# THE RISING RATE OF RESPIRATION IN DEVELOPING EGGS

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# THE RISING RATE OF RESPIRATION IN DEVELOPING EGGS

## I. Introduction

In all animal eggs in which the respiration at early developmental stages has been studied it has been found that the rate of oxygen consumption rises as development proceeds. The phenomenon of the rising rate is characteristic of that part of the development which includes cleavage stages, gastrulation, and early differentiation. During this period there is practically no change in mass. Nothing is taken in from the outside in the case of marine eggs except water, inorganic salts and oxygen; water and oxygen in fresh-water eggs; and only oxygen in terrestrial eggs. During the succeeding stages of development, which for many animals may be designated as the larval period, the rate of respiration, insofar as it has been studied, does not exhibit any special character, but may rise, fall, or remain constant, or, in some cases, alternately rise and fall. For this reason, a rising rate of respiration is observable only in those forms where the eggs can be studied shortly after fertilization. Where only relatively late stages are obtainable this phenomenon cannot always be demonstrated. Thus, in the chick, first studied by Hasselbalch (1900), the rate of respiration per unit mass of embryo drops throughout the period of incubation. As is well known, the chick egg undergoes considerable development in its passage down the oviduct of the hen, having already completed gastrulation at the time of laying. Similarly, the rate of oxygen consumption of the eggs of Ascaris was found

by Fauré-Fremiet (1913) to decrease slowly from the 24th hour of development on. The respiration at earlier stages was not reported.

The rising rate of respiration has been demonstrated in the eggs of a wide diversity of forms. Among the earliest work was that of Godlewski (1900) who reported an increasing respiratory rate in developing amphibian eggs. In 1908, Warburg showed that the respiratory rate of sea urchin eggs slowly rose during early cleavage; his data stop at the 32-cell stage. In the same year, Buglia reported an increasing rate in the eggs of the sea-hare, Aplysia. None of these early measurements were very accurate, however, and could only serve to indicate the general trend of the respiration. In 1915, Warburg applied the Barcroft manometric technique to eggs, thus enabling for the first time gas exchange measurements to be made at short intervals of time. In his 1915 paper, Warburg repeated his earlier work on the sea urchin, verifying his previous finding and extending the measurements as far as gastrulation (the 24th hour after fertilization). His data show that the rate of oxygen consumption rises in an almost linear fashion from the one-cell stage to the gastrula. The total increase over the whole period was almost 400 percent. The manometric technique has since come into almost universal application. The rising respiratory rate was first shown in fish by Hyman (1921), working with Fundulus; in insects by Fink (1925); in Chaetopterus by Whitaker (1933); in Ciona, Dendraster, Strongylocentrotus, and Urechis by Tyler and Humason (1937). These results have been verified by a

number of other workers. Irregularities in the rate have been found in fish and insect embryos. In the former, according to Hyman (loc. cit.), the rate alternately rises and falls during development, with peaks at 2, 4, and 10 days after fertilization. This is confirmed by the recent measurements of Trifonova (1937) on the developing perch. In insects, the respiratory rate drops during pupation (Fink, loc. cit.) and during diapause (Bodine, 1929).

The present work was undertaken in order to elucidate the nature of the respiratory increase and its relationship to developmental processes in the eggs of two marine invertebrates --Urechis caupo and Strongylocentrotus purpuratus. Such a study is of importance in connection with the problem of the energy changes in development which has been investigated in recent years principally by Tyler (see review, in press). It was first necessary to measure the oxygen consumption over a longer period than has heretofore been done in these forms, in order to determine the way in which the rate of respiration varies throughout development and where, if at all, the rate finally ceases to increase. In order to determine the nature of the substrate burned at various developmental stages, the respiratory quotient was measured in Urechis. To examine the relationship of respiration to synthetic processes the nitrogen metabolism of this form was also studied, especially with regard to the possible synthesis of protein. To investigate the relationship of the rising rate to increase in nuclear material, experiments on the effect of polyspermy were performed. To ascertain its relationship to

morphological changes, the effect on respiration of reversibly stopping development at a particular stage was studied. To decide what limits the rate at different stages, experiments were performed in which the respiration was stimulated by dinitrophenol at various times in development. The substrate burned by the dinitrophenol-stimulated respiration was determined.

It will be shown that the changes in the rate of respiration which occur as development proceeds bear no immediate relationship to simultaneously occuring morphological changes, but can be related in part to changes in the available substrate within the egg and in part to changes in the respiratory catalysts. The latter may mean either that new catalysts are synthesized as development proceeds or that all are present at the start but must be brought into action by being placed in more favorable relationship with the substrate. The evidence points to the second alternative. Evidence will be presented to indicate that metabolic changes in the embryo precede the corresponding morphological changes. The hypothesis will be suggested that metabolic changes are involved in the process of morphological determination and that the rising rate of respiration is related to the increasing spatial differentiation of elements associated with the process of determination of embryonic parts.

## II. Materials

The greater part of the work to be presented was done with the eggs of Urechis caupo (Fisher and MacGinitie, 1928). This worm occurs in the mud flats at several places along the coast of California. The animals used for these experiments were collected at Anaheim Slough, near Long Beach, California and at Morro Bay, California. The worms inhabit U-tubes in the mud, two to three feet deep. These tubes are almost invariably below the average low-tide level. Because of this fact, the animals can be collected only at exceptionally low tides and at certain times of the year. They are easily kept in the laboratory over periods of many months. The usual practise in this laboratory is to place them in glass tubes on the bottom of aquarium tanks through which fresh sea water is continuously run. They can be kept for shorter periods of time (1-2 weeks) in jars of sea water through which air is constantly bubbled. Males and females are kept in separate tanks. Under laboratory conditions the animals do not spawn, so that eggs and sperm can be obtained the year around.

The sea urchin, Strongylocentrotus purpuratus, which was also used, was collected from the rocks along the shore at Corona del Mar, California. The animals spawn intermittently over a period extending from December to June. At other times of the year and during the early parts of the inter-spawning periods they contain no mature germ cells. They can be kept in the laboratory in tanks with continuously changing sea water. Where sea water is not available they can be maintained for a short time in the cold, if kept moist. Since a large part of

the present work was done where running sea water was not available, Urechis was used wherever possible, it being more easily maintained under these conditions.

## III. General Methods

Handling of the eggs.

The eggs of Urechis are contained in six modified nephridial sacs. They are removed by the insertion of a fine-pointed pipette into one of the six gonopores. The tension on the sac forces the eggs into the pipette. Eggs so obtained are placed in a finger bowl and washed in several changes of filtered sea water. This is accomplished by filling the dish with sea water, allowing the eggs to settle, and siphoning off the supernatant. Washing is necessary in order to remove the slime which often comes off the surface of the animal during the removal of the eggs and the excess jelly which seems to surround them. This material, aside from being undesirable where respiration measurements or analyses are being made, often prevents fertilization. After washing, the eggs are fertilized with several drops of a dilute sperm suspension. Sperm is removed from the males in the same manner as are the eggs from the females. The sperm is kept "dry"--i.e., undiluted--until ready for use, since sperm suspensions rapidly lose their fertilizing ability when diluted. Only suspensions in which at least 95 percent of the eggs were fertilized were used in the experiments. With Urechis, 100 percent fertilization is easily obtained. Following fertilization, the eggs are again

washed several times to remove excess sperm. One of two procedures is then followed: if the eggs are to be used immediately for respiration or analysis, they are concentrated by allowing them to settle in a beaker or a tube which is then made up to the desired volume. If later developmental stages are required, the eggs are partitioned among a number of finger bowls and allowed to develop at room temperature until the desired stage is reached. They are then pipetted off (if the swimming stages are used only top-swimmers are taken), washed again, and concentrated to a small volume. In the case of swimming embryos, the washing and concentrating are done in a pair of No. 3 sintered glass filters. It was found that the strong swimming movements of the larvae prevent their being driven down in a centrifuge unless forces are applied which are sufficient to injure them. Such treatment results in irreversible deformation of the embryos and weakening, or loss, of the swimming action.

