källvik passage the variation recorded was 2.30‰ (5.25–2.95). This indicates a deep penetration of oligohaline water during the spring discharge, and suggests that the constricted Källvik strait represents a rather abrupt discontinuity in the local inshore salinity gradient. It is thus a hydrographic barrier which may affect the distribution of *N. diversicolor* more adversely than it does that of the associated marine species. If, as seems likely, *N. diversicolor* produces its larvae during the spring lowering of salinity, when there is still a large net outflow past Källvik, there is a good chance that even if the weakly-swimming larvae were injected into the plankton by turbulence, they would either be killed by low salinity or swept seaward. Other marine species with a later breeding season or a longer planktonic existence might be able to pass into the Stadsfjärd when the hydrographic barrier is minimal during the summer. It would be interesting to know if a more gradual salinity gradient leading into some other Baltic river system might not permit *N. diversicolor* to extend its range into salinities as low as it seems to tolerate in estuaries elsewhere. If such a river can be found, it might provide a test of the ideas just expressed.

Conditions in the Krogarvik, the shallow bay close by the Zoological Station, might also prove very illuminating in respect to the possibility that *N. diversicolor* is either limited by inability to regulate osmotically during periods of minimal salinity combined with low temperature in spring, or that its reproduction or larval survival are hindered. Unfortunately, the determinations of salinity at the bottom of the Krogarvik obtained by Segerstråle in the years 1929–32 and 1945–50 have not been continued until 1954. *N. diversicolor* was common in this bay in the summer of 1954, and the largest individuals, which had certainly over-wintered, were found near the head of the bay in water so shallow that they must have been

close beneath the ice (unless they had migrated from deeper levels, an undemonstrated possibility). It is perhaps significant that the winter of 1953–54 was unusually free of snow, and it may be that the Krogarvik experienced little reduction of salinity at the spring thaw. I am informed by Professor Palmgren that the taking of salinity samples at the bottom of the Krogarvik will be resumed, and if the winter results can be correlated with the abundance of *N. diversicolor* in each following summer, it may be possible to determine from this natural experiment what salinities can be tolerated by these worms at winter temperatures. Studies of osmoregulatory ability under refrigeration in summer might be helpful, but probably not conclusive because of the ability of some animals to adjust their metabolism to seasonal changes in temperature; studies made in winter would be more informative.

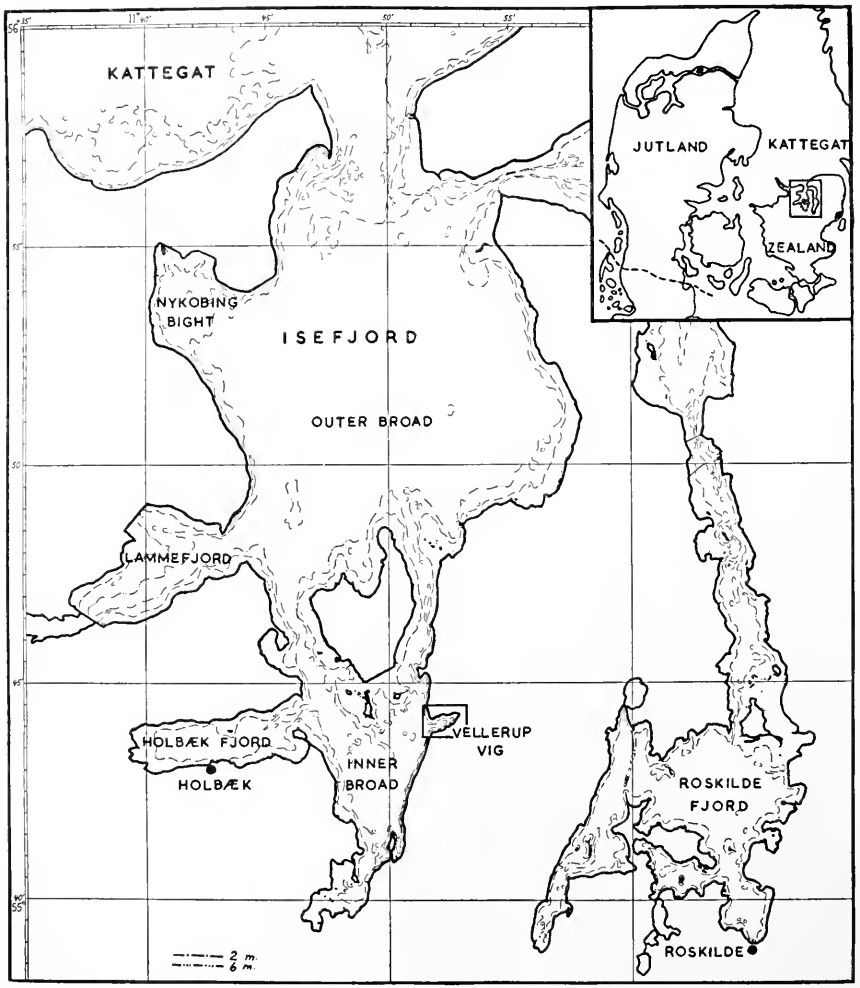

FIGURE 2. Map of the Isefjord, Denmark.

Part II. Observations in the Isefjord, Denmark

General description of the area

In a geographical sense the Isefjord (Fig. 2), in the northern part of the island of Zealand to the west of Copenhagen, is intermediate between British waters and the Baltic, while its mean surface salinity of 20‰ is midway between salinities at Tvärminne and Plymouth. The hydrography of the Isefjord has been summarized by Nielsen (1951). His account shows that this body of water is characterized by a stability of salinity greater than that of the south Finnish coast. The Isefjord has an area of 280 square kilometers and is so shallow (mostly less

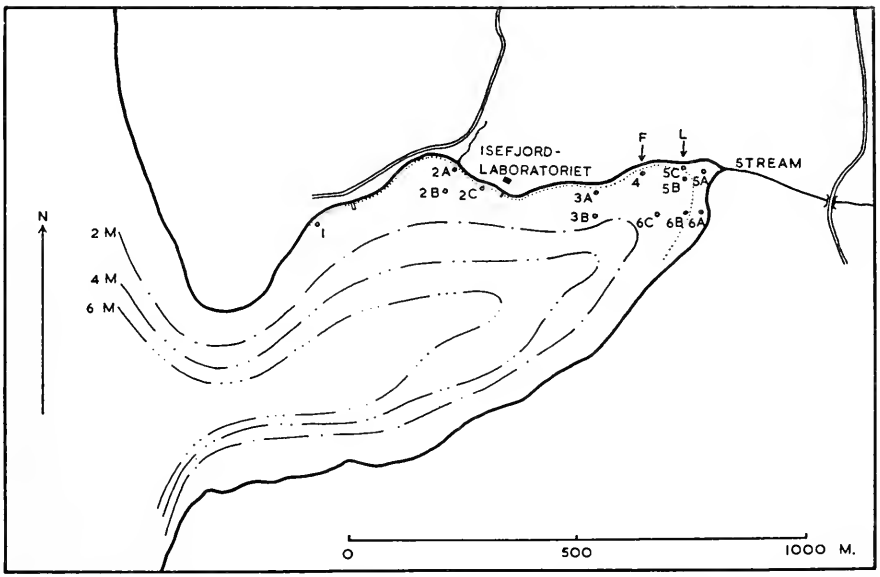

Figure 3. Vellerup Vig, showing sampling stations. Details in Table II. Arrows marked F and L show limits of *Fucus* and *Littorina* on the shore.

than 10 meters) that despite the presence of a sill at its mouth, salinity stratification rarely occurs. According to Nielsen's observations, mean surface salinity in the Inner Broad (southern part of the fjord) is 20.18‰, with extremes noted of 17.79 and 22.23‰, while at depths of 4–6 meters the corresponding values are 20.54‰ (19.03–23.88). The Isefjord is thus polyhaline in the sense of Välikangas (1933). In comparison with conditions near Tvärminne it is notable that the lowest salinity of 17.79‰ was obtained in March, immediately beneath the ice, and that the difference in salinity between surface and 5 meters at that time was only 4.74‰. The Isefjord freezes easily and may have an ice cover for three months in cold winters, although there is evidently not such a marked seasonal lowering of salinity beneath the ice as is experienced in Finnish waters. The reason for this is doubtless the slight fresh water drainage into the Isefjord, which has a very small watershed (800 sq. km.) and receives only small streams. So low is the fresh water input that the Inner Broad, the head of the fjord, actually has a mean salinity 0.5‰ *higher* than the Outer Broad which lies nearer the

TABLE II

Field observations, Vellerup Vig: salinities at low and high water at sampling stations (see Fig. 3) densities of Nereis diversicolor and N. southerni (approx. number per $\frac{1}{8}$ sq. meter), and principal associated biota

Key to symbols

F—*Fucus vesiculosus* (autochthonus)
L—*Littorina littorea* (on upper midtidal rocks)
Mt—*Mytilus edulis*
M —*Mya arenaria*
C —*Cardium edule*

Station (see Fig. 3)	Character of station	Salinities ‰		Worm density		Associates
		L. W.	H. W.	*N. div.*	*N. south.*	
1	At L. W. M.; muddy sand	18.40	—	6	1	F L Mt — —
2A	Subtidal, by stream mouth; sand and clay	1.63	17.58	83	4	F L Mt — —
2B	50 meters out; muddy sand, near rocks	15.41	18.40	2	33	F L Mt C —
2C	Subtidal, away from stream; sand and clay	—	—	3	22	F L Mt — —
3A	Subtidal; muddy sand	10.96	13.67	3	41	F L Mt — —
3B	50 meters out; fine sand	12.33	17.21	0	19	— — — M C
4	Subtidal, at limit of *Fucus;* sand	7.73	9.59	75	2	F L Mt — —
5A	Intertidal, under flowing fresh water at low tide; gravelly	0.03	0.62	6	0	— — — — —
5B	Subtidal, at foot of fresh water outflow; gravelly	0.29	0.83	39	0	— — — — —
5C	Intertidal; muddy sand, at limit of *Littorina* on rocks	1.25	4.13	76	0	— L — — —
6A	Intertidal; muddy sand at head of bay	2.32	9.37	134	0	— — — — —
6B	Subtidal; muddy sand	1.88	—	47	1	— — — M —
6C	100 meters from H. W. M.; fine sand	4.05	9.52	5	16	— — — M C

Kattegat. Tides are small, about 20 cm. in mean range, but massive ingression or expulsion of water during gales commonly causes deviations in water level of ± one meter. The Isefjord is thus an area of moderately reduced but exceptionally stable salinity, and it is of interest to learn where *N. diversicolor* finds its optimum within it.

Field observations

The present study has been restricted to the head and northern shore of Vellerup Vig, a small bay on the eastern shore of the Inner Broad (Fig. 3). The

intertidal shore is mostly narrow, rather steeply sloped, varying from muddy sand to coarse gravel, covered with stones in more exposed places, and interrupted by extensive patches of reeds (*Phragmites*). A soft flat muddy shore about 25 meters wide is exposed by low tides at the head of the bay. Below low-water mark the bottom is nearly flat, of fine muddy sand with some clay and scattered large boulders which support *Fucus vesiculosus*, *Mytilus edulis*, and *Littorina littorea*, except in the less saline head of the bay. *N. diversicolor* occurs quite generally along the midtidal shore beneath stones resting on muddy sand, but is not abundant sub-tidally except in certain areas which are clearly those freshened by inflowing streams. A good-sized stream enters the bay at its head and a small one just to the west of the Isefjord Laboratory.

In making collections, most worms had to be dug under water, hence direct sampling of quadrats was not feasible, and the method was adopted of filling a cubical box 25 cm. in each dimension by several spadefuls of substratum taken

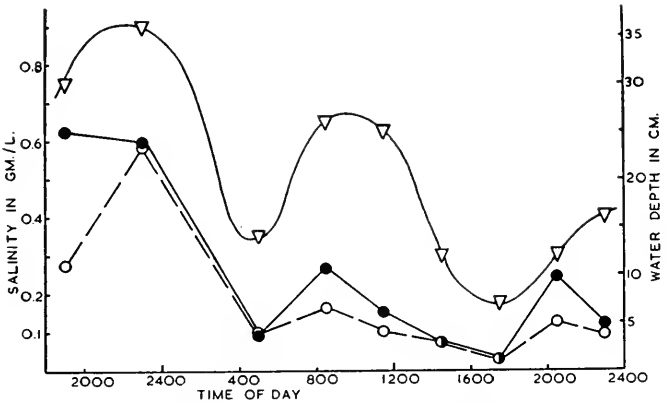

FIGURE 4. Variation in water level and salinity over fringe of population of *N. diversicolor* at station 5A (see Fig. 3) at stream mouth, Vellerup Vig, August 28–29, 1954. Triangles, depth of water in cm.; black circles, salinity at bottom; open circles, salinity at surface.

within an area a few feet across at each station. On the assumption that the digging was effective to about half the depth of the box, the full box would represent an area sampled of ⅛ square meter. The material obtained was sifted through a pair of sieves of ¼- and ⅛-inch mesh. Most of the smallest worms were lost, but the counts obtained are comparable among themselves. Figure 3 shows the positions of collecting stations, and the results of the field observations are summarized in Table II. It is evident that *N. diversicolor* is highly concentrated in the head of the bay and near the mouth of the small stream near the laboratory.

A second nereid, which has been shown to be less tolerant of reduced salinity (Jørgensen and Dales, in press), is also abundant in Vellerup Vig and is being described as a new species, *Nereis southerni*, by Abdel-Moez and Humphries (in press). The populations of *N. southerni* and *N. diversicolor* overlap only very slightly. I am informed by Dr. Erik Rasmussen that *N. southerni* has been increasing in abundance in Vellerup Vig during the past few years, and that it

seems to have replaced *N. diversicolor* in areas where the latter used to be of regular occurrence. This fact forces one to exercise caution in seeking to establish the salinity optimum of *N. diversicolor*. The apparent optimum shown in the present studies can only be a restriction of the species to that part of its potential range where it escapes critical interspecific competition. The same problem has been encountered in the case of *N. diversicolor* at Millport, Scotland (Smith, 1955). It is, nevertheless, clear that *N. diversicolor* at Vellerup exhibits a marked tolerance for water of very low salinity, even extending its distribution into areas exposed to outflowing fresh-water at low tide.

At the mouth of the stream at the head of the bay *N. diversicolor* was found in reduced numbers, but still commonly, living intertidally beneath flowing fresh-water for part of each tidal cycle. In order to gain a rough picture of the salinities endured by this vigorous fringe of the population at the extreme of its habitat, water-levels and salinities were measured at intervals over a 27-hour period, and the results are shown in Figure 4. Although no great regularity is revealed, it is evident that these worms can live beneath salinities of less than 1‰ (0.65–0.03‰) for at least a day at a time. It should be noted that the summer of 1954 prior to these observations had been the rainiest experienced in many decades, so that salinities at this spot had probably been even lower than those recorded. On the other hand, it is likely that an occasional high stand of water caused by wind may have provided a temporary respite in the form of a saline wedge forced in beneath the outflowing fresh-water at this point. Figure 4 indicates some salinity stratification near the stream mouth.

Discussion

N. diversicolor collected from the head of Vellerup Vig has been used by the writer in studies of chloride regulation and also by Jørgensen and Dales (in press). Worms from this spot are quite capable of living in fresh water in the laboratory, and apparently some of them endure it for a greater or less time in nature. In my studies of the level of chloride regulation after adaptation to low salinities, I have found no difference between *N. diversicolor* from Finland and from Denmark. This finding of similar regulatory ability is in marked contrast to the distribution of the species in the respective localities. In the Finnish area of prevailing low salinity we find *N. diversicolor* restricted to the more saline part of the potentially available range, even less able to invade oligohaline waters than *Mytilus, Cardium, Mya, Balanus improvisus*, and others. But in the Isefjord, where prevailing salinities are three times higher, *N. diversicolor* is strongly concentrated in the least saline portions of the available range, into which it advances further than any of its marine associates, thus behaving quite like its relatives in British estuaries and unlike those of the inner Baltic Sea.