For the eggs of Strongylocentrotus, the procedure, with a few modifications, was the same as that outlined above. The animals are opened by cutting away the lower part of the test with scissors along a line about 1 cm. below the equator. The gonads, in the case of females, are removed with a spoon or spatula and placed in a net of fine bolting cloth suspended in sea water. The eggs extrude from the ovaries and drop through the cloth, leaving the ovarian tissue and visceral fragments behind. In the case of males, the testes are placed in a Syracuse dish containing a small amount of sea water and the sperm diluted up when needed.

In culturing sea urchin eggs in large quantities it was

sometimes found advisable to agitate the eggs in order to insure a sufficient supply of oxygen. This was accomplished by placing the culture dishes on a mechanical agitator adjusted so as to impart just enough motion to keep the eggs rolling along the bottom of the dishes.

In most of the experiments, the final washing of the eggs was done in buffered, carbonate-free sea water, as described by Tyler and Horowitz (1937a), and this was used as the final suspension medium. Buffered sea water was used in order to eliminate changes in pH occasioned by the removal of $CO_2$ and carbonates by the alkali in the manometer vessels. Aside from the possibility that changes in pH might influence the respiration directly, constancy of pH was especially necessary in the dinitrophenol experiments. Tyler and Horowitz (1937b) have shown that the action of substituted phenols is dependent on the pH of the external medium.

Carbonate-free sea water is prepared by acidifying sea water to pH 2.5 (6 cc. 1 M HCl per liter of sea water). This is then equilibrated with the atmosphere by bubbling air through it for 24 to 36 hours. To this acidified, equilibrated sea water analytically pure glycylglycine is then added to a concentration of 0.015 M, plus sufficient alkali to bring it to the desired pH of ca. 8.0. As shown by Tyler and Horowitz, glycylglycine buffers in the range pH 7.5-8.5 and is non-toxic to eggs in the concentration employed.

The reliability of respiration data on marine eggs obtained in carbonate-free sea water has recently been questioned

by Laser and Rothschild (1939). These authors report that in experiments with Psammechinus miliaris eggs the rate of respiration in Warburg vessels containing alkali is lower than in vessels to which alkali has not been added. They ascribe this effect to an inhibitory action of bicarbonate-lack on the respiration, resulting from the fact that the alkali, in removing $CO_2$ from the suspension medium, displaces the carbonic acid/bicarbonate equilibrium toward the side of carbonic acid. This finding, if confirmed, must result in a radical change in the present methods of conducting respiration experiments. In the present work, no measurements were made in which alkali had been omitted from the vessels. Since most of the measurements, however, were made in carbonate-free sea water it was deemed advisable to compare the respiration of eggs in carbonate-free and in ordinary sea water, alkali being present in both cases. This is shown in Table I. Both of these media are bicarbonate-deficient, in the sense of Laser and Rothschild. They differ widely, however, in their bicarbonate content, even in the presence of alkali. Whitaker (1933a) has reported that $CO_2$ equilibrium between sea water not containing eggs and the alkali in Warburg vessels is not attained even after 20 hours. (The vessels were shaken for the first 4 hours). He points out that in the presence of eggs the attainment of equilibrium must be even slower due to the production of carbon dioxide, and that a steady state in $CO_2$ exchange may be reached between eggs, sea water, and alkali. In view of the variability of the eggs themselves, the differences in Table I cannot be regarded as significant, except for the 3rd and 4th

Table I

Comparison of rates of oxygen consumption in sea water and carbonate-free sea water. Values given in $mm.^3$ $O_2$ per $10^5$ eggs per hour. Temperature, 20° C.

| | Urechis | | Strongylocentrotus | |
|---|---|---|---|---|
| Hours from fertilization | s.w.[1] | $CO_3^{--}$ free s.w. | s.w. | $CO_3^{--}$ free s.w. |
| 3 | 5.61 | 7.65 | 7.36 | 8.17 |
| 4 | 6.42 | 8.19 | 9.62 | 9.23 |
| 5 | 8.01 | 8.73 | 10.44 | 10.61 |
| 6 | 11.71 | 11.60 | 11.40 | 12.59 |
| 7 | 16.00 | 16.14 | 12.52 | 13.03 |
| 8 | 19.90 | 19.84 | | |
| 9 | 23.80 | 23.70 | | |

[1] From Tyler and Horowitz (1938a), corrected to $10^5$ eggs and 20° C.

hours in the case of Urechis. Here the respiration in carbonate-free sea water is higher than in ordinary sea water, contrary to the finding of Laser and Rothschild. To what extent the respiration would have been altered had no alkali been used cannot be stated positively. Nevertheless, it seems extremely likely that a bicarbonate effect, if such exists, would manifest itself in suspensions differing so widely in bicarbonate content as those presented in Table I. The opposite view entails the assumption of a high bicarbonate threshold, below which the respiration is unaffected by changes in the bicarbonate concentration, and above which the rate of respiration rapidly increases with increasing

concentrations. No such threshold, to the present writer's knowledge, has ever been demonstrated.

Manometric

The respiration measurements were made by the Warburg manometric technique. This technique is described in Dixon (1934). Four types of manometer vessels were used in the experiments (see Figure 1):

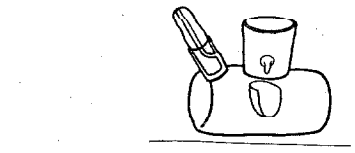
(1). Large cylindrical vessels, taking approximately 7 cc. of egg suspension and with vessel constants around 1.1 were used for ordinary measurements.

(2). Small cylindrical vessels, described by Tyler (1936) were used when only small amounts of material were available. These took 1.1 cc., with vessel constants around 0.2.

(3). Conical vessels of the usual type, with one side-arm, were used in the dinitrophenol experiments and in other instances where it was desired to add a substance to the egg suspension without interrupting the experiment. These held approximately 4.5 cc. of suspension, with vessel constants near 1.2.

(4). Conical vessels with two side-arms were used in the measurement of respiratory quotients. Their use will be described in connection with the experiments. They took 4 cc. of suspension, with vessel constants for oxygen of ca. 1.6 and carbon dioxide constants of ca. 1.9.

All measurements were made at 20° C. The water bath was kept



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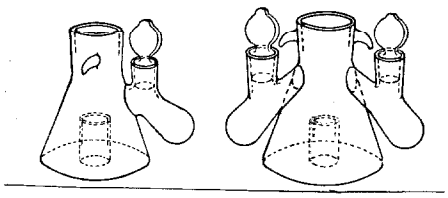

3 4

FIGURE 1

Types of manometer vessels used in these experiments. Types 1 and 2 are similar in shape; type 1 is larger and is without the small stopcock shown in the drawing. Relative sizes of vessels not as indicated.

(Vessel 1,2 reproduced from Tyler, 1936a; 3 and 4 from American Instrument Co. Bulletin 801.)

constant within 0.01 degree. The rate of shaking of the manometers was varied from 70 to 90 round-trips per minute, depending upon the size of the vessels, the concentration of eggs, and the nature of the material. The amplitude of shaking (half arc) was varied between 2 cm. and 4.5 cm. It was found, in agreement with other workers, that sea urchin eggs are very sensitive to violent shaking. The eggs of Urechis, on the other hand, are more resistant in this as in other respects to experimental treatment, and little difficulty was found in causing them to develop normally in the manometer vessels. In all the experiments, the general practise was to examine the eggs at the end of the run and to eliminate from the calculations those cases where there was an indication of damage to the embryos due to shaking or other factors.

The eggs were distributed to the manometer vessels by means of automatic, calibrated pipettes. These pipettes can be filled rapidly to a known volume, thus insuring accurate sampling of the egg suspension. In an actual test to determine the pipetting error the following results were obtained:

Table II

Variability in weight of water delivered by automatic pipette No. 3. Temperature, 20° C.

| Sample | Wt. (grams) | ΔW |
|---|---|---|
| 1 | 1.080 | 0.003 |
| 2 | 1.080 | 0.003 |
| 3 | 1.073 | 0.004 |
| 4 | 1.071 | 0.006 |
| 5 | 1.079 | 0.002 |
| 6 | 1.075 | 0.002 |
| 7 | 1.080 | 0.003 |
| Mean | 1.077 | 0.0033 |

Mean deviation = 0.306 percent

After placing the vessels in the bath, a period of 10 to 20 minutes was allowed for equilibration. This is necessary for the attainment of temperature equilibrium and of $CO_2$ equilibrium between the alkali in the vessels and the other contents.

Counting

Counts of the number of embryos were made in most of the respiration experiments and in the analytical work. This was done in the following manner: from the egg suspension to be pipetted into the vessels, or to be used for analysis, a sample of known volume was removed and diluted with a known volume of sea water. Successive aliquot portions were then removed from the diluted sample, the embryos killed by addition of a drop of Bouin's solution, and counted directly under the microscope. A pipette drawn out to a fine point was used to pick the embryos up, one by one, as they were counted. The average of a number of such counts was taken, and from this the number of embryos per cc. of the original suspension was calculated. The sampling error involved in this method is concerned almost entirely with the samples actually counted and is equal to $\sqrt{m/N}$, where m is the mean number of eggs per sample and N is the number of samples. The percentage error is equal to the reciprocal of the square root of the total number of eggs counted.[1] The error in the sample taken for dilution is neglible,[2]

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[1] Given a large population, distributed at random, from which successive small samples are drawn, the probability $P_n$ that a given sample will contain exactly n individuals is given

in comparison with the error in the sample taken for counting.

Since, on the average, 500 eggs (embryos) were counted in a

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by the Poisson (1837) exponential function,

$$P_n = \frac{m^n e^{-m}}{n!}$$

where m is the mean number of individuals per sample and e is the base of natural logarithms. It is desired to know the standard deviation of the mean, $\sigma_m$, of the distribution so obtained. It can be shown that the second moment about the mean of a Poisson distribution is equal to the mean (see Rietz, 1927). Since the standard deviation is equal to the square root of the second moment, we have

$$\sigma = \sqrt{m}$$

The standard deviation of the mean is defined by

$$\sigma_m = \frac{\sigma}{\sqrt{N}}$$

where N is the number of samples taken. For a Poisson distribution, then

$$\sigma_m = \sqrt{m/N}$$

The percentage error is

$$\frac{\sigma_m}{m} = \frac{1}{m}\sqrt{m/N}$$

$$= \sqrt{\frac{1}{mN}}$$

$$= \frac{1}{\sqrt{M}}$$

where M is the total number of individuals counted.

2 This follows from the fact that in the sample taken for dilution M is a very large number (of the order of $3 \times 10^4$) and $1/\sqrt{M}$ is therefore very small.

determination, the sampling error is less than 5 percent.

## IV. Rate of Oxygen Consumption During Early Development of Urechis

### Historical

The respiration of the unfertilized egg of Urechis caupo has been studied by Tyler and Humason (1937). The rate of oxygen consumption was found to vary over a wide range, depending upon the length of time the animals had been kept in the laboratory and upon the locality from which they originated. The range of variation, as reported by these authors, was from 1.38 $mm.^3$ $O_2$ per hour per mg. egg N to 5.07 $mm.^3$; or, taking $1.40 \times 10^{-5}$ mg. as the nitrogen content of one egg (see Part VII), from 1.93 to 7.08 $mm.^3$ $O_2$ per hour per hundred thousand eggs. In the same paper Tyler and Humason plot the oxygen uptake of fertilized Urechis eggs for the first 10 hours of development. It was found that fertilized eggs respired at a constant value, regardless of the pre-fertilization rate. Their curve shows a rapidly rising rate of oxygen consumption during development, the increase amounting to 500 percent between the first and tenth hours. The only other data of a similar nature to be found in the literature are in the paper of Tyler and Horowitz (1938a). In this paper the respiration of fertilized Urechis eggs was plotted for the first 19 hours of development. Two curves are presented, one for the first 12 hours and one for the 12th to the 19th hours inclusive. The two curves are not contin-

uous, however, since the temperatures at which the eggs were reared differ in the two cases. The curves show, however, that the rate of respiration is still rising rapidly at the 19th hour.

It was desirable for the present investigation to determine the oxygen consumption of developing Urechis eggs under constant conditions and over a longer period of time than that plotted in the above-mentioned papers. It was of interest to learn where the rate of oxygen uptake ceased its rapid acceleration and to determine the general shape of the curve. More particularly, it was desired to know whether sharp changes occurred and whether or not these were associated with specific morphological changes.

Normal development

Fertilization in Urechis has been described by Tyler (1931). Cleavage and early development have been investigated by Tyler (loc. cit.) and by Newby (1932). The development is very much like that of Thalassema (Torrey, 1903), and is similar to that in annelids. Cleavage is of the spiral type. After c. 8 cleavages (256-cells) a ciliated blastula is formed which breaks through the fertilization membrane. The swimming blastula subsequently invaginates by the inpushing of the cells surrounding the antipole. The blastopore does not form the anal opening, as in echinoderms, but elongates and migrates to the future ventral side of the larva. Here it constricts off, leaving an opening which forms the mouth. The enteric cavity becomes di-

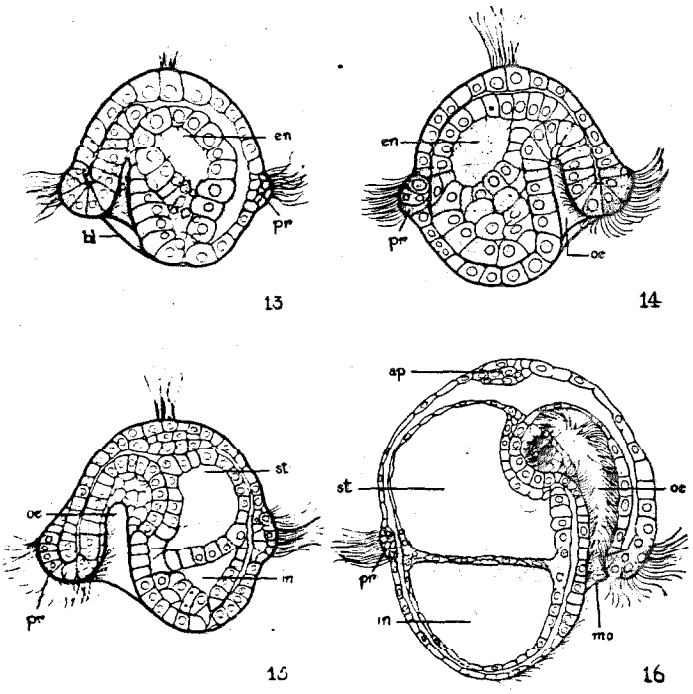
vided into esophagus, stomach, and intestine by the outgrowth of layers of cells from the wall of the cavity at about the 24th hour from fertilization (see Figure 2). The anus subsequently breaks through. Following the 24th hour, the trochophore larva enlarges and becomes relatively thin-walled (Tyler, 1931). A summary of the most important stages is presented in Table III.

Table III

Summary of the development of the eggs of Urechis caupo. Temperature 20° 1° C.

| Hrs./min. from fertilization | Stage | |
|---|---|---|
| 0/30 | 1st polar body | |
| 0/50 | 2nd polar body | |
| 1/15 | 2-cell | From Tyler (1936b) |
| 1/44 | 4-cell | |
| 2/9 | 8-cell | |
| 2/39 | 16-cell | |
| 8/0 | Swimming blastula | |
| 14/0 | Formation of enteron | |
| 16/0 | Formation of mouth and esophagus | |
| 24/0 | Formation of stomach and intestine | |

The larval period lasts several months, at least under laboratory conditions (MacGinitie, personal communication). The metamorphosis into the adult is very slow. A nine-months old worm, reared by MacGinitie, was only 3 mm. in length, but had dug a burrow for itself in the mud on the bottom of the dish, similar to those dug by the adult worms.