The factors which determine optimal conditions for a species are complex, and the distribution of *N. diversicolor* in relation to salinity has not, by this study, been reduced to any simpler terms. We have seen that the relative position of the species in the regional salinity gradient is quite different in the Baltic Sea than it is elsewhere, but it is quite possible that the differences in distribution are caused by special hydrographic conditions (in particular, the spring reduction of salinity characteristic of the inner Baltic) rather than by any fundamental or

"racial" differences between the worms themselves. The gradient between the polyhaline waters of the Isefjord and the oligohaline conditions near stream mouths is interrupted by no such zone of hydrographic instabliity as the Källvik Strait imposes between the mesohaline and oligohaline waters of the Tvärminne region. It is possibly quite significant that the most unfavorable hydrographic conditions both in the Tvärminne area and in the Baltic as a whole occur at or near the probable time of reproduction of *N. diversicolor*. We have also noted that although the Isefjord as a whole has a mean salinity of about 20‰, which lies within the optimum salinity range of *N. diversicolor* as seen at Millport (11–22‰), the Vellerup population finds its apparent optimum at a much lower absolute salinity. But in both instances the apparent salinity optima may be in part the result of interspecific competition. The studies at Tvärminne and Vellerup, despite their limited scope, emphasize the fact that an ecological optimum must be related to the total life history and physiological capabilities of the species concerned.

The travel involved in this series of studies was initially made possible by a Fulbright grant to the writer as an exchange lecturer in the Zoology Department, University of Glasgow. I am indebted to the U. S. Educational Commission in the United Kingdom and to Prof. C. M. Yonge and others at Glasgow who facilitated arrangements for the European trip. My stay at the Zoological Station, Tvärminne, Finland was made especially pleasant by countless kind and helpful efforts on my behalf by Professor Pontus Palmgren (Director), and by Dr. Ernst Palmén. I am indebted to Dr. Sven G. Segerstråle for much information, not all of which has been specifically acknowledged, as well as for his critical reading of the manuscript of this paper and the correction of a number of errors in it. Dr. Palmén, Dr. Vilho Perttunen, and Mr. Kari Lagerspetz shared the labor of dredging, and other staff members and students whom space prevents listing helped in many ways. In Denmark, Dr. Erik Rasmussen most kindly made available the facilities of the Isefjord Laboratory at Vellerup, and aided in collecting as well as with information about the locality. Salinity determinations were performed in the laboratory of Dr. C. Barker Jørgensen, Zoophysiological Laboratory, University of Copenhagen. To those mentioned and to others in Denmark who helped me I express my thanks.

Summary

1. A comparative field study of the distribution of *Nereis diversicolor* near Tvärminne, south Finland and in the Isefjord, Denmark shows that although the species penetrates into nearly fresh water in the Isefjord, its distribution near Tvärminne is limited at summer salinities of over 4‰.

2. The apparent salinity optimum of *N. diversicolor* on the border of the polyhaline Isefjord is lower than on the "marine-dominated" beach at Millport, Scotland, but in both instances there may be a restriction of the potential range because of interspecific competition.

3. The paradoxical situation is seen near Tvärminne of *N. diversicolor* being among the first of the characteristic brackish-water fauna to drop out as salinity

lessens, whereas in the Isefjord as well as in British estuarine situations it penetrates further toward fresh water than the rest of its associates.

4. It is suggested, on the basis of hydrographic conditions in the Tvärminne area, that salinities prevailing in summer cannot be the limiting factor for *N. diversicolor* in the Baltic Sea, but that critically low salinities occurring in spring while temperatures are still very low may adversely affect the osmoregulation and/or reproduction of the species. Furthermore, there may be a hydrographic (as well as a purely physiological) barrier to the spread of the species into oligohaline waters, in which connection the time of breeding and the lack of a long planktonic stage in the life history may be important.

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STUDIES ON SHELL FORMATION. IV. THE RESPIRATORY METABOLISM OF THE OYSTER MANTLE[1, 2]

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One of the major problems in the study of shell formation is the relation of respiratory pathways to the deposition of the $CaCO_3$ and the organic matrix. In addition to the participation of respiration in the synthesis of organic constituents, respiration may also be involved in the transport of organic and inorganic substances through the cells of the mantle tissue to the site of shell deposition. Further, CO_2 from respiratory intermediates may provide a source of shell carbonate. Since the respiratory pathways of the shell-forming tissues of mollusks are unknown, the first requirement, and one of the objectives of this series of studies, is their identification. The present study concerns oxidative phases, and includes assays of oxidative enzymes and the effects of various substrates and respiratory inhibitors on oxygen consumption. Decarboxylation reactions will be the subject of another report.

It has long been recognized that the mantle is intimately concerned in processes of shell formation. Recently Hirata (1953) demonstrated that the isolated mantle of the oyster has the capacity to deposit both the organic and inorganic portions of the shell, and that this capacity is maintained for a relatively long period after isolation. The fact that the mantle has within itself the mechanisms of shell formation means that *initial* studies of the respiratory reactions can be confined to this structure. Once the respiratory activities of the mantle are established, one will then be in a position to relate these to the deposition of shell substances.

Methods

The earlier portions of the respiratory studies were carried out on oysters from East Bay, Pensacola, Florida, during the period February to May, 1952, and continued on oysters from Core Creek, Beaufort, N. C., during July to November, 1952. Specimens of *Crassostrea virginica* (formerly *Ostrea virginica*), 8.0 cm. to 11.5 cm. in length (average 9.6 cm.), were collected in deep water and maintained

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below low tide level at the laboratory. Temperature and salinity ranges were as follows: Beaufort: temperature, 17.2°–31.0°; salinity 24.6–35.2; Pensacola: temperature, 10.0°–27.5°; salinity, 18.8–27.0. Enzyme assays were performed on Beaufort oysters from Core Creek and North River during May to July, 1953. Since the salinity near the laboratory where the oysters were maintained was higher than at the collecting sites, all oysters were held from 10 to 14 days before being used. During this period the temperature range was 20°–30° and the salinity range was 27.6–34.1.

Respiration measurements

Immediately after taking the specimens from the water the mantles were removed; cut into strips; drained for 10 seconds with one end touching filter paper; and weighed on a torsion balance. Respiratory measurements were carried out using Warburg's direct method on single strips of mantle weighing 150–220 mg. in 5-ml. flasks with sea water as the medium. The total fluid volume including tissue was 1.1 ml. The mantle is sufficiently thin that diffusion of oxygen is not limiting. Flasks were gassed with O_2 bubbled through sea water. All measurements were made at 25.0° except where noted. The pH of the medium was routinely measured prior to and frequently following runs. In the majority of cases the pH was not altered appreciably during the course of the respiratory measurements even with shaking for periods of 5–7 hours duration in the presence of alkali. Occasionally, however, there was a marked increase in the alkalinity from an initial pH of about 8 to 9.1–9.5. In a few cases a change of several tenths of a pH unit in the acid direction was also observed.

All substrates and inhibitors were made up in sea water. Adjustment of pH was made with HCl and NaOH. In studying the effect of a compound on respiration, O_2 consumption was measured during a 2- to 3-hour control period prior to the addition of the experimental compound from the side arm. After addition, measurements were continued for 3 to 4 hours. Oxygen consumption remained constant throughout the seven-hour period of these measurements but decreased slightly during the next few hours. The procedure recommended by Robbie (1948) was followed in using NaCN.

Enzyme assay methods

Aconitase. Spectrophotometric method of Racker (1950) using supernates from fresh homogenates. Homogenates were prepared by grinding a pooled sample of mantle tissue in a mortar followed by homogenization in 0.1 *M* K phosphate buffer, pH 7.4, in a glass homogenizer. The tissue was kept ice cold throughout. The supernatant following 10 minutes of light centrifugation was used for the enzyme assays. The increase in optical density at 240 mμ resulting from the conversion of citrate to unsaturated cis-aconitate was followed at intervals of 30 seconds. A unit of enzyme activity is the amount causing an increase in optical density of 0.001 log unit per minute at 22°–26°. Tissue activity is expressed as units of enzyme per mg. dry weight.

Isocitric dehydrogenase. Spectrophotometric method of Mehler *et al.* (1948) using an acetone powder extract as prepared by Wenner *et al.* (1952), with slight

modifications, in glycylglycine buffer, pH 7.2. The reaction is started by the addition of isocitrate, and the increase in optical density at 340 mμ resulting from the reduction of TPN is read at 15-second intervals against a control cuvette containing all components except isocitrate. Enzyme activity is expressed in units per mg. of acetone powder, one unit being the amount of enzyme required to increase the optical density 0.01 log unit per minute at 22°–26°.

Succinic dehydrogenase. Spectrophotometric method of Cooperstein, Lazarow and Kurfess (1950). Assays were carried out on homogenates prepared with 10 volumes of 0.03 *M* Na phosphate buffer, pH 7.4, followed by further dilution, giving a final concentration of 1 : 30 in the cuvette. The increase in optical density at 550 mμ resulting from the reduction of cytochrome *c* was read at 30-second intervals. Measurements were made immediately following preparation of the homogenate and before clumping of particles became marked. Enzyme activity is expressed as the decrease in the logarithm of the molar concentration of oxidized cytochrome *c* per minute for a 1 : 150 tissue dilution.

Cytochrome oxidase. Spectrophotometric method of Cooperstein and Lazarow (1951). The tissue was prepared as for succinic dehydrogenase except that the final dilution was 1 : 300. The decrease in optical density at 550 mμ resulting from oxidation of cytochrome *c* was read at 30-second intervals. Enzyme activity was calculated as the decrease in the logarithm of the molar concentration of reduced cytochrome *c* per minute for a 1 : 100 tissue dilution.

Fumarase. Spectrophotometric method of Racker (1950) using a fresh homogenate and also an acetone powder extract as prepared by Wenner *et al.* (1952). The increase in optical density at 240 mμ resulting from the formation of the unsaturated fumarate from malate at pH 7.4 provides an index of enzyme activity. The calculation of unit enzyme activity is the same as for aconitase.

Malic dehydrogenase. Spectrophotometric method of Mehler *et al.* (1948) using an acetone powder extract. The reaction was started by the addition of oxaloacetate, and the decrease in optical density at 340 mμ resulting from the oxidation of $DPNH_2$ was read at 15-second intervals. A pH of 7.2 was maintained with glycylglycine buffer. The calculation of enzyme activity is the same as for isocitric dehydrogenase.

Oxaloacetic decarboxylase. The manometric method of Vennesland *et al.* (1947) was employed using fresh homogenates at pH 4.5. Corrections were made for spontaneous breakdown of the substrate. Activity is expressed as μl. CO_2 per mg. dry wt. per hr. as calculated from CO_2.

Results

Endogenous respiration

Before considering the individual enzymes of the mantle and the effect of respiratory substrates, attention will first be given to endogenous respiration including the respiration of the different portions of the mantle. The possibility of metabolic differences between mantle areas is suggested by structural differences, differences in calcium turnover rates (Jodrey, 1953), and by differences in the rate of calcium deposition between areas of the inner shell surface (Wilbur and Jodrey, 1952). A difference in CO_2 content of mantle areas of the clam *Venus mercenaria* has also been reported (Dugal, 1939).

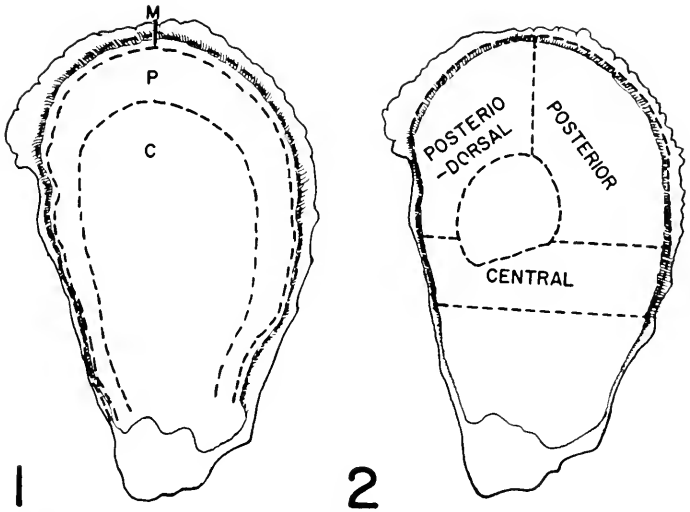

FIGURE 1. Mantle marked off to show zones used for respiratory measurements on Florida oysters. M, marginal zone; P, pallial zone; C, central zone.

FIGURE 2. Mantle marked off to show regions used for respiratory measurements.

For experimental purposes the oyster mantle may be considered to consist of three concentric zones (Fig. 1): a thin outer folded margin (M); an area attached to the shell and here termed the pallial zone (P); and a larger, thinner central zone (C). Measurements of endogenous respiration were carried out on the three zones (Table 1). The pallial zone exhibited the highest rate and the central the lowest. Differences between zones were significant at the 1% level. In subsequent experiments mantle regions (Fig. 2) including all three zones were utilized for respiratory measurements. The endogenous respiration in North Carolina oysters was the same for the three mantle regions. In Florida oysters the three regions appeared to exhibit differences in respiration, but the data, which were obtained under conditions of temperature and season quite different from the North Carolina oysters, are not sufficiently extensive to warrant discussion. In order that any such difference would not figure in the results, experimental and control tissues were always taken from corresponding mantle regions.

TABLE I

Endogenous respiration of mantle zones

Mantle zone	μl. O_2/mg. wet wt./hr.
Marginal	0.13 ± 0.03
Pallial	0.15 ± 0.03
Central	0.12 ± 0.02

Mantles were divided into three zones as shown in Figure 1 and the oxygen consumption of the parts measured simultaneously. Florida oysters. Environmental temperatures, 10°–14° C. Measurements were carried out at constant temperatures between 18° and 21°. Salinity 24–25 parts per thousand. Figures show means and standard deviations, $\sqrt{\frac{\Sigma d^2}{n-1}}$.