FIGS. 13–16.

*ap*, apical plate; *bl*, anterior portion of original blastopore; *en*, enteron; *in*, intestine; *mo*, mouth; *oe*, œsophagus; *pr*, prototroch; *st*, stomach. Normal embryos from artificially activated eggs; drawn from total mounts of preserved specimens.

FIG. 13. Sixteen-hour trochophore.
FIG. 14. Twenty-hour trochophore.
FIG. 15. Twenty-four-hour trochophore.
FIG. 16. Forty-eight-hour trochophore.

FIGURE 2

(Reproduced from Tyler, 1931)

Results

The way in which the rate of respiration varies during the development of Urechis is shown in Figure 3. This represents cu. mm. $O_2$ consumed per hundred thousand embryos per hour, plotted against time, the moment of fertilization being taken as zero. Each point is the average of 3 to 6 measurements. Figure 4 is the summation of the curve of Figure 3 and shows the total oxygen consumed by a hundred thousand embryos from the second hour after fertilization to time t, represented as the abscissa. The data from which the curves were plotted is to be found in the Appendix.

Figure 3 is a composite of a number of separate determinations made at successive intervals along the abscissa. It is impossible, unless special precautions are taken, to extend respiration measurements over a period longer than 8 to 10 hours without incurring the danger of significant errors due to the growth of bacteria in the Warburg vessels. This has been clearly demonstrated by Tyler, Ricci, and Horowitz (1938) in experiments with unfertilized Arbacia eggs. These authors showed that the rising rate of oxygen consumption exhibited by unfertilized eggs, reported by a number of different investigators, was due entirely to the respiration of bacteria contaminating the vessels. The disintegration of the eggs provided substrate on which the bacteria were able to multiply. Since even with developing eggs it is impossible to exclude the death of a few of the embryos in the course of a run, none of the present measurements were extended over longer than 8 hours. To obtain later stages, sets

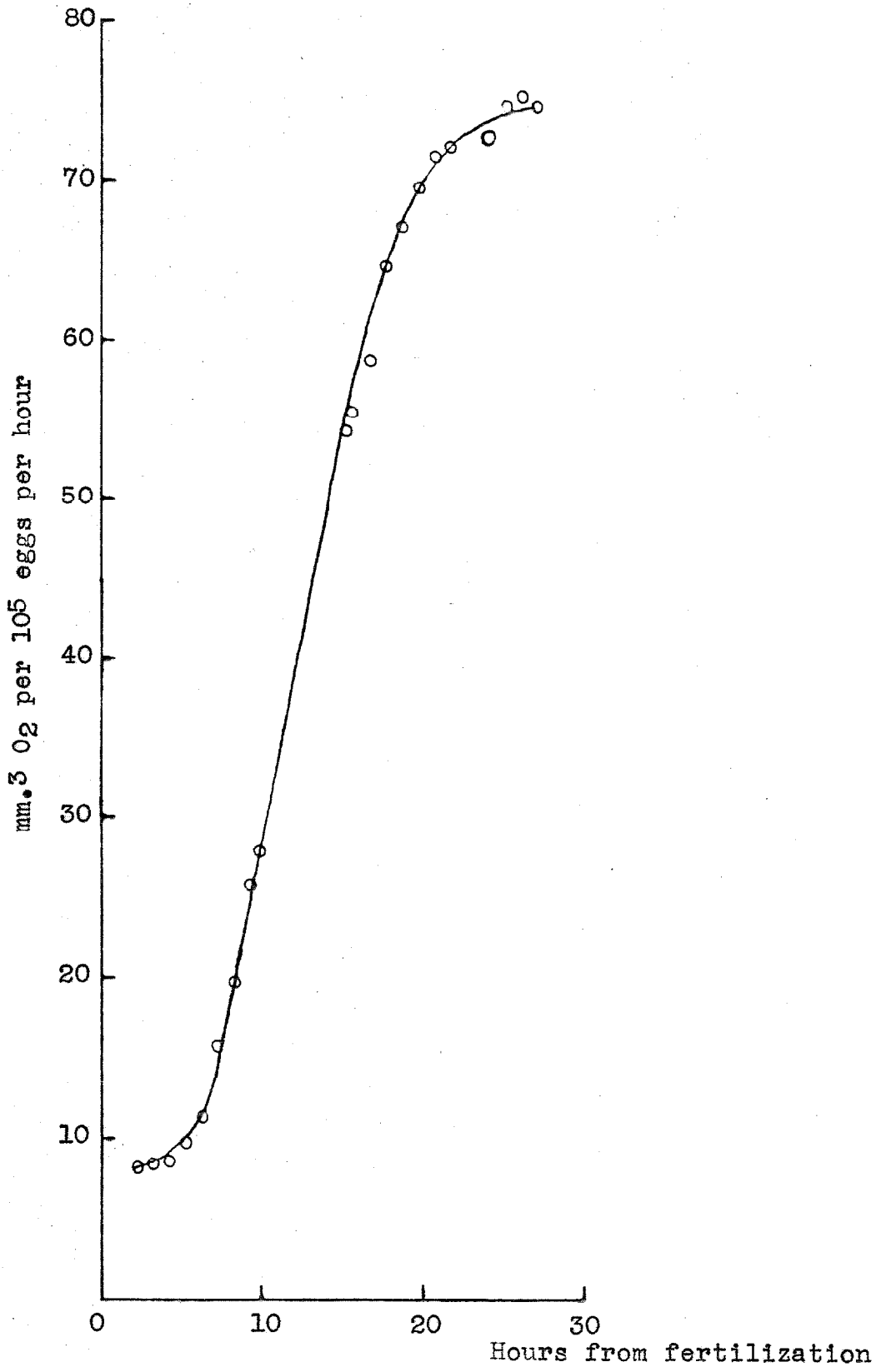



FIGURE 3

Rate of respiration of the developing eggs of Urechis caupo.

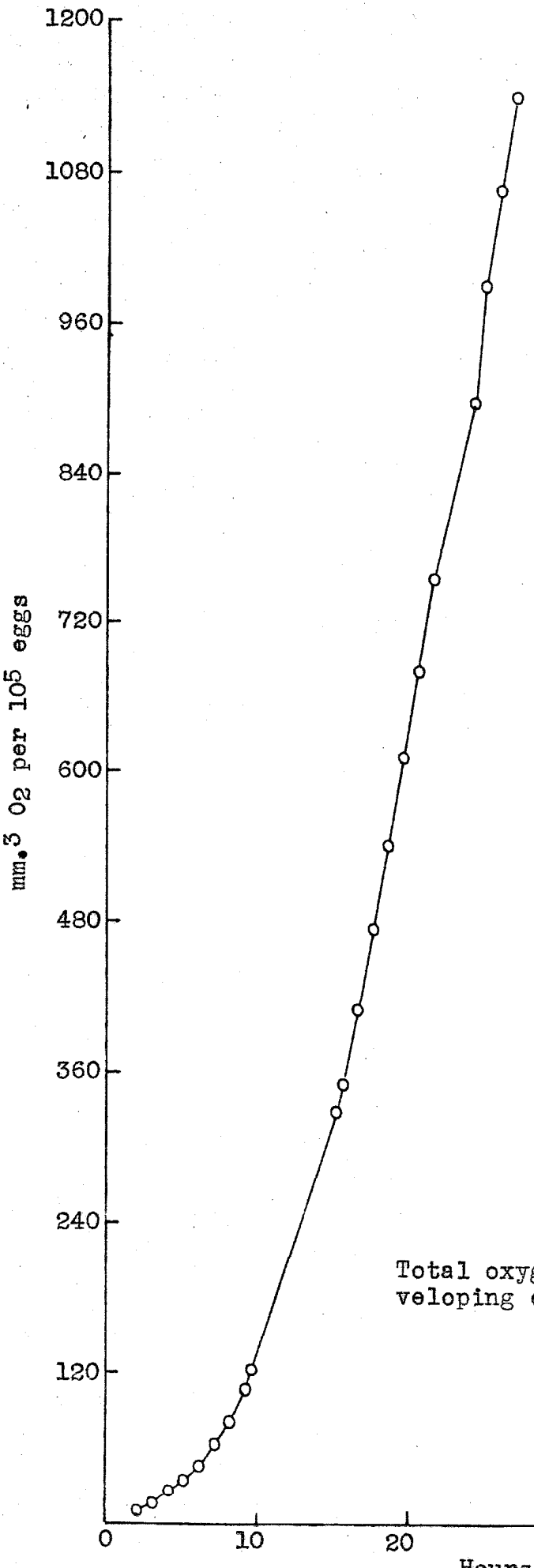



FIGURE 4

Total oxygen consumption of developing eggs of Urechis caupo.

of eggs were reared as described previously, under identical conditions, and were then used for respiration measurements. Care was taken to avoid including dead embryos, only top- swimmers being used. The absence of any significant discontinuities in the resulting data (Figure 3) justifies this procedure.

The points of Figure 3 fall along a slightly skew logistic curve. Several investigators have made mathematical analyses of oxygen consumption curves of developing embryos in an attempt to ascertain the cause of the rising rate. Bialaszewicz and Bledowski (1915) found that an empirical equation of the type $y = kt^2 + u$ fitted their data, where $y$ is the rate of oxygen consumption of fertilized eggs (frog) at time $t$ from fertilization and $u$ is the unfertilized rate. Gray (1925) fitted his data for the respiration of the sea urchin, Echinus, with the function $y = Ae^{kt}$, where $e$ is the base of natural logarithms. More recently, Atlas (1938) has fitted the respiration of two species of frog also with the equation $y = Ae^{kt}$; a change of constants must be made after the 47th hour. After the onset of rapid growth by the embryo, Atlas finds that the rate of respiration is fitted by the expression $y = Ae^{KL-c}$, where $L$ is the length of the embryo.

In the present instance, the respiration of Urechis eggs could be fitted by a function of the type

$$y = \frac{K}{1 + ke^{m_1 t + m_2 t^2 + m_3 t^3}},$$

the general equation for sigmoid curves. Nothing is to be gained by calculation of the constants. Except for purposes of accurate

interpolation, the value of empirical equations for the rate/time relationship seems highly questionable. The type of equation selected to represent the data is to a certain extent arbitrary. This is illustrated in the above example, where both a parabola (Bialaszewicz and Bledowski) and an exponential function (Atlas) are taken to depict the respiration of the frog's egg. There is, unfortunately, no known objective method for selecting the best type of mathematical expression to fit a set of data. In addition, it hardly needs emphasis that an empirical equation representing the mathematical relationships of a set of variables does not, of itself, give any insight into the causes of the phenomenon under consideration. For example, the curve of Figure 3 is typical of monomolecular autocatalytic reactions. Following the example of some authors (e.g., Robertson, 1923) one would be forced to conclude that the rate of respiration of fertilized Urechis eggs is proportional to the concentration of a certain substance in the eggs which increases in an autocatalytic manner. Schlenk (1933), as a result of his studies of the respiration of trout eggs, has accepted a similar idea as a working hypothesis. This view may be correct[1], but in the absence of any information of a more direct nature it is clearly only one of a number of possible hypotheses. Similar remarks apply to Atlas' suggestion, prompted by the mathematical

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[1] Warburg (1934) and his coworkers have shown that the rate of reduction of cytochrome in yeast cells equals the rate of respiration. Since the reduction of cytochrome is dependent upon the presence of hydrogen transporters (e.g., the yellow enzyme) in the cell, the S-shaped curve could be explained by the autocatalytic growth of one or more hydrogen transporters.

relationship of respiratory rate to tail growth (see above), that the increase in respiratory function is due to the increase in cell number. It is impossible, from the mathematical expression, to determine whether respiration is dependent on cell number, or cell number on respiration, or whether both phenomena are related through some third factor operating in the organism. A similar relationship could be drawn from the present data. Reference to Table III (p. 14) shows that from the second maturation division on, cell divisions succeed one another at close to 30-minute intervals. Since the number of cells at a given instant is equal to $2^g$, where $g$ is the number of cell divisions that have occurred, it follows that the number of cells is an exponential function of time. As indicated above, the rate of respiration is also an exponential function of time. By eliminating $t$ between the two equations, one obtains an expression relating respiration to the number of cells, covering at least the interval in which the rate of division has been determined. It is obvious, however, that such an expression has no demonstrative value.

More significance is to be attached to theoretical, as opposed to empirical, functions for the oxygen consumption data. If from a set of assumptions an expression can be derived which accurately describes the phenomenon under consideration, this constitutes evidence for the correctness of the initial assumptions. They may then be used as a working hypothesis for further investigation. Such a theoretical construction has been attempted by Gray (1927) to account for the rising rate of respiration in

Echinus eggs. He assumed the respiration to be divided into two fractions, one associated with the "growing elements" of the egg and the other with the "non-growing elements." The respiration of the "non-growing" elements remains constant, whereas that of the "growing elements" increases proportionally with the increase in these elements. From these considerations Gray obtained the equation $R = P + Qe^{kt}$, where R is the relative rate of oxygen consumption at time t from fertilization, P is the respiration of the "non-growing elements", and Q is the respiration of the "growing elements". The equation is satisfactory only for the first seven hours of development. One must assume, on the basis of Gray's argument, that after the seventh hour either the original conditions cease to operate, or new factors come into play.

More, perhaps, is to be gained by a direct study of the respiration curve itself, in order to determine whether its shape is related to stages of morphological development. This, however, will be deferred until further experiments have been described, particularly with regard to the respiratory quotient and the heat production.

The data of Figure 3 go as far as the 27th hour of development. The respiration after this time was also investigated. After repeated attempts to measure the oxygen consumption of 30-40 hour old larvae, however, it became clear that an unknown factor was disturbing the results. It was impossible to obtain reproducible measurements from day to day. The respiration showed a tendency to be distinctly higher than previously, but with

no consistency as to the extent of the increase. This was entirely unexpected, inasmuch as the measurements up to the 30th hour were readily reproducible. The suddenness of the break which seemed to occur at the 27-30th hours of development, beyond which the extreme variability in the results appeared was especially remarkable. For this reason it seemed likely that the inconsistencies were due not to any inherent variability in the experimental material, but rather to variations in the external conditions.

It was decided to investigate the possibility that the larvae normally begin to feed at about the 30th hour. It should be noted that the intestinal tract of the trochophore is open and differentiated into esophagus, stomach, and intestine at the 24th hour from fertilization. Feeding might explain the results obtained, since the bacterial population of the dishes in which the embryos were reared might vary considerably from day to day and from dish to dish, depending upon the number of dead eggs on the bottom, the length of time of storage of the sea water, etc. To determine the time at which ingestion begins, embryos were reared in flasks in which powdered carmine was suspended. The flasks were immersed in a thermostat kept at 20° C. Under the microscope the larvae are sufficiently transparent to observe dye particles within the intestine of the living form. It was found that the first embryos began to ingest carmine at the 27th hour following fertilization. By the 32nd hour the intestinal tracts in over 80 percent of the embryos were quite full of the dye particles. The details are listed in Table IV.

Table IV

Percentage of Urechis embryos showing carmine in the intestinal tract at various times from fertilization. Temperature, 20° C.

| Hrs./min. from fertilization | Percent |
|---|---|
| 27/0 | 2 |
| 28/0 | 10 |
| 29/30 | 25 |
| 33/10 | 85 |
| 35/30 | 90 |

It was thus established that the time at which variability in the rate of oxygen consumption occurs is identical with the time at which the embryos were observed to ingest visible particles from the environment. There seems no reason to doubt that under natural conditions the embryos would begin to ingest diatoms and other microorganisms at the corresponding developmental time.