A further aspect of endogenous respiration deserves mention, namely, the effect of short-term starvation of isolated mantles. Right mantles with the attached shell were isolated (Hirata, 1953) and kept for periods of one to 7 days in running sea water. The endogenous respiration of the three regions shown in Figure 2

TABLE II

Respiratory enzymes in mantle

Enzyme	Mantle	Mammalian tissue		Reaction
Aconitase	none	mouse liver rat kidney rat liver	33 50 25*	$\text{citrate} \overset{\pm H_2O}{\rightleftharpoons} \text{aconitate} \overset{\pm H_2O}{\rightleftharpoons} \text{isocitrate}$
Isocitric dehydrogenase	0.36	mouse liver rat kidney rat liver	10.8 66 22*	$\text{isocitrate} + \text{TPN} \overset{+2H}{\rightleftharpoons} \text{oxalosuccinate} + \text{TPNH}_2$
Succinic dehydrogenase	0.03	rat liver	2.96	$\text{succinate} + \text{ferricytochrome c} \overset{\pm 2H}{\rightleftharpoons} \text{fumarate} + \text{ferrocytochrome c}$
Cytochrome oxidase	0.61	rat liver rat kidney	11.85 23.16	$\text{ferrocytochrome c} + 1/2\ O_2 \longrightarrow \text{ferricytochrome c} + H_2O$
Fumarase	2.0± 0.3	mouse liver rat kidney rat liver	132 62 77*	$\text{fumarate} \overset{\pm H_2O}{\rightleftharpoons} \text{malate}$
Malic dehydrogenase	3.3	mouse liver rat kidney rat liver	256 173 87*	$\text{malate} + \text{DPN} \overset{+2H}{\rightleftharpoons} \text{oxaloacetate} + \text{DPNH}_2$
Oxaloacetic decarboxylase	592± 187	mouse liver	3.2	$\text{oxaloacetate} \overset{\pm CO_2}{\rightleftharpoons} \text{pyruvate}$

Aconitase: supernatant from light centrifugation of 1:20 homogenate in 0.1 *M* K phosphate buffer, pH 7.4, 0.1 ml.; 0.05 *M* phosphate buffer, pH 7.4, 2.4 ml.; 0.03 *M* sodium citrate, 0.5 ml. *Isocitric dehydrogenase:* 0.25 *M* glycylglycine buffer, pH 7.2, 1.0 ml.; 6.6×10^{-4} *M* TPN, 0.45 ml.; 0.05 *M* $MnCl_2$, 0.05 ml.; 1:15 acetone powder extract, 1.0 ml.; 0.01 *M* sodium isocitrate, 0.5 ml. *Succinic dehydrogenase:* 0.1 *M* sodium succinate in 0.1 *M* Na phosphate buffer, pH 7.4, 0.5 ml.; 0.03 *M* NaCN in 0.17 *M* phosphate buffer, pH 7.4, 0.2 ml.; 1:10 homogenate in 0.03 *M* phosphate buffer, pH 7.4, 1.0 ml.; 100 mg.% cytochrome in 0.17 *M* phosphate buffer, pH 7.4, 1.0 ml.; water to 3.0 ml. *Cytochrome oxidase:* 60 mg.% cytochrome in 0.03 *M* phosphate buffer, pH 7.4, 3.0 ml.; 1:10 homogenate in 0.03 *M* phosphate buffer, pH 7.4, 0.1 ml. *Fumarase:* 0.5 ml. of 1:40 homogenate in double distilled water; 0.1 *M* potassium malate, 1.5 ml.; 0.1 *M* K phosphate buffer, pH 7.4, 0.75 ml.; double distilled water, 0.25 ml. *Malic dehydrogenase:* 0.25 *M* glycylglycine buffer, pH 7.2, 1.0 ml.; 1.7×10^{-3} *M* $DPNH_2$, 0.2 ml.; 1:15 acetone powder extract, 0.1 ml.; 0.01 *M* sodium oxaloacetate 1.0 ml.; water to 3.0 ml. *Oxaloacetic decarboxylase:* 0.1 *M* acetate buffer, pH 4.7, 0.25 ml.; 6.6×10^{-4} *M* TPN, 0.05 ml.; 10^{-2} *M* $MnCl_2$, 0.1 ml.; 1:10 homogenate in 0.1 *M* acetate buffer, 0.5 ml.; 7.6×10^{-2} *M* oxaloacetate, 0.1 ml. (1 mg. per flask).

Figures for mantle enzymes other than fumarase and oxaloacetate represent pooled samples of 9 or more mantles. Starred figures on mammalian tissues obtained in the present study. Other data on mammalian tissues from Wenner *et al.* (1952) except succinic dehydrogenase and cytochrome oxidase which are from Cooperstein *et al.* (1950) and Cooperstein and Lazarow (1951), respectively.

was then measured. Surprisingly, the respiration per mg. wet weight did not change significantly with the period of isolation, nor was it different from that of mantles taken directly from unstarved oysters. Such metabolic stability, especially in the absence of added substrate, is indeed remarkable.

Enzyme assays

Table II presents the results of the assays of mantle enzymes, together with values for the same enzymes in certain mouse and rat tissues for comparison. Isocitric, succinic, and malic dehydrogenases were present and confirm earlier unpublished experiments on succinic and malic dehydrogenases carried out in collaboration with Dr. Henry Kritzler and Mr. Arthur A. Hirata using the Thunberg technic. Fumarase was found in fresh homogenates but not in acetone preparations. Assays of freshly prepared homogenates gave no indication of aconitase activity.

Close comparisons between the *in vivo* activity of the mantle and mammalian enzymes (Table II) are not justified even though the same assay methods were used for both. Under the conditions of assay, however, tricarboxylic cycle enzymes in mantle have a considerably lower activity than in the mammalian tissues.

TABLE III

Effect of substrates on mantle respiration

Substrate	0.005 *M*	0.01 *M*	0.025 *M*	0.05 *M*
Isocitrate	——	26±8	——	——
Citrate	−1±5	2±4	——	——
Succinate	10±5	15±9	18±8	26±3
Malate	13±5	20±5	16±7	——
Oxaloacetate	13±8	——	20±7	22±5
Pyruvate	8±10	3±7	——	——

Figures show mean percentage change in oxygen consumption and standard deviation following the addition of the substrates to mantle.

On the other hand, this is not the case for oxaloacetic decarboxylase. This enzyme had an activity in the mantle which is two orders of magnitude higher than that of mouse liver. This finding may be of special significance for the mantle tissue which is concerned with processes of carbonate deposition. (See paper by Wilbur and Jodrey which follows the present paper.)

Substrate effects

Several substrates of the tricarboxylic acid cycle have been added to pieces of mantle (Fig. 2) and their effects on oxygen consumption measured (Table III). Isocitrate, succinate, malate, and oxaloacetate stimulated respiration, the effect becoming greater as the concentration was increased above 0.005 *M*. The maximal stimulation amounted to 20–26%. Oxidative enzymes for these four substrates were demonstrated in the previous section (Table II). Citrate was without effect, and this result is correlated with the failure to demonstrate aconitase. In the absence of aconitase citrate would not be converted to isocitrate which can

be oxidized by the mantle. Pyruvate also did not alter oxygen consumption appreciably. The possible "sparking" action of acetate was not studied.

The question of penetration of the substrate arises in cases of the negative results with pyruvate and especially with citrate which would form calcium and magnesium salts in the sea water medium.[4] Ronkin (1950) found that the effect of respiratory inhibitors on the gill of the mussel *Mytilus* was increased by decreasing the pH of the medium, presumably from increased penetration of the undissociated molecule. The effect of pH was examined with succinate, pyruvate, and citrate (Table IV). Pyruvate and citrate exhibited no significant effect on the mean respiration over the range pH 6.0–8.4. However, certain mantles were stimulated and others were inhibited as indicated by the standard deviation values. The stimulating action of succinate remained constant between pH 6.6 and 8.4 and was abolished below pH 6.6, although the level of endogenous respiration was

TABLE IV

Effect of pH on mantle respiration

Substrate	pH				
	6.0–6.2	6.3–6.5	6.6–7.4	7.5–7.9	8.0–8.4
Succinate, 0.05 *M*	2.1 ±11(6)	0.1 ±6.8(4)	24 ±11(6)	26 ±7.5(12)	27 ±9.2(7)
Pyruvate, 0.01 *M*	−6.2 ±5.7(2)	——	−2.2 ±11(2)	——	2.5 ±10(2)
Citrate, 0.01 *M*	2.1 ±6.1(11)	——	1.5 ±12(5)	——	1.6 ±20(5)
Sea water	0.15	0.20	0.21	0.18	0.17

Oxygen consumption was first measured for 2–3 hours in sea water, the pH of which had been altered by the addition of HCl or NaOH. Substrates made up in sea water of the same pH were then tipped in and the measurements of oxygen consumption were continued for several hours. The pH was again measured at the end of the experiment. The mean percentage change and standard deviation of the respiratory rate resulting from the addition of substrate are given. O_2 consumption in μl/mg. wet wt./hr. Figures in parenthesis show the number of cases. At pH 6 the calculated dissociation is 74% for succinate and 80% for citrate. Data were not available for a calculation for pyruvate.

maintained very nearly at the normal level to a pH of 6.0–6.2. This latter result poses an interesting problem in that one might expect endogenous respiration to be depressed to a greater degree if succinate is in the respiratory cycle.

We have mentioned that isolated mantles can be maintained in good condition for several days without added substrate. Nine such mantles isolated for 2 to 7 days have been compared with 7 freshly dissected mantles with respect to their respiration in the presence of 0.05 *M* succinate. The mean respiration (μl O_2/mg. wet wt./hr.) was 25 and 23, respectively ($P < 50$), demonstrating that both groups respond similarly. This result is not especially surprising in view of the unchanged endogenous respiration.

Respiratory inhibitors

In the two previous sections the presence of succinic dehydrogenase and cytochrome oxidase in mantle has been demonstrated. One can therefore expect that compounds known to inhibit the succinoxidase system will affect mantle respira-

[4] However, isocitrate, which would be expected to behave similarly, apparently penetrates.

tion. The action of such enzyme inhibitors and the copper-complexing agent diethyldithiocarbamate has been examined and the results are presented in the following sections.

Sodium cyanide. Endogenous O_2 consumption of mantle in the presence of 2×10^{-4} *M* NaCN averaged only 7% of the control value without cyanide. The inhibitory action of cyanide was completely reversible after an exposure of 1¼ hours (Fig. 3) but only partially reversible after longer periods.

Methylene blue, which typically reverses at least a portion of cyanide inhibition (Thunberg, 1918), increased the oxygen consumption in a saturated solution in sea water from the cyanide level of 7% to a new level of 10%, and thus was

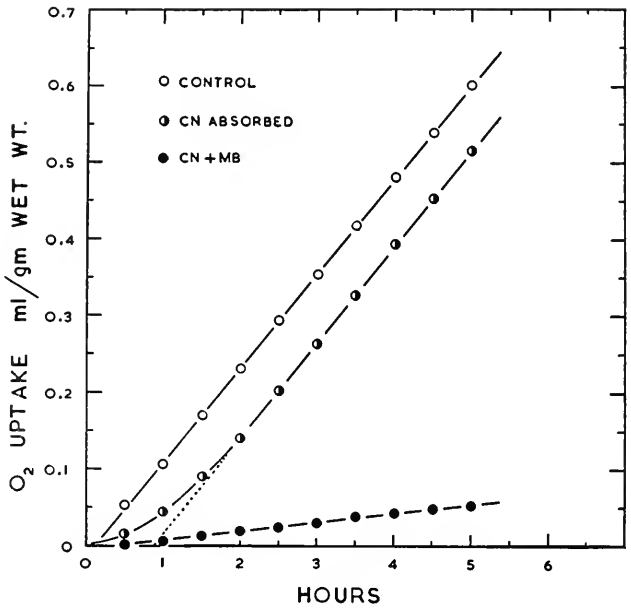

FIGURE 3. The effect of cyanide on mantle respiration. Lower curve: mantle in 2×10^{-4} NaCN; saturated solution of methylene blue (final concentration 1.4×10^{-5} *M*) tipped in at one hr. The change from 7% of control respiration to 10% after the addition of methylene blue is not perceptible on the graph. NaCN present in center well to prevent absorption of cyanide by KOH. Middle curve: mantle in 2×10^{-4} *M* NaCN for 1¼ hr. in Warburg flask prior to 0 time on graph. At 0 time KOH without cyanide was added to center well of flask to absorb HCN. Oxygen uptake returned to the control value as cyanide concentration decreased.

without appreciable effect. This result is not due to failure of penetration since uptake of methylene blue by the cells of the whole mantle can be clearly seen under the microscope. Methylene blue when added to normal mantles neither altered the endogenous respiration nor the increased oxygen consumption produced by succinate. The absence of an increased succinate effect in the presence of a saturated solution of methylene blue (6.3×10^{-5} *M*) was shown in 12 experiments in which the average respiratory increase amounted to 21.2% as compared with 20.5% for 0.005 *M* succinate alone. From this result it appears that the cytochrome system is not limiting in succinate oxidation.

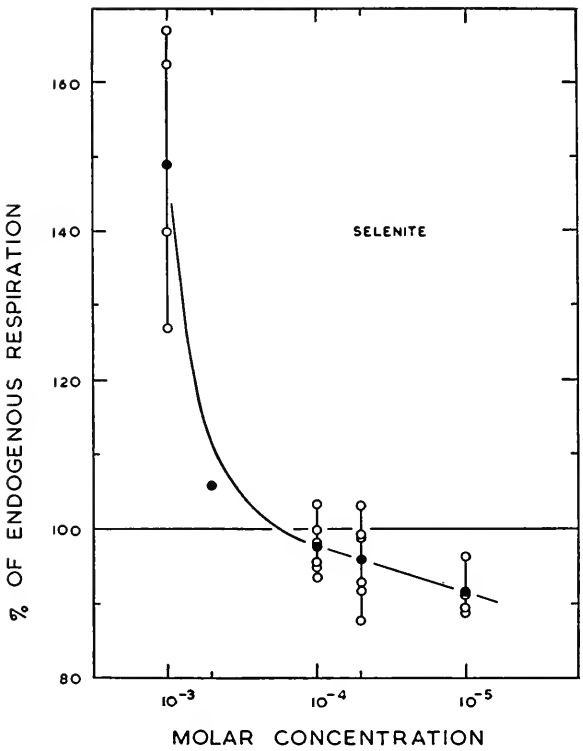

FIGURE 4. The effect of sodium selenite on mantle respiration. Solid circles show mean values. Open circles give values for individual experiments. Endogenous O_2 uptake was measured for 1–2 hrs.; selenite was then tipped in and measurements were continued. Note that the abscissa is in log units.

TABLE V

Effects of diethyldithiocarbamate and ethyl carbamate on mantle respiration

	Mean per cent inhibition		
	10^{-4} *M*	10^{-3} *M*	10^{-2} *M*
Diethyldithiocarbamate	8	32*	13
	16	27	14
	10	22	7
	21	26	0
Ethyl carbamate	4	3	5
	−1	1.5	4

The figures represent individual experiments and show the percentage change in O_2 consumption. North Carolina oysters.

* Maximum inhibition of 57% was found in Florida oysters.

Sodium selenite. Selenite has been studied in view of its inhibition of succinic dehydrogenase (Stotz and Hastings, 1937). The results of selenite on endogenous respiration are summarized in Figure 4 which demonstrates a dual effect of this compound. A slight inhibition occurred with 10^{-5} *M* with progressively less inhibition as the concentration was increased; and at 10^{-3} *M* a mean increase in respiration of 49% was observed. Measurements using selenite alone in sea water showed that the autoxidation of this compound would not account for the observed increase in oxygen consumption.

Selenite in a concentration of 10^{-5} *M*, which depressed endogenous respiration slightly, had a variable effect on respiratory increase from added succinate. In some cases the selenite reduced or abolished the succinate effect; in others it did not.

Sodium malonate. Malonate characteristically inhibits succinic dehydrogenase competitively. Mantle tissue treated with 0.025 *M* malonate prior to the addition of 0.005 succinate showed a 38% inhibition of the succinate effect which was significant at the 5% level. Malonate (0.025 *M*) was without effect on endogenous respiration. Cleland (1950) found only slight inhibition of the succinoxidase of unfertilized oyster eggs by malonate.

Sodium diethyldithiocarbamate. Endogenous respiration was inhibited by diethyldithiocarbamate, the maximum inhibition occurring at 10^{-3} *M* concentration with less inhibition at lower and higher concentrations (Table V). Ethyl carbamate (urethane), an inhibitor of dehydrogenase activity (Keilin, 1925; Stotz and Hastings, 1937), did not affect endogenous respiration in the same concentrations used for diethyldithiocarbamate. Assuming penetration of ethyl carbamate, the difference in action of the ethyl and diethyldithio compounds points to specificity of the sulfur-containing compound and, further, suggests that a copper catalyst may be an important part of the respiratory system.

Discussion

Our objective in this series of experiments has been to establish a background of respiratory data for an understanding of sources of energy and carbonate for shell formation. In so doing we have first described the general respiratory picture of various parts of the mantle of Florida oysters and have found that the pallial zone immediately central to the folded outer margin has a slightly but significantly higher endogenous respiration than either the marginal or central area. It is this pallial zone of the shell which consistently showed the most rapid deposition of calcium in North Carolina oysters previously studied (Wilbur and Jodrey, 1952). As interesting as this apparent correlation may be, the comparison of two sets of data taken at different times and on oysters from different geographical locations must be viewed with caution.