Some evidence of a relationship between feeding and oxygen uptake had been obtained in measurements on trochophores. It was found in some cases that week-old larvae presented an abnormal appearance (see Figure 5) in being somewhat shrunken. These were interpreted as starved embryos. Measurements of the oxygen consumption showed that these larvae respired at a very low rate. One batch of 115 hour-old trochophores consumed only 43 $mm.^3$ of oxygen per $10^5$ larvae per hour. This corresponds to the respiration of 13 hour-old embryos.

The relationship of oxygen consumption to assimilation

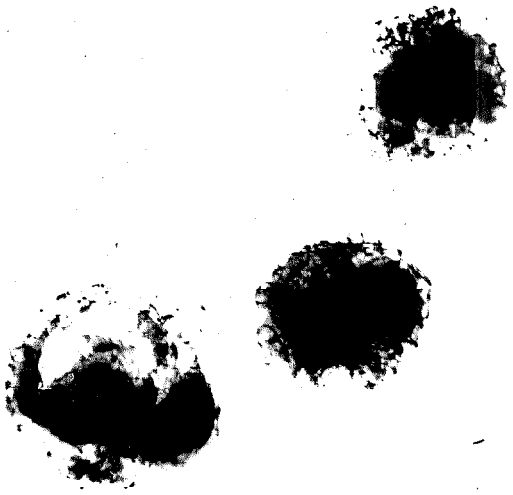

FIGURE 5

Relative sizes of normal and "starved" embryos. The two trochophores on the right are shrunken embryos, 80 hours old. The one in the lower left, taken from the same dish, is of normal size.

of food was finally established in an experiment in which the embryos were given substances approximating their natural food and the respiration measured at the same time. Trochophores were reared in finger bowls in the usual way, except that special precautions were taken to keep bacterial growth at a minimum--the sea water was changed daily, dead eggs were removed from the bottom of the dishes, etc. They were maintained under these conditions for a week in order to deplete as far as possible their own stored food supply. Four hours before the start of the measurements 15 cc. of nutriment in the form of a boiled, filtered extract of sea urchin eggs was added to half of the dishes. At the end of the four hours it was observed under the microscope that the embryos had ingested quantities of the suspended particles. The intestinal tracts of the controls were for the most part empty. Both sets of embryos were then washed, as described above, and placed in the Warburg vessels. The oxygen consumption is tabulated in Table V. It is seen that

Table V

Effect of feeding on respiration of Urechis larvae. Experimental set given extract of sea urchin eggs 4 hours before start. Measurements begun 172 hours after fertilization. Values expressed as $mm.^3$ $O_2$ per $10^5$ larvae per hour. Temperature, 20° C.

| Hours from fertilization | Controls | Controls | Feeding | Feeding |
|---|---|---|---|---|
| 174 | 26.0 | 25.0 | 45.5 | 42.5 |
| 176 | 36.9 | 35.0 | 50.3 | 51.5 |

the fed trochophores consume, on the average, 54 percent more oxygen per hour than do the controls.

The respiration of polyspermic eggs

In order to test the often suggested hypothesis that the increasing rate of oxygen consumption of developing eggs is related to the increase in the amount of nuclear material, it was of interest to measure the respiration of polyspermic eggs. Tyler and Schultz (1932) have described a method for the artificial production of polyspermy in Urechis eggs by means of treatment with acid-sea water. Warburg (1910) made a few measurements on the respiration of polyspermic sea urchin eggs in order to discover whether the increase in rate of oxygen consumption upon fertilization was due to the bringing in of respiratory catalysts by the spermatozoan. He did not describe his method for the production of polyspermy. The results he obtained showed a slightly higher rate of respiration in the polyspermic eggs than in the controls, but Warburg did not consider the difference significant. It is impossible to tell from Warburg's short experiment whether the respiration is influenced by the amount of nuclear material, since even assuming that polyspermic eggs in the 2-cell stage respire at the same rate as monospermic eggs in the 4-cell stage, the difference would be too slight to measure accurately. The differences would become apparent only at later stages. Brachet (1934b) has measured the oxygen consumption of polyspermic frog eggs obtained by treatment with sodium iodide. He found a marked difference in the respiration of polyspermic and normal eggs, the polyspermic eggs consuming 70 percent more oxygen during the second hour after fertilization than the controls. After 4-5 hours, however, the rate of oxygen

consumption was equal in the two lots. Brachet does not indicate whether more than two sperms were involved in the polyspermy, nor whether the polyspermic eggs continued to divide after the second hour.

To produce polyspermy in Urechis, the eggs were inseminated, and one minute later HCl was poured into the suspension to a concentration of 0.001 M. The eggs remained in this solution for 25 minutes, during which time the fertilization process was reversed without ejection of the sperm, as described by Tyler and Schultz (loc. cit.). The eggs were then removed to ordinary sea water and reinseminated; the controls were also inseminated at this time. In general, 70-90 percent of the eggs so treated were bispermic, as indicated by cleavage into 3 or 4 cells at the first division.

The results of the oxygen consumption measurements for two experiments are shown in Table VI. It can be seen from the table that the bispermic eggs respire at a lower rate than the controls throughout the entire period of the measurements. Examination of the eggs at the end of the runs showed that in all cases a large percentage (20-70 percent) of the bispermic eggs had not cleaved, but had developed into unicellular--probably multinuclear--swimmers. This, presumably, was a result of the shaking of the Warburg vessels. For this reason it is difficult to draw any conclusions from these data regarding the respiration of polyspermic eggs at later developmental stages. The observed lower rate of respiration of the bispermic eggs at the earlier stages may have been due to the same factors

Table VI

Oxygen consumption of mono- and bispermic Urechis eggs. Values expressed as $mm.^3$ $O_2$ consumed per $10^5$ eggs per hour. Temperature, 20° C.

| Hours from fertilization | Experiment A | | Experiment B | |
|---|---|---|---|---|
| | Monospermic | Bispermic | Monospermic | Bispermic |
| 3 | 9.28 | 7.87 | 9.79 | 7.63 |
| 4 | 10.31 | 8.53 | 10.05 | 7.47 |
| 5 | 11.26 | 9.41 | 12.06 | 9.48 |
| 6 | 13.88 | 10.87 | 14.36 | 12.10 |
| 7 | --- | 15.22 | 19.16 | 15.61 |
| 8 | 20.70 | 17.21 | 22.99 | 18.72 |

which prevented cleavage. It has been shown by Tyler and Horowitz (1938a) and by Brachet (1938) that treatments which prevent or retard the cleavage rate also retard the increase in respiratory rate. It may, on the other hand, be a direct result of polyspermy, with the resulting abnormal nucleoplasmatic ratio and deranged mitotic activity, in which case one would be forced to conclude that the rising rate of respiration of developing eggs is not a direct result of the increasing quantity of nuclear material. The present data are not sufficient to permit an answer to this problem.

## V. The Respiratory Quotient

### Introduction

An analysis of the rising rate of oxygen consumption in eggs would be incomplete without measurements of the carbon dioxide production. The ratio of carbon dioxide produced to oxygen consumed is the so-called respiratory quotient (R.Q.). From this quantity it is possible to make certain important deductions concerning the metabolic processes of the egg. The value of the respiratory quotient enables a distinction to be made between respiration concerned with the combustion of foodstuffs and "respiration" concerned simply with the addition of oxygen to substances within the egg; knowing the nitrogen excretion, it makes it possible to distinguish between the combustion of carbohydrate, fat, and protein; and, in some cases, it reveals whether an interconversion of these foodstuffs is occurring. Finally, from the respiratory quotient and the oxygen consumption it is possible to calculate the aerobic heat production.

### Historical

Very little of a reliable nature is known concerning the R.Q. of marine eggs. Most of the information to be found in the literature was obtained by the use of methods which cannot be considered as giving more than approximately correct values. Since the significance of the R.Q. depends to a large extent on relatively small differences, no very precise conclusions can be drawn from them. Only those values which may be regarded as

fairly accurate will be considered here. The rest, which are mostly of historical interest, are adequately reviewed in Needham's (1931) treatise.

In Warburg's 1915 paper, he reports measuring $CO_2$ production in sea urchin eggs. He found the value of the R.Q. to be 0.9 for fertilized eggs. He made no measurements at later developmental stages. The method used was similar to that to be described in the next section, except that oxygen consumption and $CO_2$ production were measured on different sets of eggs. This introduces a source of error, but Warburg considered the accuracy of his results to be within 5 percent. A more serious criticism lay in his use of a bicarbonate-free salt solution as the suspension medium, rather than carbonate-free sea water. The salt solution employed (Warburg, 1914) was lacking in magnesium. Herbst (1897) showed that developing eggs are adversely affected by magnesium lack. Shearer extended Warburg's work in 1922, but used a less accurate method for the estimation of $CO_2$. His method consisted simply in taking the difference between the pressure changes in vessels with and without alkali for absorption of $CO_2$. No correction was made for $CO_2$ retention by the cells and medium. Shearer himself called attention to the roughness of the procedure and laid no stress on the results obtained. Nevertheless, his values, which cover the first hour following insemination, agree with Warburg's in being around 0.9. Rapkine (1927) measured the $CO_2$ production of sea urchin eggs for the first 40 hours of development. He did not use a manometric method, but analysed for dissolved gases in the medium.

No correction was made for retention by the eggs. His values for the R.Q. at the beginning of development are much higher than those of Warburg and Shearer, and are, in fact, higher than can be accounted for by ordinary metabolic processes. They range from 1.6 to 4.2. After the 14th hour of development the values vary between 0.5 and 1.0, with peaks at 24 hours and 40 hours. Runnstrom (1933) has studied the respiratory metabolism of freshly fertilized sea urchin eggs; his value is identical with that of Warburg and of Shearer (0.9). Needham (1933) has measured the respiration of the eggs of the shore crab, Carcinus. He used artificial, bicarbonate-free sea water to eliminate difficulties encountered in $CO_2$ determinations due to the bicarbonate in ordinary sea water. He found an R.Q. of 1.0 at Stage I of his arbitrary classification. At Stage II the quotient had fallen to 0.72, but by the time of hatching it had risen again to 0.80. Amberson and Armstrong (1933) have measured the R.Q. of developing Fundulus eggs. Although a marine fish, the eggs are capable of developing in distilled water. Amberson and Armstrong used this medium in order to eliminate bicarbonate errors. They found a falling R.Q. as development proceeds. The quotients are 0.90 for the first day, 0.77 for the second to fourth days, and 0.72 for the fifth to twelfth days.

Rather more is known of the R.Q. in terrestrial and fresh-water eggs than in marine eggs, since bicarbonate may be readily eliminated from the medium. Among the invertebrates, the eggs of the pond snail, Lymnea (Baldwin, 1935) and of the grasshopper (Boell, 1935) have recently received attention.

Baldwin found an average R.Q. of 1.05 for the cleavage and early developmental stages of Lymnea. There was no indication of a significant change over the period studied. Boell reported a falling R.Q. in the pre-diapause Melanoplus egg. The values he obtained were 0.95 for the first week and 0.70 for the third week, with no reliable data for the second week. Among the vertebrates, the chick has naturally been the subject of a large number of investigations. The most accurate measurements for the early stages are probably those of Needham (1932). He determined the R.Q. of the blastoderm and yolk sac isolated from the rest of the egg, since the values obtained by previous workers, using the whole egg, had been confused by the large amount of inert yolk, white, and shell, with their unknown alkali reserves. He obtained values close to 1.00 for the period from the second to sixth days of development. Measurements for later stages have been obtained by Hasselbalch (1900), Lusanna (1906), and Murray (1927). The best values are probably those of Murray. He found that the R.Q. fell from 0.82 to 0.70 during the last 10 days preceding hatching. The values he obtained for earlier stages are probably unreliable, for the reasons stated above. Brachet (1934a) has measured the R.Q. of developing frog eggs. He found that the R.Q. rises from 0.66 in the segmenting egg to approximately unity at gastrulation, where it remains until hatching. The eggs of the rainbow trout have been studied by Schlenk (1933). He found that the gas exchange during the first two weeks of development was too slight to be measured by the method he employed. For the 14th to the 30th days, however, he

obtained an R.Q. of 0.65, which rose in the succeeding two weeks to 0.72.

The chief interest of these investigations is in the changing value of the R.Q. throughout development. Needham (1931, p. 986 ff.) has made the generalization that the early stages of development are characterized by high values of the R.Q., and that the R.Q. falls off as development proceeds. From this, he derives the concept of a "succession of energy sources" in ontogeny--these being carbohydrate, protein, and fat in the order named. From the investigations cited above, it can be seen that a falling R.Q. does in fact obtain in most cases. The outstanding exception is the frog. In Lymnea, any decrease would probably have been obscured by the synthesis of fat, which according to Baldwin (loc. cit.) occurs during development in this form. The data for the trout and for the sea urchin are too fragmentary or too inaccurate to enable conclusions to be drawn from them.

In view of the fundamental significance of the respiratory quotient in metabolic studies, it is surprising that so few reliable data exist for marine eggs. The chief difficulty in the determination is probably, for reasons to be explained, in the high bicarbonate content of sea water. It is believed that this difficulty has been removed in the present work.

The main interest in the eggs of marine invertebrates from the standpoint of a succession of energy sources in ontogeny is that they are homolecithal, in contrast with those of fish, amphibia, insects, and birds. The yolk is more evenly distrib-

uted throughout the egg and is a part of every cell. The transformation of yolk into living tissue occurs within the cells and without any visible increase in the size of the embryo. In the telolecithal types, on the other hand, the yolk is spatially distinct from the embryonic part of the egg, serving as a reservoir at the expense of which the embryo grows. The succession of energy sources postulated by Needham could be explained by a selective action of the yolk enzymes or to the relative ease of transportability of the various foodstuffs from yolk to embryo, rather than to developmental necessity. In homolecithal eggs the question of ease of transportability would appear not to enter. If the succession of energy sources can be shown to occur in these eggs, then it would seem likely that the source of energy is dependent upon a succession of enzymes within the egg and is probably related more or less directly to developmental changes.

Special methods

The method used for the measurement of the respiratory quotient was a modification of the method of Meyerhof and Schmitt (1929), sometimes referred to as the First Method of Dickens and Simer (1930). The principle of this method is as follows: two sets of Warburg vessels are run, in the usual manner, except that they contain acid in a special side-arm which can be tipped into the main compartment at any desired time; in the first set of vessels, the acid is mixed with the other contents immediately after equilibration, driving off the $CO_2$ held in the

tissues, medium, and alkali; the positive reading so obtained gives the total $CO_2$ present at the start of the measurements. In the second set of manometers oxygen consumption measurements are made in the usual manner; at the end of the experimental period the acid is tipped in and the resulting positive pressure read; this gives the amount of $CO_2$ present at the end of the experiment. The difference between the $CO_2$ present at the start and at the finish, divided by the observed oxygen consumption gives the respiratory quotient.

The advantages of this over other methods[1] are, first, that the oxygen consumption and $CO_2$ production are measured on the same eggs; and, second, that the method takes into account the $CO_2$ chemically bound in the eggs and medium. This last is most important where glycolysis is suspected, since the production of lactic acid in the course of an experiment drives off quantities of $CO_2$ which would otherwise invalidate the results; the overall effect would be an abnormally high value for the R.Q. The reverse occurs in non-glycolysing tissues with a high alkali reserve. Here the $CO_2$ produced in respiration would be bound in the cells; the effect would be an abnormally low value for the R.Q. The latter is the situation most frequently encountered in embryonic material.

For the present work, vessels with two side-arms as described on page 11 were used. The eggs were placed in the main compartment, 0.5 N NaOH in one side-arm, and 3 M citric acid in the other. The alkali absorbed $CO_2$ in the course of the run.