While all steps of the tricarboxylic cycle have not been examined in mantle, enzyme assays and O_2 consumption measurements using several substrates have demonstrated that the major portion of the cycle is present. One apparent difference is the absence of aconitase which catalyzes the conversion of citrate to cis-aconitate and isocitrate. The cycle could operate, however, with isocitrate rather than citrate as the first compound. The isocitrate could then be converted to oxalosuccinate through the action of isocitric dehydrogenase. In homogenates of

the egg of the oyster *Ostrea commercialis* citrate increases oxygen consumption (Cleland, 1951), indicating that in this material citrate is metabolized. While the precursors of the tricarboxylic acid in mantle are not known, pyruvate may well be involved even though its action on respiration could not be clearly demonstrated using strips of mantle. Cleland (1950, 1951) has found that glycolysis and pyruvate occur in the egg of the oyster and the tricarboxylic acid cycle appears to be present for the oxidation of the pyruvate.

The tricarboxylic acid cycle in addition to providing sources of energy would generate CO_2 which might furnish shell carbonate. The respiratory processes described may be expected to apply to other functions of the mantle as well: ciliary movement; mucus formation and secretion; electrolyte regulation; and so on. A direct attack on the relation of respiration to shell formation can be made by measuring the effect of respiratory substrates and inhibitors and combinations of these on shell deposition. (See following paper by Wilbur and Jodrey.)

The very nearly complete blockage of respiration by 2×10^{-4} *M* cyanide points to heavy metal catalysis. However, methylene blue had a negligible effect in reversing the inhibition, suggesting that the cytochrome system may not play a major role in oxidation in this tissue, and, further, that the system concerned may have a redox potential higher than that of methylene blue. A copper catalyst may be involved as indicated by the inhibition of respiration by diethyldithiocarbamate. This can scarcely account for all the oxygen consumption, however, since the maximum inhibition observed was 57%. Maximum inhibition of O_2 consumption by sodium diethyldithiocarbamate occurred at an intermediate concentration of the inhibitor, respiration being less depressed at higher and lower concentrations. Apparently the progressive binding of copper causes the oxygen consumption to decrease to a minimum with a second opposing reaction occurring either as a result of still further decrease in copper concentration or as another effect of the inhibitor. Selenite also exhibited a dual effect on endogenous respiration. With this compound the inhibition at low concentrations was slight and at higher concentration (10^{-3} *M*) there was a stimulation of endogenous respiration. The decrease could be attributed to succinate inhibition, although malonate was without effect on endogenous respiration. Whether selenite at higher concentrations acts in a manner similar to dinitrophenol which also stimulates oxygen consumption in mantle (Maroney and Wilbur, unpublished results) remains to be studied.

Two pieces of evidence in addition to the methylene blue and the diethyldithiocarbamate findings raise the question as to the quantitative importance of the tricarboxylic acid cycle in mantle respiration. Lowering the pH of the medium below 6.6 completely prevented the increased respiration which normally occurs with added succinate without decreasing respiration below the usual endogenous level. Also, malonate left the endogenous respiration unaltered. Both procedures would be expected to decrease O_2 consumption by inhibiting succinate oxidation.

The stability of the oxidative mechanisms of the oyster mantle is noteworthy. The endogenous respiration remained relatively constant in mantles isolated for several days in sea water without added substrate, and these mantles responded to added succinate in the same manner as freshly dissected mantles. The endogenous respiration was also but little changed by altering the pH of the sea water medium from 8.4 to 6.0. Such constancy of respiratory processes would seem to indicate a highly developed control of the intracellular environment. The experi-

mental variations just mentioned may have their counterparts in conditions encountered by the animals such as periods without feeding and variation in the pH of the mantle environment when bathed by sea water as compared with periods when the valves are closed, although in the normal environmental situation the extremes of pH studied here are probably not reached (Dugal, 1939).

Summary

1. The oyster mantle has been studied with respect to endogenous respiration, activity of various oxidative enzymes and response to intermediates of the tricarboxylic acid cycle, and respiratory inhibitors.

2. The endogenous respiration has been found to be similar for different mantle regions, though small differences were present. Endogenous respiration was not significantly altered by changes in the pH of the sea water medium between 6.0 and 8.4 or by isolation of mantles in sea water without added substrate for periods of a few days.

3. Isocitric, succinic and malic dehydrogenases, fumarase, and cytochrome oxidase were present in the mantle. Aconitase could not be demonstrated. Oxaloacetic decarboxylase was found in very high concentration. Its presence may be of significance with respect to the formation of shell carbonate.

4. Isocitrate, succinate, malate, and oxaloacetate stimulated respiration. Enzyme assays and the effects of added substrates indicated the presence of the major portion of the tricarboxylic acid cycle in mantle tissue, although all steps have not been examined. Citrate and pyruvate were without effect on respiration over the range pH 6.0–8.4.

5. Malonate inhibited succinate oxidation partially but did not alter endogenous respiration. Selenite reduced respiration slightly at 10^{-5} *M* but brought about a mean increase of 49% at 10^{-3} *M*. Cyanide, 2×10^{-4} *M*, inhibited respiration almost completely. The inhibition was completely reversible after 1¼ hours but only partially reversible after longer periods. Methylene blue was ineffective in reversing cyanide inhibition.

6. Sodium diethyldithiocarbamate produced a maximum inhibition of 57% at 10^{-3} *M*. Higher and lower concentrations were less effective. Ethyl carbamate (urethane) was without effect at the concentrations employed with diethyldithiocarbamate. The results suggest a copper respiratory catalyst in mantle.

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The possible relation of carbonic anhydrase to calcification of mollusc shell has been discussed at length by Stolkowski (Ann. Inst. Oceanogr., *26:* 1–113, 1951). This investigator and his collaborators have shown that benzene sulfamide inhibits shell regeneration in the snail *Helix aspersa* and calcification in the ciliate *Coleps hirtus* and the larvae of the sea urchins *Paracentrotus lividus* and *Arbacia aequituberculata;* but it is not certain that the effect of the inhibitor is limited to carbonic anhydrase.

STUDIES ON SHELL FORMATION. V. THE INHIBITION OF SHELL FORMATION BY CARBONIC ANHYDRASE INHIBITORS [1, 2]

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The formation of mollusc shell involves the deposition of crystalline material, which is largely calcium carbonate, in an organic matrix. The source of the carbonate has been uncertain, although its origin from metabolic CO_2 has been suggested (Robertson, 1941; Sobotka and Kann, 1941). The enzyme carbonic anhydrase is present in the shell-forming mantle tissue of many mollusks (Freeman and Wilbur, 1948); and if the rate-limiting reaction in carbonate deposition were either $CO_2 + H_2O \rightarrow H_2CO_3$ or $CO_2 + OH^- \rightarrow HCO_3^-$, we might expect this enzyme to accelerate deposition (Meldrum and Roughton, 1933). This could be tested by applying carbonic anhydrase inhibitors which should retard carbonate formation. Also, if shell carbonate has its origin in metabolic CO_2, carbonate deposition should be accelerated by supplying the mantle with a substrate that can be readily decarboxylated, provided, of course, that carbonic anhydrase is in excess.

These possibilities have been examined in the oyster, *Crassostrea virginica* (formerly *Ostrea virginica*) by measuring the influence of carbonic anhydrase inhibitors and metabolic substrates on the deposition of radioactive calcium in the shell.

Methods

Oysters were collected in the area of Beaufort, N. C., and maintained in natural waters near the laboratory for several days prior to use at temperatures between 22.8° and 30.0°. The salinity range was 27.6–35.1 except for the period of one week of very low salinity due to a hurricane and during which the oysters were not used. All experiments were carried out within the range 21.5°–25.6°. Individual oysters were prepared as previously described (Wilbur and Jodrey, 1952) and placed in large flat dishes of aerated sea water containing carbonic anhydrase inhibitors for periods of 40 minutes or 7–8 hours. Ca^{45} (4 μc./l.) of high specific activity was then added to the sea water, and the activity of Ca^{45} deposited in the posterior and central regions of the inner shell surface was measured directly (Wilbur and Jodrey, 1952). Isolated mantle preparations with the attached shell (Hirata, 1953; Jodrey, 1953) were employed in studying the effect of respiratory substrates on Ca^{45} deposition both in the presence and absence of carbonic anhydrase inhibitors. Experimental details are presented in the legends of tables.

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Carbonic anhydrase activity was measured in mantle homogenates by the method of Roughton and Booth (1946) as modified by Wilbur and Anderson (1948). Carbonic anhydrase inhibitors were supplied through the kindness of Dr. Emmanuel Waletzky, American Cyanamid Co. The pH of all solutions was adjusted to that of sea water (*ca.* pH 8).

Respiratory measurements on mantle tissue were carried out in oxygen in 5-ml. Warburg flasks using strips of whole mantle weighing 150–220 mg. The thickness of this tissue is such that gas diffusion is not limiting.

Results

Effect of carbonic anhydrase inhibitors on calcium deposition

The effects of 2-benzothiazolesulfonamide (Cpd. I) and 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox) (Cpd. II) on the deposition of Ca^{45} are given in Table I. Treatment of oysters with Cpd. I at a concentration of 1 : 20,000 and 1 : 50,000 for 40 minutes prior to the addition of Ca^{45} gave essentially complete inhibition of calcium deposition as shown by the ratio of the deposition with the inhib-

Table I

Effect of carbonic anhydrase inhibitors on Ca^{45} deposition in the oyster

	Ratio $\frac{\text{Ca depos.-treated}}{\text{Ca depos.-untreated}}$	Counts/min.		P
		Untreated	Treated	
2-benzothiazole-sulfonamide (Cpd. I)				
1:20,000*	0.02	912±193(12)	41±23(12)	<0.001
1:50,000*	0.07			
1:40,000	0.02	1129±345(8)	43±14(8)	<0.01
1:80,000	0.09			
1:150,000	0.26	1137±261(12)	407±119(11)	<0.05
1:160,000	0.68			
1:250,000	1.11	1189±197(4)	1315±258(4)	<0.8
2-acetylamino-1,3,4-thiadiazole-5-sulfonamide				
1:12,000*	0.60	1243±242(8)	727±178(8)	<0.2
1:25,000*	0.56			
1:100,000	0.52	1550±269(7)	923±219(8)	<0.1
1:150,000	0.71			

Each oyster, connected to a kymograph, was placed in one liter of aerated sea water containing inhibitor. After 40 minutes (starred) or 7–8 hours treatment with the inhibitor 4 μc. Ca^{45} were added. The oyster remained in the radioactive solution for 5 hours. The radioactivity of circular areas of 6.2 $cm.^2$ in diameter on the inner surface of the right valve was measured directly. The radioactivities of a posterior and a central area were averaged to give the value for each individual. The means and standard deviations are shown in the columns under the heading counts/min. The numbers in parenthesis in these same columns indicate the number of individuals. Not all the concentrations of Cpd. II for which data were obtained are given in the table. See text concerning statistical procedures.

itor and in its absence (column 2). With a pretreatment of 7–8 hours, a concentration of 1 : 80,000 caused nearly complete inhibition. At 1 : 160,000 inhibition was still marked but it was not present at 1:250,000. Cpd. II also inhibited calcium deposition. Quantitatively, however, the results are quite different with this compound in that the ratio of deposition with the inhibitor and in its absence fell within the range 0.52–0.71 for all concentrations between 1:12,000 and 1:150,000. As the concentration was increased above 1 : 150,000 the maximum effect occurred at 1 : 100,000, and higher concentrations appeared to give no greater inhibition.

Individual differences in the rate of deposition are always very considerable in oysters, and this is shown in Table I, columns 3 and 4. To demonstrate significant differences between treated and control oysters on a statistical basis the concentration groups have been paired. The P values are given in the last column. With Cpd. II the differences between treated and control groups are not highly significant for single concentrations nor for paired groups. However, when all treated and all control groups are combined the difference is significant at the 1% level.

The marked inhibition of carbonic anhydrase by Cpd. I and Cpd. II (Miller, Dessert and Roblin, 1950) has been demonstrated on oyster mantle using supernates of lightly centrifuged homogenates equivalent to one part of tissue (wet weight) in 50 parts of solution. The enzyme activity at 0° was inhibited completely at a concentration of approximately $1 : 10^7$ for Cpd. I and $1 : 10^6$ for Cpd. II.

Absence of general toxic action of carbonic anhydrase inhibitors

The inhibition of calcium deposition by Cpd. I and Cpd. II might be expected, quite apart from specific enzyme inhibition, if these compounds had a general toxic action. Accordingly, toxicity of the two carbonic anhydrase inhibitors has been studied at various concentrations by recording shell movements and by measuring the oxygen consumption of the mantle. Kymograph records of shell movements were made for all oysters in each experiment. Movements were normal in Cpd. I at 1 : 80,000 which gave almost complete inhibition of calcium deposition. At 1:50,000 abnormal behavior was shown by more frequent opening and closing of the valves followed by increased periods of closure. This behavior became more marked at higher concentrations. Shell movements remained normal in Cpd. II even at a concentration of 1:12,000. The respiration of mantle was unaffected by either compound at the concentrations shown in Table I. A slight inhibition of oxygen consumption (*ca.* 10%) was produced by both compounds at 1:10,000, however. The results on shell movements and oxygen consumption clearly show that concentrations of the carbonic anhydrase inhibitors which had a marked inhibitory action on calcium deposition exerted no general toxic action on the oyster.

Respiratory substrates on calcium deposition

In the Introduction it was suggested that if shell carbonate is derived from metabolic CO_2, then by supplying substrates that can be decarboxylated, deposition of calcium carbonate should be accelerated. The isolated mantle-shell preparation provides an opportunity to put this to test since substrates can be added directly to the mantle and the deposition of Ca^{45} on the shell measured. Succinate, malate,

and oxaloacetate were used. Previous studies had shown that all three substrates increased the respiration of mantle approximately to the same degree (Jodrey and Wilbur, 1955). Oxaloacetate, in contrast to succinate and malate, is decarboxylated spontaneously and also through the action of the enzyme oxaloacetic decarboxylase found in high concentration in the mantle. Succinate and malate as substrates of the tricarboxylic acid cycle should be converted to oxaloacetate and other compounds which undergo decarboxylation (Jodrey and Wilbur, 1955).

Oxaloacetate was found to increase calcium deposition 4-fold (Table II). The rate of deposition of the isolated mantle was thereby increased approximately to one-half that of the whole oyster. It would be interesting to determine whether a similar effect will result by supplying CO_2 alone. Succinate and malate had no significant effect. The reason for this result is not clear.

TABLE II

Effect of respiratory substrates on Ca^{45} deposition in the mantle-shell preparation of the oyster

Treatment	Ca^{45} depos., counts/min.		P	Ca depos.-treated / Ca depos.-untreated
	Untreated	Treated		
Succinate	569±91(12)	627±80(12)	<0.7	1.1
Malate	455±67(12)	568±58(12)	<0.3	1.2
Oxaloacetate	533±79(16)	2223±214(23)	<0.001	4.2
Mantle-shell prep. no substrate	521±42(40)		Ratio $\frac{\text{mantle-shell prep.}}{\text{intact oyster}} = 9.4$; $P < 0.001$	
Intact oyster	5060±88(67)			

Each mantle-shell preparation was placed in 500 ml. of aerated sea water containing the sodium salts of substrates, 0.01 *M*, for 60–75 minutes. Four μc. of Ca^{45} were then added and the preparations remained in the solutions for an additional 11 hours. Radioactivity of the shells and method of expressing data as in Table I. Intact oysters connected to kymographs remained in one liter of sea water containing 4 μc. of Ca^{45} for 5 hours. For comparison with mantle-shell preparations the counts from the shells of intact oysters were multiplied by $2 \times \frac{11}{5}$ to correct for the difference of Ca^{45} activity in the sea water and the time of exposure.

Carbonic anhydrase may be expected to assume increased importance as more CO_2 passes through the system to form carbonate. This should be demonstrated by an increased effect by carbonic anhydrase inhibitors at high calcium deposition rates. To test this the effect of 1:40,000 and 1:80,000 2-benzothiazolesulfonamide was measured with Ca^{45} using the mantle-shell preparation with oxaloacetate added (high deposition rate) and in the absence of added substrate (low deposition rate). In the absence of substrate there was a decreased calcium deposition in the presence of the inhibitor (43% decrease), but the difference between treated and control preparations was not statistically significant ($P < 0.2$; 17 treated specimens; 17 untreated). When oxaloacetate was added, increasing the deposition rate 5.5-fold, the same concentrations of inhibitor produced a significant decrease in calcium deposition amounting to 49% ($P < 0.001$; 16 treated specimens; 23 untreated).

Discussion

The principal findings of this study are a marked increase in calcium deposition when oxaloacetate was made available to the mantle and a marked decrease in calcium deposition produced by carbonic anhydrase inhibitors. A third finding, related to the first, confirms an earlier study (Jodrey, 1953) which reported that the rate of calcium deposition of the isolated mantle-shell preparation is only a fraction of that of the whole oyster. These three results point to certain features of shell formation about which we have had no clear indication heretofore, namely: (1) the origin of shell carbonate; (2) the relation of carbonic anhydrase to shell deposition; and (3) the interrelation of the mantle and the rest of the organism with respect to shell formation. We may now consider these aspects of shell formation briefly.

The increase in calcium deposition which occurred when a source of CO_2 was made available, while somewhat indirect as evidence, strongly suggests that at least a part of the shell carbonate has its origin in the CO_2 of metabolic processes. An alternative source of CO_2 would be the bicarbonate of sea water. This would have to pass into the mantle or other parts of the organism and be converted to CO_2 if carbonic anhydrase were to act as a catalyst. That such a source is important becomes unlikely in view of the low rate of deposition in the isolated mantle-shell preparation immersed in sea water.

The probable importance of carbonic anhydrase catalysis in the conversion of CO_2 to carbonate is indicated by the reduction in the rate of calcium deposition when the oyster is treated with carbonic anhydrase inhibitors. At low rates of calcium deposition, as in the case of the isolated mantle-shell preparation without added substrate, the hydration of the CO_2 is sufficiently rapid without carbonic anhydrase and thus a carbonic anhydrase inhibitor was without significant effect. When the rate was increased by supplying oxaloacetate, however, a clear-cut inhibition occurred. Since the uncatalyzed reaction would always be present, inhibitors should never give complete inhibition. This was the case with experiments using Cpd. II on whole oysters and Cpd. I on the mantle-shell preparation with added oxaloacetate. When whole oysters were treated with Cpd. I, on the other hand, inhibition of calcium deposition was essentially complete. These results point to an inhibition by Cpd. I of factors other than carbonic anhydrase. Such factors are apparently located in parts of the organism other than the mantle since the same inhibition did not occur in the isolated mantle.[4]

Carbonic anhydrase will be important in carbonate formation only when the hydration of CO_2 becomes limiting.[5] Mollusks which are depositing carbonate at a low rate would have no need for the enzyme; and it is not surprising that some mollusks have little or none (Freeman and Wilbur, 1948).

[4] Cpd. I reduced the deposition rate of the whole oyster below that of the mantle-shell preparation. This has no significance for our present discussion, however, since the relative contribution of the mantle *per se* to calcium deposition in the whole organism is unknown.

[5] Theoretically, carbonic anhydrase could also accelerate the solution of shell $CaCO_3$ brought about by the action of weak acids other than carbonic acid, provided that the second of the two following reactions were limiting (Meldrum and Roughton, 1933): $CaCO_3 + 2HA \rightarrow H_2CO_3 + CaA_2$; $H_2CO_3 \rightarrow CO_2 + H_2O$. However, these reactions occur in lamellibranchs when the valves are closed (see Dugal, 1939); and it seems unlikely that the enzyme would be of much importance in this process.

Because the deposition of calcium by the isolated mantle in the absence of substrate is low, it does not follow, of course, that the same low rate obtains in the whole organism. Nonetheless, in view of the marked effect of oxaloacetate on the isolated mantle it seems likely that in the intact organism CO_2 or substrates that can be decarboxylated, or both, are furnished to the mantle and so increase the rate of calcium deposition. Thus, the mantle, while autonomous in one sense, would only perform effectively because the raw materials of shell are furnished by other parts of the organism.

Summary

1. The carbonic anhydrase inhibitors 2-benzothiazolesulfonamide (Cpd. I) and 2-acetylamino-1,3,4-thiadiazole-5-sulfonamide (Diamox) (Cpd. II) markedly reduced the rate of deposition of calcium in the shell of the oyster *Crassostrea virginica*. Treatment of oysters with Cpd. I, 1:80,000, for 7–8 hours reduced the rate to one-tenth the normal value and gave essentially complete inhibition at higher concentrations. The latter result indicates that inhibition by this compound is not limited to carbonic anhydrase. With Cpd. II the maximum inhibition was 50% and was produced at a concentration of 1:100,000 or higher.

2. Measurements of shell movements and oxygen consumption of mantle tissue showed that concentrations of carbonic anhydrase inhibitors which had a marked inhibitory action on calcium deposition exerted no general toxic action on the oyster.

3. The rate of calcium deposition of the oyster was more than 9 times that of the isolated mantle-shell preparation, confirming an earlier study. The addition of 0.01 *M* oxaloacetate to the mantle-shell preparation increased the deposition rate more than 4-fold, suggesting the utilization of metabolic CO_2 for shell carbonate by the organism. Succinate and malate were without significant effect on calcium deposition.

4. The experimental findings support the view that carbonic anhydrase increases calcium deposition in the oyster when the rate is sufficiently high that either of the following reactions becomes limiting: $CO_2 + H_2O \rightarrow H_2CO_3$; $CO_2 + OH^- \rightarrow HCO_3^-$. At lower rates the enzyme would not be required.

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MITOTIC RESPIRATORY RHYTHMS IN SINGLE EGGS OF PSAMMECHINUS MILIARIS AND OF CIONA INTESTINALIS

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Using the reference (Cartesian) diver technique of Scholander, Claff and Sveinsson (1952), Scholander, Claff, Sveinsson and Scholander (1952) measured respiration of single eggs of marine animals, most of which had previously been studied on somewhat larger samples by Zeuthen (1949, 1950b, 1950c; Cartesian diver method, 1950a). All authors mentioned agree that the rhythm in mitotic division of the fertilized egg is accompanied by a faint respiratory rhythm. However, in the details there are certain discrepancies, especially concerning the reproducibility of the observed rhythm. The discrepancies shall be discussed in this paper which presents new data for single eggs of *Psammechinus miliaris* and of *Ciona intestinalis*. This paper reports all results obtained.

Method

1. Diver

The diver is the same as previously used by the author (1950a), only modified so as to work with one egg instead of a hundred, or a few hundred eggs. The increased sensitivity is obtained 1) by reducing as much as possible the dimensions of the diver and 2) by increasing the sensitivity of the manometer. The diver consists of a chamber and a stopper, both made from the same glass capillary of which two pieces are selected so that one fits tightly into the other. The diver's interior communicates with the exterior through capillary spaces left between stopper and chamber. These spaces permit free equilibration of pressure, and the stopper reduces diffusion. The procedure followed for making and filling this type of diver is illustrated in Figure 1 and is briefly described in the text for that figure. The diver has been drawn too short and thick. For correct dimensions, see inserts of Figures 4 and 6. In Figure 1 also other dimensions are misrepresented.

Before the diver can be filled for an actual experiment its weight must be adjusted so that it will float when a conveniently sized air bubble is introduced into the chamber. For this adjustment a suitable air bubble is pipetted into the newly made diver's chamber, using a Holter braking pipette (step 1 in Fig. 1). The stopper is loosely inserted and the diver is set afloat. It is, however, likely to be far from buoyant. From the rate of sinking or rising in water one soon learns to judge whether or not it shall be possible to regulate it to buoyancy. If prospects are good one first compresses or expands the air bubble by applying pres-

sure or suction to the system. If the diver is brought to float this way the necessary pressure change informs about how much smaller or bigger the air bubble should be chosen next time. Most often, however, it is necessary to remove or to add air and (or) glass. Air is removed or added from the bubble in the chamber, or the stopper's air volume is altered by letting the outer end of the stopper collapse or expand some. This can be done in a surprisingly regulated way by heating in a micro-flame mounted on the stage of the dissecting microscope. If the stopper's outer end is first drawn into a solid tail, the weight of the stopper can be altered by the addition to, or removal of, glass from this tail.

The air volume which finally makes the diver float is measured as the length of an air column in a calibrated Holter braking pipette which is used for all subsequent fillings of this individual diver. The air volume of the floating diver can also be derived from direct measurements of the linear dimensions of the air column in the floating diver.

It does require some skill to prepare these tiny and very fragile divers; many must be discarded. General rules: Always hold the diver with soft and flexible instruments. A piece of rubber, *e.g.*, a rubber stopper with a slit cut in the edge or across the one end, serves as excellent forceps. Watchmaker's forceps protected in the tip with flat pieces of rubber are useful. It is better to hold the divers with the fingers than with unprotected instruments. Capillarity should be made use of whenever possible. In air tiny pieces of glass adhere to wet glass rods and can thus be moved about without much risk of breakage.

2. Manometer

The diver's equilibrium pressure was adjusted and measured using a special "sensitive manometer" (Zeuthen, 1953b). The "manometer" is really a burette (Fig. 2 e, f, g, h), by means of which accurately measured volumes of fluid can be withdrawn from, or injected into, a closed air space (Fig. 2b extending from water surface in a through the ground joint c, the three-way tap d to the water surface e) in which regulated pressure changes are thereby created. The amount withdrawn is read by the movements of the bubble g in the burette. The diver floats in a small amount of alkaline medium in a pocket (a) open to the air space. The "manometer" can be adjusted to the nearest 1/25 mm. water pressure ($\backsim 4 \times 10^{-6}$ atm.) which is somewhat better than the sensitivity of the present diver.

At the beginning of the experiment the pressure in b is adjusted by mouth through n, m, l with d turned 90° relative to the position shown in the figure. With m closed, the manometer k is useful for the fine regulation of the initial flotation pressure in b; n is a CO_2-trap, l is an air brake. During actual measurements the three-way tap d is in the position indicated in the figure.

The volume of the "burette" from 0 cm. to 70 cm. is less than 1% of the volume of b, extending to e. Linear movements of g in the (very uniform) "burette" set up practically proportional pressure changes in b. The system can be reset when g has been moved across the scale. This introduces small changes in the value of h (equation below), which can, however, usually be neglected. The gas exchange is calculated from the formula

$$\frac{\mu l\ O_2}{\text{min.}} = \frac{x \cdot v \cdot V_D \cdot (B + h - e) \cdot 273}{(V + v) \cdot 10{,}300 \cdot (273 + t°)} = x \cdot K$$

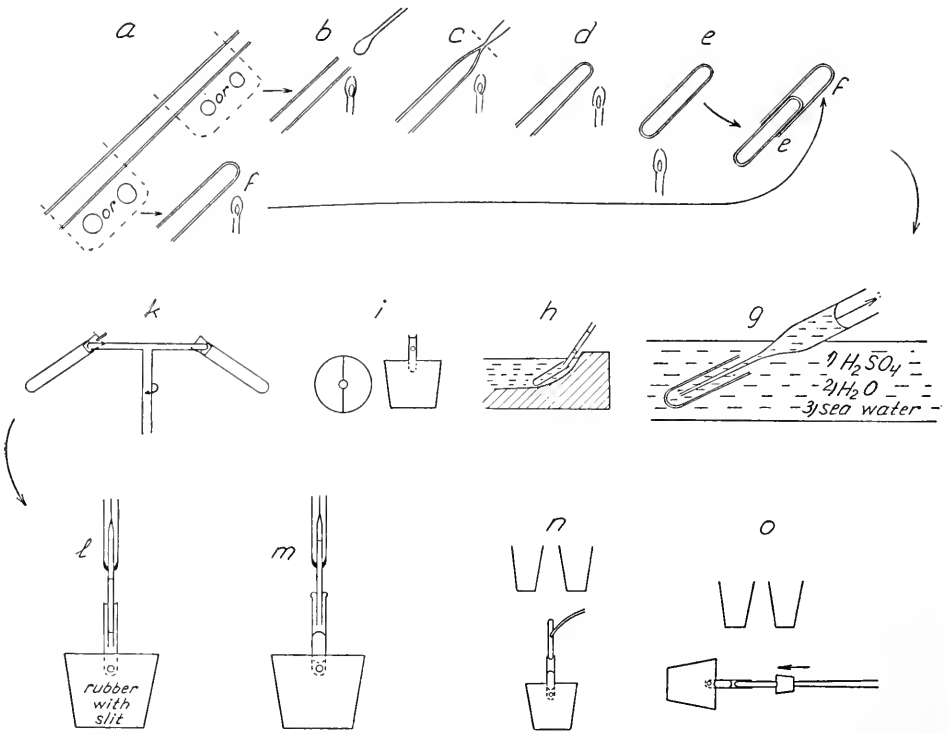

FIGURE 1. Method of making the diver. a. Pull a capillary (Thüringer glass) so as to obtain outside diameter *ca.* 0.2–0.25 mm; inside diameter = outside × 0.87–0.92. If divers made from a given capillary tend to become too heavy or too light, use more thin-walled or more thick-walled glass. Select two pieces of which I fits tightly when pushed a certain distance into II (use head lupe). The cross section of the capillary should be perfectly circular or it should deviate from circular in such a way that I and II can easily be oriented to fit each other. The alternatives are demonstrated. b–e. A micro-flame is mounted on the stage of a dissection microscope. The stopper is sealed—first at what is to be its inner end (upper right, b)—by making contact with a glass rod, pulling, cutting (square line in upper part of c indicates cut) and gently sealing the cut end. This way, a minimum of glass weight is used for sealing. Repeat in what is to be the outer end of the stopper (lower left of e), and seal the far end of the chamber (f). g. The chamber is rinsed in concentrated sulfuric acid and then successively in H_2O and in sea water, always by sucking large amounts of fluid into a pipette, which is introduced deep into the submerged chamber. h–m. The chamber is placed on the slanting side of a dish filled with sea water and the cell is dropped from a Holter braking-pipette into the open end of the diver chamber. The chamber is removed to a holder (i) which is an ordinary laboratory rubber stopper with a slit in it to hold the diver. The rubber stopper is selected to fit the jacket of a small hand centrifuge (k). The cell drops by its own weight to the bottom of the diver chamber or it is spun down with the centrifuge. *Ciona* eggs having a heavy coat of testa cells tend to stick to the glass. They can be pushed down with a small glass rod. The evaporation in the centrifuge is negligible ($< 5\%$). Air is introduced with a braking pipette (l) and, using a second pipette, the water in the upper end of the neck is replaced with alkali, isotonic with the sea water (m). For these operations the pipettes are mounted horizontally and the diver is moved vertically by the use of a special stand (*cf.* Holter, 1943). n. The rubber stopper holding the diver chamber is now placed under the dissection microscope so that the diver is in a vertical position. The stopper (for the diver) is brought to adhere by capillarity to the wet surface of a glass rod. While in the position indicated the glass rod is

in which x is the movement (in mm. per minute) of the bubble g in the "burette," v is the volume (μl) per mm. "burette," V is the volume of b extending to e (c. 100,000 μl; $v/V = 2 \times 10^{-5}$; in $(V + v)$ v can be skipped); V_D is the air volume of the floating diver. B is the barometric pressure in mm. H_2O (760 mm. Hg $\sim$ 10.300 mm. H_2O); h is the initial equilibrium pressure of diver (mm. H_2O) read on k as the difference in height of the menisci (with under-pressure, h negative); e is the vapor tension of water (or of n/10 NaOH and of 2% proteose-peptone) at $t°$ (mm. H_2O).

It should be noticed that x varies inversely with $(B + h - e)$. Therefore the sensitivity of the system should increase greatly at low pressures; and—as long as other factors are not limiting—it should approach infinity when h approaches negative values equal to $B - e$. The present experiments were carried out at total pressures $(B + h)$ varying from 323 to 578 mm. Hg.[1]

There are several possible ways of calibrating the manometer. The one here used is not claimed necessarily to be the best. The space b (from a to d, *cf.* Fig. 2) is determined by weighing with water. The pressure change induced in the whole system (b extending to e, m and meniscus in k), when g is moved from 0 to 70, is read on k, with the meniscus in the left branch adjusted to a defined level. This pressure change should be recalculated to what would have been observed if the space d, to m and k had not been included. The relative volumes of the systems: 1) b to e, m and k, 2) d to e, m and k, and 3) d to m and k (g in all cases in a stable position) are calculated from the pressure changes (read on k) induced for a defined linear movement of the left meniscus in k, when d is in the three positions defining the three spaces mentioned. The data obtained permit the calculation of the pressure change (mm. H_2O) induced in the space b, extending to e, for every mm. movement of g on the scale. In the above formula the calculated pressure change replaces $\frac{v}{V + v}$.

In one bath several "manometers" can be operated simultaneously more or less like Warburg manometers. In the present apparatus there are four manometers but for the present study only one was run at a time.

3. Bath, optical equipment

The bath was a 100-liter tank, on all sides, including the top, well insulated with cork plates. Holes were left open for stirring propeller, for manometer, for illumination from behind, and for observation. The bath was allowed to adjust itself to the room temperature and it varied in the range indicated in Table I, with maximum drifts in the course of an experiment of 0.1°–0.2°/hour.

The flotation vessel remained inserted into the bath for at least one hour before

[1] In this paper only rate curves are presented. It is only on very large-scale cumulative graphs that any scatter of the points can be demonstrated, and that the faint respiratory rhythm can be visually demonstrated. Lack of space prevents the publication of such curves.

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turned in a plane perpendicular to the paper so that the cross section of the stopper fits that of the chamber. Then the stopper is fitted into the chamber. Under the dissection microscope with the diver in a horizontal position, it can be pushed further in (o), using the rubber-protected tip of a watchmaker's forceps. The diver, thus filled, is dropped into a dish with alkaline flotation medium and transferred with a pipette to the flotation vessel (Fig. 2a).

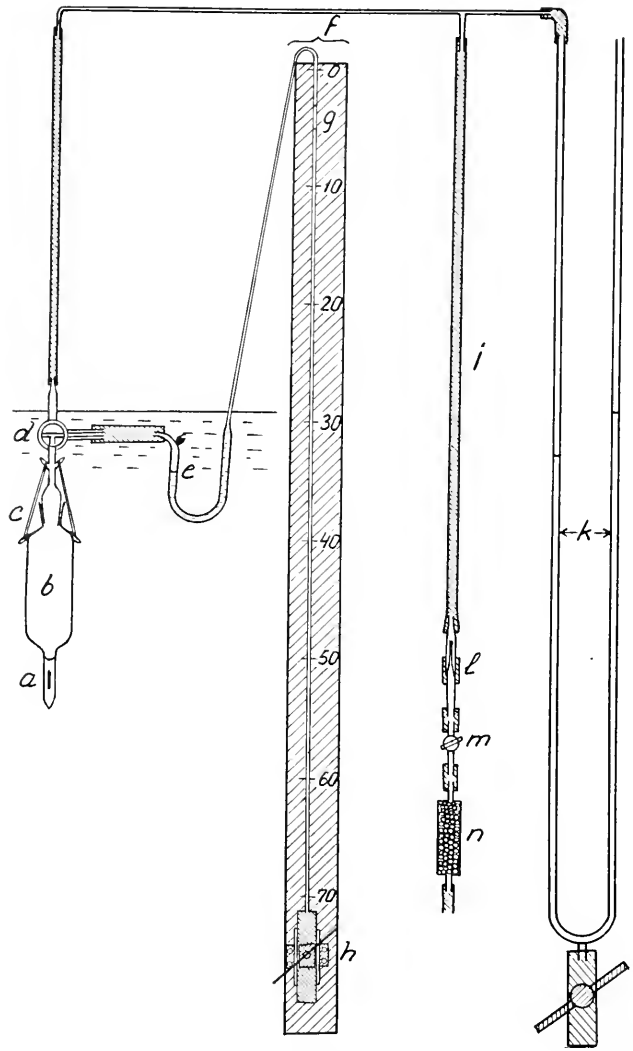

FIGURE 2. Manometer; *cf.* text.

the diver was introduced. This was done as gently as possible and a minimum of water was carried along. This way currents in the flotation medium were minimized during actual measurements, but they were not completely suppressed. It remains uncertain to which extent the initial adjustments in equilibrium pressure observed in most experiments (blanks and runs with egg) should be referred to currents in the flotation medium, to gradual establishment of diffusion steady-states inside the divers, and between flotation medium and gases in the space b, Figure 2, or to as yet unknown shortcomings of the present instrument.[2]

[2] This instrument, as also the 1950 diver, was designed for the demonstration of faint respiratory rhythms. For relative measurements it appears to be unsurpassed, but for absolute measurements it needs to be controlled for background drifts, and to be tested with objects which respire at known rates. Successful work on these lines was carried out by Mr. W. L. M. Geilenkirchen working in this laboratory. The paper will appear in a Dutch journal.

Horizontal microscopes (12.5 × 2.5) with ocular scales are firmly mounted in front of the bath, one for each manometer. The diver's equilibrium pressure is read by "turning point" determinations. The diver is kept floating at about the same level (± 0.1 mm.) throughout the experiment. Whenever a reading is due the manometer is set so that the diver rises slowly. At the same time the respiration makes the diver heavier, and it therefore turns on some level. The manometer, when first set, is not again touched before the diver has turned and the pressure has been read. Figure 3 demonstrates some trials with a respiration diver (1.35 μl), which is subjected to varying initial underpressures. Curves I

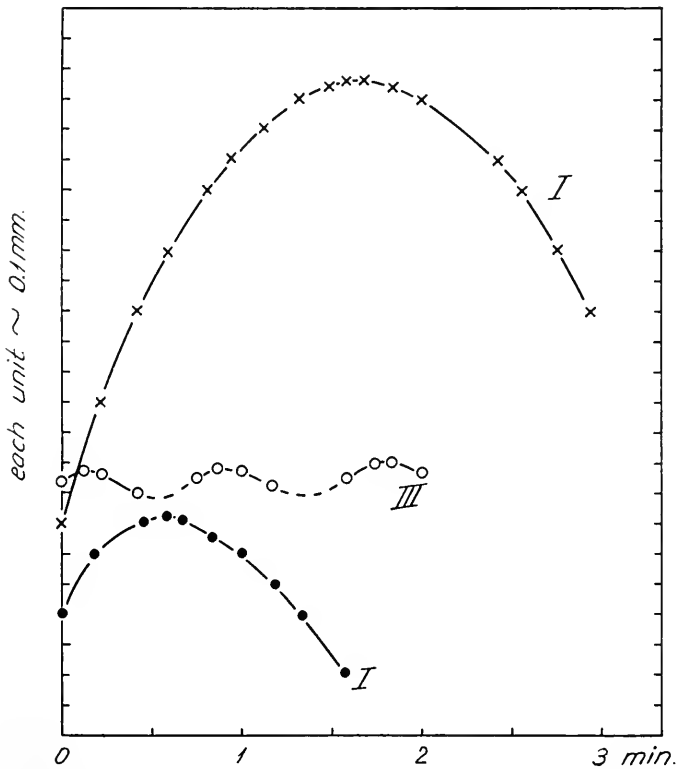

FIGURE 3. Rates of movement of a respiration diver which was subjected to varying initial under-pressures.

represent two cases in which the diver was given relatively low initial pressure and high initial rate of movement. The changing positions are plotted as a function of time. Curve III shows that the level on which the diver turns can be reproduced to about 0.03 mm. and also that the diver can be kept continuously moving up and down within a narrow zone. The results would have been even better if the observer had not had to make notes. Incidentally, one is not forced to accept a reading if the diver happens not to turn on the right level. For the present investigation the measuring periods were about 300 seconds. It is claimed that the turning point can be determined within, say, ± 3 seconds or about ± 1% of the time be-

tween successive readings or better. Close inspection of the data underlying the curves of Figure 3 indicates that about 0.005 mm. displacement of the diver was enough for the observer to decide about the direction of the diver's movement. In the optical field this corresponds to 0.15-mm. displacements. Suitable reference marks on the diver are points which in the beam of light appear like little stars. If such a point happens to form a continuation of one of the lines of the ocular scale the decision is about whether the line extended with the "star" tends to bend up or down in the optical field.

4. *Material*

The eggs of *Ciona intestinalis,* fertilized and unfertilized, were used. The testa cells were not removed; they have been found to make up for 20–30% of the res-

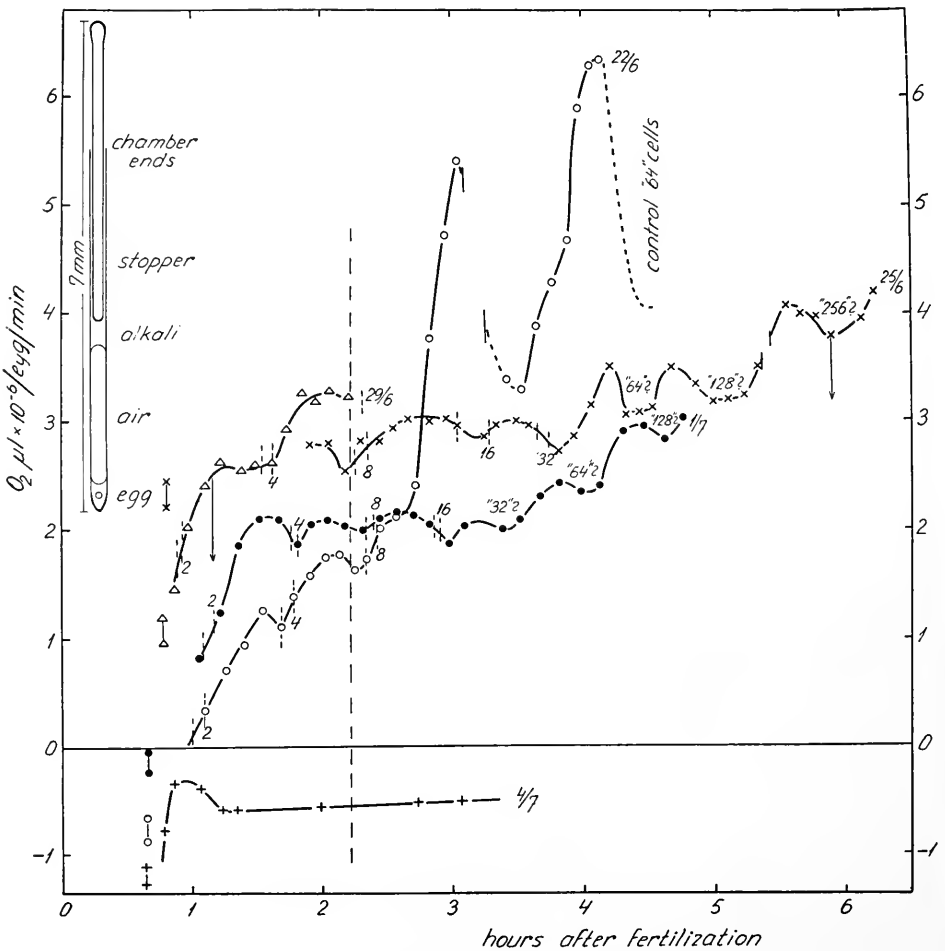

FIGURE 4. Four experiments on single eggs of *Psammechinus miliaris,* with one control experiment (4/7); *cf.* Table I and the text.

piration of the unfertilized egg (Holter and Zeuthen, 1944) and less in the case of the fertilized egg. The eggs were thoroughly washed before use. Another object used was the fertilized egg of *Psammechinus miliaris,* Z-form. The jelly was removed with a pipette, the fertilization membrane was retained. Both animals are from the littoral zone and the experiments were run in sea water, salinity 20–25‰. The divers were floating in 22‰ NaCl, made up to n/5 with isotonic NaOH; no Na-taurocholate was added.

Experiments

A. *Psammechinus miliaris.* Figure 4 shows four experiments with single eggs of *Psammechinus.* The same diver was used throughout, and an experiment with empty diver is also reported (4/7). The ordinate shows the rate of oxygen uptake per egg per minute, and the abscissa indicates hours after fertilization.

Table I

Oxygen consumption in single eggs of Psammechinus miliaris. Data pertaining to Figures 4–6

		$B+h$ (mm. Hg)	t_1^0	t_2^0	O_2/min., µl (135 min.)	Vol./egg, µl	µl O_2/µl egg/min.
	22/6	340	16.9	17.3	2.2×10^{-6}	6.25×10^{-4}	3.5×10^{-3}
	25/6	450	18.2	17.7	3.2×10^{-6}	4.9×10^{-4}	6.5×10^{-3}
I	29/6	533	19.6	19.8	3.8×10^{-6}	5.25×10^{-4}	7.2×10^{-3}
	1/7	418	18.6	19.3	2.6×10^{-6}	4.8×10^{-4}	5.4×10^{-3}
	4/7	344	21.1	—	—	—	—
	20/6	538	16.9	17.4	2.6×10^{-6}	5.0×10^{-4}	5.2×10^{-3}
II	24/6	523	17.5	17.5	2.1×10^{-6}	4.75×10^{-4}	4.4×10^{-3}
	7/7	578	19.9	20.0	3.2×10^{-6}	—	—
III	4/7	362	—	—	—	—	—
Aver.		466	18.5		2.8×10^{-6}	5.2×10^{-4}	5.4×10^{-3}

I, fertilized eggs, Figure 4. II, fertilized eggs, Figure 5. III, control experiment, Figures 4 and 5.

Two points connected with a vertical line indicate the time when the diver was initially brought to float in the flotation medium. The position of these pairs of points on the ordinate is in all cases arbitrary. Due to space limitation two curves (three in Fig. 6) have been displaced on the ordinate to the extent indicated by the length of the arrows. Gaps on these curves (and on other curves presented) indicate resetting of the manometer. Important characteristics of the 5 experiments are given in Table I. The two temperatures are at (or near) the beginning and the end of each experiment.

The diver, shown with typical filling in the insert of Figure 4, has a gas volume (V_D) of 0.063–0.065 µl, determined slightly differently, in the 5 experiments. The internal cross sectional area (A) of the diver capillary was calculated from several diameters and V_D was calculated from the formula $A\cdot(l-2r)+4/3\pi r^3$ in which l is the length of the air column from meniscus to meniscus and $2r$ is the (average) diameter of the diver capillary; l was measured on the floating diver. In the graphs

pairs of vertical stippled lines across a curve indicate the time when a division was beginning and when it was completed. The number of cells resulting from a division is indicated. After the 16-cell stage (32-cell stage on 25/6) accurate observations were no longer possible. The numbers placed by the time of later minima on the curves represent guesses. We actually know (*cf.* Zeuthen, 1951, 1953a) that "64," "128" and "256" cell stages do not exist. The cell numbers are in all cases lower than indicated. The long stippled line (2 h. 15 min.) shows the time when the absolute rates reported in Table I were measured as the distance from curve to curve (4/7) for the control experiment. Egg volumes were calculated from the measured diameter of the experimental egg itself, or from the average diameter of several control eggs.

The position of the control curve (4/7) indicates that probably in all cases there is some diffusion of gas into the diver from the surroundings. Unfortunately, the diver was broken before another control was run, and this causes some uncertainty as to the absolute rates measured. However, Borei (1948) for both *Psammechinus* forms ("Z" and "S") found an average rate of O_2 uptake of 3.1×10^{-6} μl/egg/min. (18°, 120 min. after fert., egg size 4.43×10^{-4} μl). The results here reported check well with this. Inspection of the rate curve 22/6 for slope indicates that we have reason to suspect the value (Table I) for this experiment to be too low.

The egg used on 25/6 stayed in the diver until the mesenchyme-blastula stage; the eggs used on 22/6 and on 1/7 were removed immediately after the experiment and not observed further. The egg studied on 29/6 stopped development by the time of the third division. By this time the experiment had to be discontinued because the stirring motor set up disturbing vibrations; two hours later the four blastomeres of this egg had fused into two. By this time most of the control eggs were blastulae, but a number had developed into monsters of the same type as the one in the diver.

Figure 5 presents two more experiments with single *Psammechinus* eggs, both showing asynchrony between divisions of the two first blastomeres. The egg used on 20/6 showed a mild degree of asynchrony, the second division being delayed 3–4 minutes in the one blastomere. In the following division cycle this delay could again be observed; 7 cells were directly counted—there may perhaps have been 8. In this experiment optical conditions were poor. Possible division activity was noted at times indicated (?). On the following morning this egg was a mesenchyme blastula (while still in the diver); the controls were gastrulating. The blastomeres in the egg studied on 24/6 showed pronounced asynchrony of divisions. The number of cells observed at any time is indicated, optical conditions were good. Both experiments (20/6, and especially 24/6) are inconclusive as to respiratory cycling, but they may indicate that pathological conditions which bring about asynchrony of division may at the same time increase the amplitude of the respiratory cycles above normal.

Such a situation, if correct, suggests that physiological interaction between neighboring cells which are in different phases of mitosis might result in increased cycling amplitude. This hypothesis was put to a preliminary test, with negative result, in the experiment of 7/7. For this experiment a good batch of eggs was divided into two lots and fertilized with a time interval of 17 minutes, which roughly equals half of one mitotic cycle. The jelly, and in most eggs also the

fertilization membrane, was removed shortly after the fertilization using a narrow pipette. From the lot first fertilized, 92 eggs in the 2-cell stage were selected, 44 without membrane and 48 with. From the second lot 92 eggs were isolated in the 2-cell stage, all without membrane. All 184 eggs were thoroughly mixed together and introduced into a diver (gas space: 9.8 μl). The resulting respiration curve (7/7) is indicative of simple interference between two groups of eggs, each of which shows respiratory cycling of about the magnitude shown in the typical curves of Figure 4. Thus, this experiment supplied no evidence of physiological

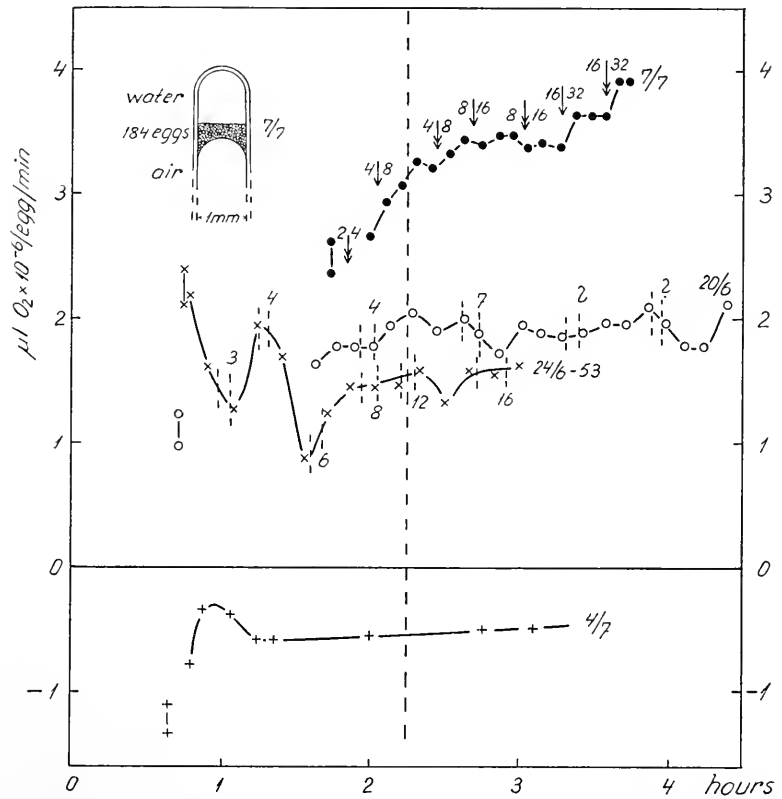

FIGURE 5. Two deviating experiments (20/6, 24/6) on single *Psammechinus miliaris* eggs. One experiment (7/7) with 184 eggs fertilized in two separate lots (92 + 92 eggs) showing absence of physiological interaction between the two groups.

interactions between two groups of cells which are much out of phase. In Figure 5 single and double arrows indicate the approximate division times of control eggs belonging to the two separate lots.

The absolute respiration rates measured two hours 15 minutes after fertilization are given in Table I. The experiment 4/7 serves as base line for the experiments on 20/6 and 24/6. The base line for the experiment 7/7 is the 0-line.

B. *Ciona intestinalis.* The diver used for these experiments looked much the same as the one used in *A;* however, all dimensions were slightly larger. V_D

measured as under A averaged 0.112 μl (variation 0.110–0.115, for Experiment 8/6 a mistake was made when the dimensions were measured—the calibration found on earlier days was accepted). In Figure 6 rate of oxygen uptake is plotted on an arbitrary time scale. Time for beginning and for end of each division is indicated with stippled vertical lines, and the resulting number of cells is indicated; (?) indicates poor conditions of observation. The experiments on 11/6 and 5/6 are for unfertilized eggs, and the experiments on 3/6, 6/6 (2 exps.) and 8/6 are on fertilized eggs which all showed beautiful development in the diver (not observed beyond the time of experiment).

The two control experiments on 10/6 were made with the same diver as used for all *Ciona* experiments, but with certain changes introduced by accident. After completion of the experiment on 8/6 some of the outer end of the diver chamber broke off. Therefore the diver became buoyant with a reduced value of V_D (0.060 μl instead of 0.112) and the diffusion barrier set up in the diver's neck after introduction of the stopper became shorter, *i.e.*, probably less efficient. Be-

TABLE II

Oxygen consumption in single eggs of Ciona intestinalis. Data pertaining to Figure 6

		$B+h$ (mm. Hg)	t_1^0	t_2^0	O_2/min., μl (1 hour)	Vol./egg, μl	μl O_2/μl egg/min.
	3/6	473	18.32	18.50	1.05×10^{-5}	2.4×10^{-3}	4.35×10^{-3}
	6/6(1)	455	18.52	18.55	1.03×10^{-5}	—	—
I	6/6(2)	506	18.55	18.75	1.22×10^{-5}	2.35×10^{-3}	4.7×10^{-3}
	8/6	463	17.88	18.22	1.14×10^{-5}	2.3×10^{-3}	4.65×10^{-3}
II	5/6	570	18.21	18.35	0.83×10^{-5}	—	—
	11/6	323	—	19.83	0.75×10^{-5}	2.0×10^{-3}	3.75×10^{-3}
III	10/6	460	—	—	—	—	—

I, Fertilized eggs. II, Unfertilized eggs. III, Control exp.

fore the accidental loss of glass the stopper fitted the diver chamber over a distance of 3.75 mm. This distance was reduced to about 3.1 mm. The diver thus modified was floating at a pressure not too different from that used in the biological experiments (*cf.* Table II) and the readings represented by the hollow circles were obtained. Then the diver was removed from the vessel and the stopper was pulled about one mm. back in order still further to reduce the diffusion barrier in the diver's neck. The diver was set afloat again and the readings represented by black circles were obtained. The (small) difference in level between the control curves and the 0-line is accepted as representing a maximum of diffusion of gas into this diver in all experiments.

In Table II the respiration intensity for each egg is measured (at time one hour) by the distance between experimental and control curves. Also the cell volume is given, based on direct measurements of two cell diameters or on an average diameter for at least four control eggs.

The respiration intensity here measured for single eggs in early cleavage stages

(1.0–1.2 $\times 10^{-5}$ μl/egg/min.) compares well with results reported in 1944 for single *Ciona* eggs by Holter and Zeuthen (*ca.* 1×10^{-5} μl/egg/min. at 22.5°), also when temperature differences and possible volume differences between eggs are taken into account. The present experiments were run at a 4° lower temperature than used in 1944 but with eggs which on an average seem to have been slightly bigger than in 1944. Also for the unfertilized eggs the over-all rates check with the earlier report.

Discussion

Out of a total of 10 experiments on single dividing marine eggs (4 *Ciona* eggs, 6 *Psammechinus* eggs) 6 showed respiratory cycling of much the same kind (*Psammechinus* 25/6, 29/6, 1/7, Ciona 3/6, 6/6 (2), 8/6). A seventh (*Psammechinus* 22/6) showed the same kind of cycling during divisions 2 and 3. The atypical experiments are the following: *Psammechinus:* one experiment (24/6) showed strong respiratory variation before the 8-cell stage, and one (22/6) considerable variations between 16- and 64-cell stages; one experiment (20/6) was inconclusive. One *Ciona* experiment (6/6 (1)) showed considerable early variations, but was interrupted early.

Concerning the deviating experiments it should be stressed that sometimes (*cf.* control 4/7) readings within the first hour or so are uncertain. The reasons are imperfectly understood. However, the always possible presence of such technical difficulties places considerable doubt on all non-reproducible deviations from the general findings, and the author is therefore unable to decide whether the results on 6/6 (1) and on 24/6 should be referred to errors in the technique or to the respiring cells themselves. On 22/6 the curve for the early part of the experiment seems to indicate stability of the system. However, by about the time of the 4th division the respiratory rate suddenly increased steeply and the respiration ended up so intense that the scale was used up after four more readings. Then the manometer was reset, *i.e.*, the pressure in the system was lowered and the diver was equilibrated with the indicator air bubble back in the upper end of the scale. The two first readings after this showed low rate of respiration but then the rate accelerated much the same way as before. Again, one is unable to decide between the egg or deficiencies in the apparatus as the cause. A leakage developing in stop-cock d, Figure 2, and re-developing after the stop-cock was operated with the resetting of the manometer, *might* perhaps result in effects as those recorded.

For *Ciona* two experiments on unfertilized eggs are available. One of the two (11/6) might perhaps be fitted with a cycling curve, but the author feels that the least committing interpretation is absence of cycling. There are independent reasons for not depending too much on the experiment on 11/6. It was run at such a low pressure (*cf.* Table II) that the manometer had to be reset too often, as demonstrated by the many gaps on the curve.

Had all runs on *Ciona* eggs, fertilized and unfertilized, been fitted with smooth, non-cycling curves, we should evidently have found more scattering around the curves for dividing, fertilized eggs than for non-dividing, unfertilized eggs. This suggests that the respiration in fertilized eggs is subject to variations not present in unfertilized eggs, and where possible the curves are drawn to suggest that these variations are rhythmic and timed with the mitoses.

It seems doubtful whether the present techniques—as also the one of Scholander *et al.* (1952)—is reliable enough accurately to record the respiratory variations in every run, and many more control experiments with unfertilized eggs would have been necessary if we were to predict the frequency with which an experiment with fertilized egg would fail to demonstrate a rhythm actually present. It is strongly emphasized that in this situation the degree of reproducibility of the rhythmic phenomenon itself remains our main control. The type of cycling respiration which is demonstrated throughout the curves for the experiments on 25/6, 29/6, 1/7, in part of the curve 22/6 (all for *Psammechinus*) and on 3/6, 6/6 (2) and 8/6 (for *Ciona*) the author considers as reproducible as can be expected in view of the technical difficulties involved. They largely confirm results he has previously obtained on single frog eggs and in mass runs on different marine eggs. All deviating observations, discussed in detail above, he considers interesting but quite inconclusive.

Scholander *et al.* (1952) published a material of 19 runs on four different species of single dividing eggs which were studied for rhythmic respiration. As far as can be gathered from the description of Scholander *et al.* a few eggs show definite respiratory cycling, one even very strong cycling; 10–11 eggs show definite cycling with an amplitude much the same as found by the present author. However, the authors state that it almost vanishes after the two or three first divisions. Five experiments are inconclusive as to cycling, or it is concluded that there is no cycling. Of these 5, the 4 were with Urechis eggs which show the lowest rate of respiration of the eggs studied. The most constant cycling was in single Dendraster eggs which on an average respire more intensely than any of the other three species of eggs studied. One exceptional case of an egg (*Strongylocentrotus purpuratus*) showing abnormally intense cycling is suggestive. However, this egg was definitely sick. It developed very slowly and in an asynchronous way (*cf.* Fig. 5 of this paper).

On the basis of this material Scholander *et al.* concluded that respiratory cycling is observed, but (p. 197): "We must emphasize, however, that cell division in individual cells very often takes place without any demonstrable cycling. Quite regularly, therefore, the energy requirements for the different phases are apparently fitted nicely together within the limits of a steady constant flow of oxidative processes." In the absence of experiments with objects which can be expected to show non-rhythmic respiration the reproducibility of any type of rhythmicity is the only control, and to the present author the stated absence of such reproducibility in the material of Scholander *et al.* does not invite conclusions as the above quoted. First, proof is required that the method will reproduce constant rhythmicity, if present, in every run. Substantiated doubts whether the method of Scholander *et al.* will do this have been expressed on a previous occasion (Zeuthen, 1953a). To the present author all evidence, viewed together, indicates as good reproducibility as for technical reasons can be expected of the phenomenon of respiratory cycling in cleaving eggs.

Scholander *et al.* also conclude (p. 197) that: "In our experiments the cycling is always strongly damped. It almost vanishes after the two or three first divisions." They further write (p. 197): "Zeuthen (1949) has found that later on cycling increases very markedly. Even if it can be demonstrated that this late cycling also occurs in single eggs, there will still be a minimum of cycling after

the second or third division, and hence there can be no simple correlation between the wave amplitude and the steadily increasing number of cell divisions." The views previously expressed by Zeuthen (1949) are these (p. 316): Up to the stage of division 4–5 in *Psammechinus miliaris*, division 6–7 in *Dendraster excentricus* and in *Strongylocentrotus franciscanus*, limit unknown for frog and Urechis the extra respiration is a function of the constant mass of the embryo rather than of the number of cells dividing. But after this stage has been passed the extra respiration becomes more closely a function of the number of cells dividing than of the mass of the embryo. The "extra respiration" of each division was

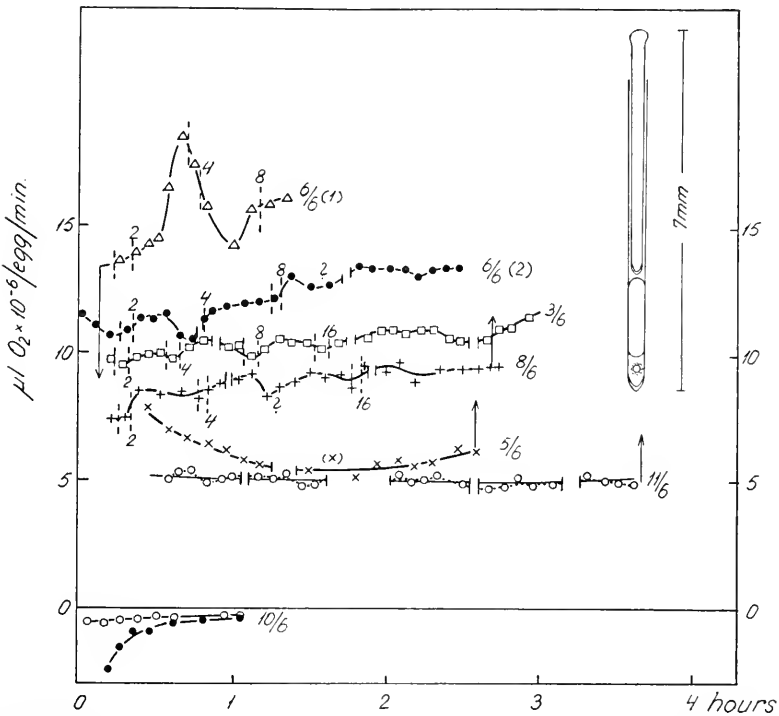

FIGURE 6. Four experiments (3/6, 6/6 (1), 6/6 (2), 8/6) on fertilized dividing *Ciona* eggs, two (5/6, 11/6) on unfertilized eggs, and two control runs (10/6) without eggs.

defined as the small areas, from one minimum on the respiration curve to the next, between the respiration curve and a smooth auxiliary line drawn to touch all minima.

The first half of the above statement, about constancy of the respiratory cycling in the early divisions, is confirmed by the present experiments. The author finds no evidence of decreased cycling after the first 2–3 divisions. On the other hand, the increased cycling with the late divisions is only slight. However, it was commented already in 1949 that in this latter respect different eggs behave differently. In the situation now established it should be pointed out that within the single egg asynchrony of divisions begins with divisions 4–5 and tends to become pro-

gressively worse later, even though a statistical rhythm persists throughout cleavage (*cf.* Zeuthen, 1951; Holter and Zeuthen, 1955). Slightly increased respiratory cycling by the time when asynchrony sets in is therefore indicative of definitely increased cycling in the single blastomeres.

Further discussing a point raised by Scholander *et al.*, Claff (1953) expressed doubts about one of Zeuthen's main summary points (1951) which is (p. 66): "In the segmenting egg the oxygen consumption follows a rhythm which throughout segmentation correlates with the mitoses." However, he quotes evidence only from 1949 and 1950, not from the paper from which the summary point is taken. This evidence (Zeuthen, 1951; Holter and Zeuthen, 1955) is the following: Mitotic rhythmicity occurring throughout 10 successive cycles was demonstrated in batches from single females of *Psammechinus microtuberculatus.* It was stated that increasing rates of metabolism occurred whenever a considerable majority of the cells were in mitotic stages characterized by having nuclei with nuclear membranes. For single eggs it was stated that asynchrony between divisions progresses from

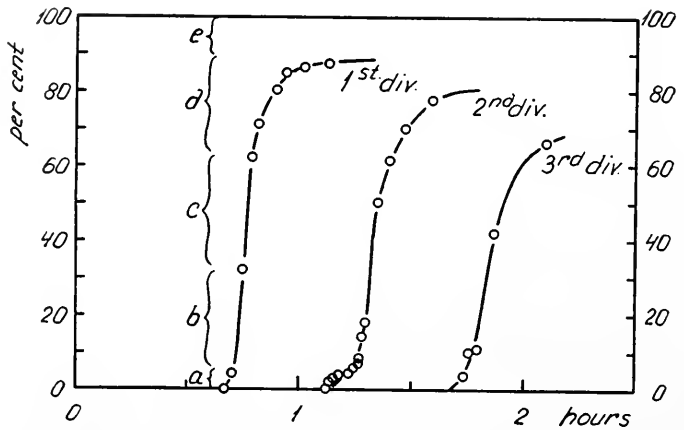

FIGURE 7. Unequal distribution of division frequencies in the 1953 Kristineberg material.

16-cell stage on, but a manifest statistical mitotic rhythm persists throughout cleavage. Scott and Fox (1952), quoted by Claff in support of his views, do not disprove the presence of a statistical division rhythm throughout cleavage in batches of Arbacia eggs, and they did not measure respiration. Actually, their findings can very well be interpreted to agree with Zeuthen's data (1951) demonstrating a statistical division rhythm.

Nevertheless, it is of course true that the degree of synchronization in batches cannot be expected always to be equally good. During the summer 1953 the author had intended to do mass runs on *Psammechinus miliaris,* Kristineberg Z-form, and to study the influence of inhibitors on the respiratory rhythmicity. However, respiratory cycling was only occasionally observed. This was nothing new or surprising because it happens whenever the egg material shows poor synchronization of cleavage. Usually this difficulty can be overcome by selection of suitable females, but not this year.

The degree of synchronization of divisions was then investigated in the follow-

ing way. From a batch of fertilized eggs (one female) showing more than 90% fertilization, with nicely lifted membranes, 100 fertilized eggs were selected with a Holter braking pipette within three minutes after fertilization. The curves of Figure 7 give the relation between time after fertilization and per cent of cells entering the first, the second, and the third division. The three curves demonstrate progressively more and more unequal distribution of division frequencies, with 30% being excessively delayed already in the first division; the number of eggs which at all divide decreases with each new division. The eggs entering the first division were separated into five groups (a–e incl.), a representing those which first enter division, d those which cleave with most delay. Group e (seven cells) was discarded. These eggs divided directly into four cells. Whereas group a developed nicely to swimming blastulae, groups b, c, d stopped development at earlier stages and the earlier, the more first division was delayed. Six hours after fertilization all eggs in group a (four eggs) were young blastulae (swimming next morning); in group b (28 eggs) after six hours fourteen were young blastulae, six less advanced "morulae" and eight were eight-cells. Next morning thirteen were swimming blastulae, the others had not advanced further. Six hours after fertilization, group c (31 eggs) were "morulae" and did not develop further; group d (30 eggs) all remained in division stages with anything from one to eight cells. This type of experiment was repeated several times with comparable results, but the one here reported was the most striking. These observations present very interesting problems but could not be followed up because our time at the zoological station was up. It should be stated that the spawning season was not yet over; also that exceptional climatic conditions this year (extreme heat with surface temperatures approaching 25°) influenced the eggs strongly. Everybody complained about "poor material."

The curves of Figure 7 demonstrate heterogeneities in the 1953 Kristineberg material which unquestionably represent extreme tendencies which may or may not exist in all batches of sea urchin eggs. The 1947 *Psammechinus* material used at Kristineberg by the author in his first experiments on respiratory rhythms in dividing eggs was definitely better than the 1953 eggs. Nevertheless, already in 1947 it was found useful (*cf.* Zeuthen, 1950a) to select small batches of eggs for experiments as those individuals in a population (one female) which first enter first division. This secured for the 1947 respiration experiments relatively synchronously developing small groups of eggs. In later experiments on other sea urchins and on Urechis no such selection was made for the reason that representative small samples of whole batches from "good females" (picked on basis of quality of membrane lifting and of percentage fertilization) showed reproducible respiratory cycling throughout cleavage. The author accepts as likely that the eggs of those species for which the nicest respiratory cycling was observed in mass runs may also have been those which showed the highest degree of synchronization of development in whole batches. On this basis he deems *Dendraster excentricus* (1949) and *Urechis caupo* (1949, 1950c) the best material with which he has worked. Still, it was for the Naples *Psammechinus microtuberculatus* that respiratory cycling and mitotic activity was demonstrated to correlate throughout cleavage (Zeuthen, 1951).

In the following the situation demonstrated for the 1953 Kristineberg material

shall be considered extreme and atypical. We shall accept that usually (*cf.* Blum and Price, 1950 for *Arbacia punctulata*) there is a normal distribution in time of the frequencies with which eggs in a population from one female enter every new division and we shall further, for the sake of simplicity, assume that all eggs in a small population show identical respiratory variations only with some displacement of the curves in time. How much, then, shall the excursions on the respiration curves found for single eggs be blurred in records for the whole population?

In Figure 8, I, the curve for *Psammechinus*, July first, was accepted as common for all eggs in a small population of 5. This curve was re-plotted five times (a) at time intervals of three minutes, which means that there is a difference in time of twelve minutes between the fastest and the slowest egg. A full cycle lasts anywhere from twenty-eight to forty minutes. The five curves are then mixed (b, heavy curve) to give the respiration curve for the population for comparison with the curve for a single egg. In Figure 8, IIb, the same procedure is repeated, only with the curves plotted five minutes apart (twenty minutes between fastest and slowest). In the cases of Figure 8, I, II, the divisions are equally, not normally, distributed in time, *cf.* distribution diagrams; a more natural situation is constructed in Figure 8, III. In this case the two heavy curves from Figure 8, Ib, IIb, are mixed with one curve for a single egg. The resulting heavy curve for this population is indicated. In this population there is reasonably good approximation to a normal distribution of identical stages in time (*cf.* diagram to the right in 8, III). The striking fact is that although in Figure 8, III, the fastest and the slowest cells are separated by a time corresponding to 5/7 of the shortest cycles, all respiratory variations for the single eggs are recorded in the population, but of course with some damping of the wave amplitude, especially in the case of the shortest cycles. In Figure 8, III, the abscissa is chosen so that zero corresponds to zero consumption. The respiratory variations observed by the author for single frog eggs (1946) and later for early divisions in small populations of marine eggs are very much alike when shape and amplitude (*ca.* 5% of total rate) are concerned. Linderstrøm-Lang's calculations (1946) demonstrated that the records for a single frog egg may have been subject to some damping caused by diffusion in this fairly big system. The true variations may have occurred with an amplitude of 7–11%. In the case of the marine eggs the diffusion factor can hardly have been of significance (Zeuthen, 1950c), but as demonstrated in this paper there may have been damping of about the same order of magnitude (slightly different from one group of experiments to another) as in the frog egg experiments, however due to incomplete synchronization. It is now considered that the curves here published for single marine eggs (Figs. 4 and 6) represent very close approximations to actual intracellular variations in the rate of oxidation, typical probably of many types of dividing egg cells.

Figures 4 and 6 show that it is no longer possible to describe the respiration of dividing eggs as following a sine curve. Clearly, in *Psammechinus miliaris* and in *Ciona intestinalis* there is a rather sharp dip in the rate of oxygen consumption reaching a minimum around the time of every new division, especially divisions 2–5 inclusive, and rapidly again increasing after the division. With later divisions the sharp dips change into broad valleys. The respiratory rate tends to remain on a high level for some time prior to the next dip, especially so in the early

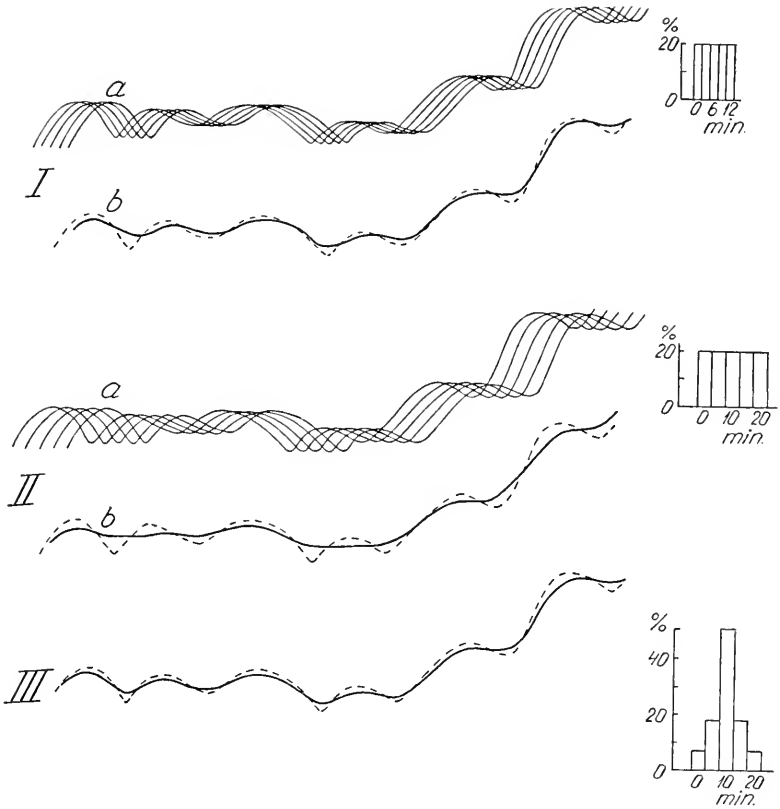

FIGURE 8. Construction of average curves for populations (heavy curves Ib, IIb, III) of eggs which are assumed to respire according to identical curves, only with some displacement in time. The dotted curves in Ib, IIb, and III are identical with the one curve replotted 15 times in Ia, IIa. The diagrams to the left in the figure show the frequencies with which the cells in the populations I, II and III enter every new division.

divisions. The cell division sometimes begins already when the respiration is decreasing, more often when it is minimum or already increasing. This confirms most of the previous results of the author (frog (1946) sea urchins (1946, 1949, 1950b, 1951) the ciliate *Tetrahymena piriformis* (1953b)) and of Scholander *et al.* (1952) (sea urchins, *Urechis*). In sea urchins, the respiratory rate (*cf.* Zeuthen, 1951; Holter and Zeuthen, 1955) seems to be minimum around late telophase, it increases sharply during inter- and prophase, remains level around mid-mitosis and drops again in late mitotic phases. Sometimes, however, the division has been observed to occur early in the phase of decreasing respiratory rate. This was for a few (not all) runs on several hundreds of *Urechis* eggs (1950c); however, in this case conditions for optical observations were admittedly much poorer than in runs with single eggs. If confirmed, this finding warns us that the respiratory cycling may appear to be more closely associated with other mitotic events than with the cytoplasmic fission (*cf,* discussion in Zeuthen, 1951; Holter and Zeuthen, 1955).

This view is corroborated by the following facts: A single fertilized frog egg which altogether failed to divide showed normal respiratory cycling (1946) and colchicin-treated fertilized eggs of *Psammechinus microtuberculatus* (1951) which did not divide showed respiratory cycling with increased amplitude and extended period.

The observed respiratory rhythm shows inverse relation to the state of development of the Mitotic Apparatus (M.A.) of Mazia and Dan (1952). The M.A. is a structure which is tied together by S-S-bridges. This may be highly important, suggesting some connection between SH-groups and respiratory cycling. However, for several reasons it is too early to make any deductions from this observation.

The work was supported by a grant from the Eli Lilly Foundation. The author is indebted to the late Professor Nils Holmgren and to Dr. Gunnar Gustafsson, both of Kristinbergs Zoologiska Station, for the many ways in which they facilitated this work, and for the hospitality they offered.

Summary

1. In single fertilized marine eggs (*Psammechinus miliaris, Ciona intestinalis*) the rate of respiration shows a temporary decrease (5–10%) and reaches a minimum every time the egg divides. The findings were reproduced in 6½ experiments out of 10. The method is not yet well enough controlled to permit definite statements as to whether or not deviating experiments should be referred to insufficient technique or to the eggs themselves.

2. The respiratory rhythm demonstrated resembles the one previously observed in the frog egg, in the *Urechis* egg and in several echinoderm eggs.

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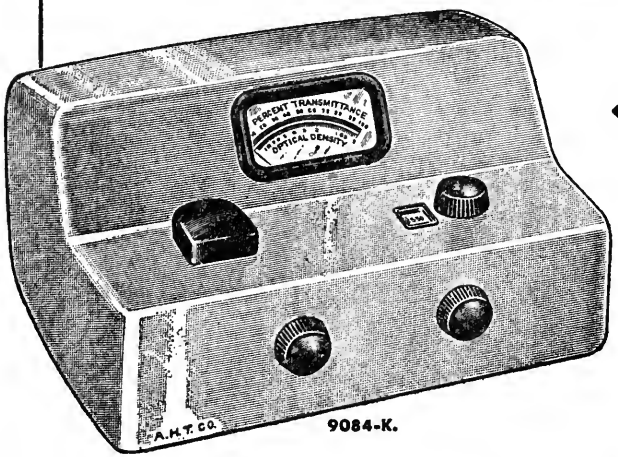

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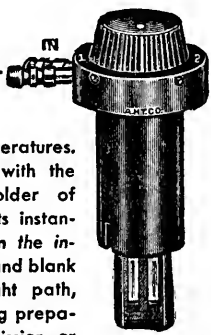

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