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[1] E.g., the direct method and the "improved method" of Warburg; see Dixon (loc. cit.) for discussion of other methods.

At the proper time both side-arms were tipped into the main compartment, cytolysing the eggs and releasing the $CO_2$. Measurements of the pH of the mixed contents showed it in no case to be higher than 2.0, thus insuring complete displacement of the bound $CO_2$.

In order to obtain the best accuracy with this method it is necessary to reduce the $CO_2$ content of the alkali and the suspension medium to as low a value as possible. For this purpose buffered, carbonate-free sea water, as described previously, was employed. The alkali used in the vessels was diluted up from concentrated, carbonate-free NaOH. If these precautions are not observed the blank $CO_2$ is so large as to obscure the relatively slight amount of $CO_2$ formed by the eggs. Along with these measures it is important to run the determinations for a sufficient period to insure enough $CO_2$ production for accurate measurement. Two hours usually suffice with a moderate concentration of eggs. The values of the R.Q. so obtained were taken as representing the middle of the two-hour interval.

The Bunsen coefficient, $\alpha_{CO_2}$, which enters into the calculations, varies, as is well known, with the electrolyte concentration. The value for sea water at 20° C. as used in these calculations was 0.778 (Runnstrom, 1933).

## Results

Figure 6 shows the $CO_2$ production of developing Urechis eggs plotted against time; the corresponding oxygen consumption is plotted for comparison. Each point is the average of several

## EXPLANATION OF FIGURE 6

Oxygen consumption and carbon dioxide production in developing eggs of Urechis. Curve A: rate of oxygen consumption. Curve B: rate of carbon dioxide production.

## EXPLANATION OF FIGURE 7

Rate of heat production in developing eggs of Urechis, calculated from oxygen consumption and respiratory quotient data.

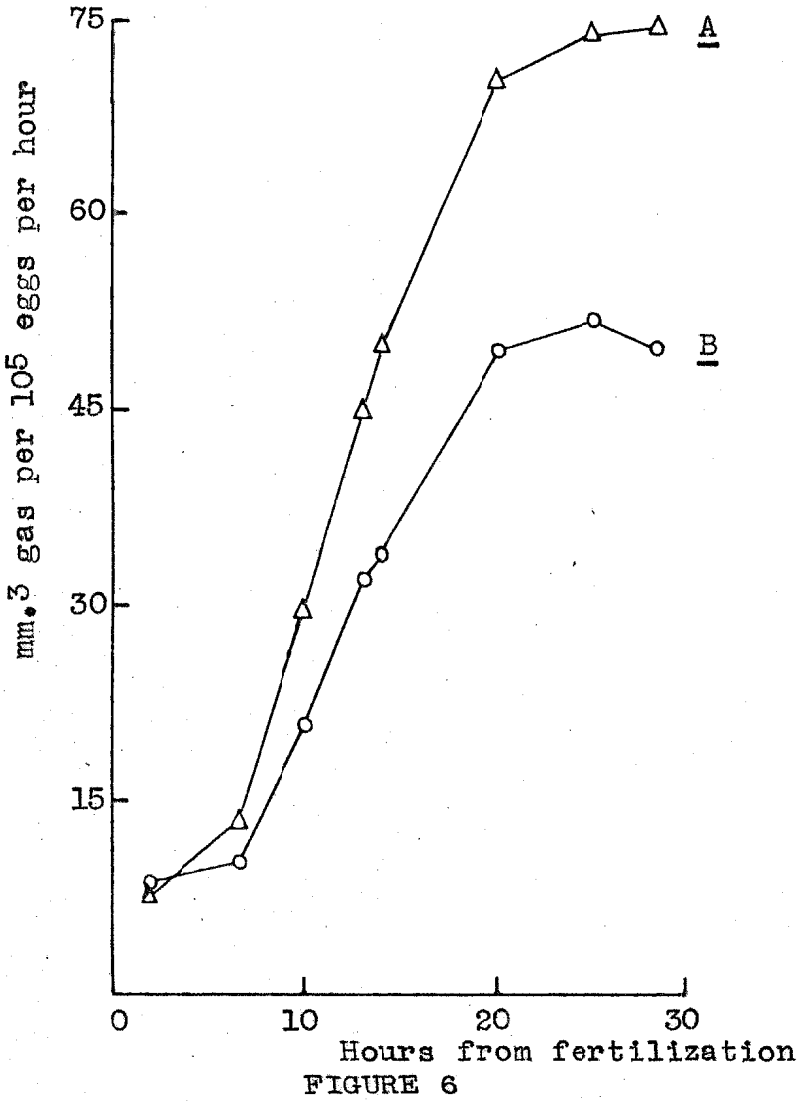

FIGURE 6

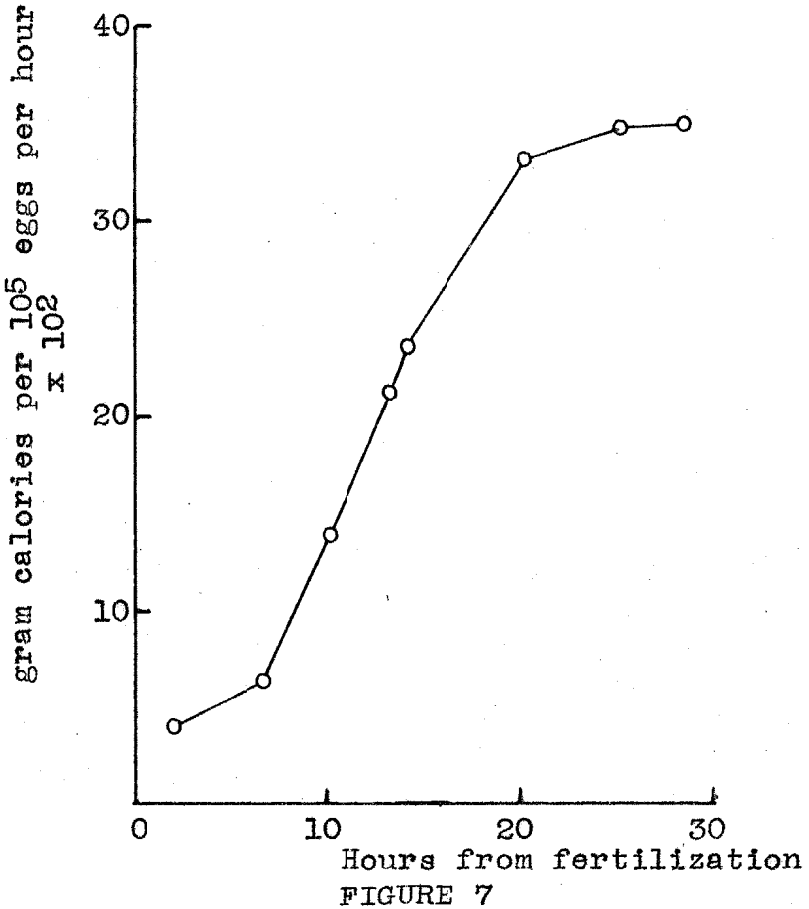

FIGURE 7

values. It is seen that the two curves start at the same level at the second hour from fertilization, but diverge as development proceeds. The data from which the curves were plotted and the resulting R.Q.'s are tabulated in Table VII. From a value of 1.07 at the second hour, the R.Q. drops to 0.76 at the seventh hour, and to 0.71 at the thirteenth hour, where it remains

Table VII

Respiratory quotients of developing Urechis embryos. Temperature, 20° C.
$X_{O_2}$ = mm.$^3$ $O_2$ consumed per vessel during the experimental period.
$X_{CO_2}$ = mm.$^3$ $CO_2$ produced per vessel during the experimental period.

| Hrs./min. after fertilization | $X_{O_2}$ | $X_{CO_2}$ | R.Q. | Ave. |
|---|---|---|---|---|
| 2/0 | 72.3 | 80.2 | 1.11 | |
| | 71.2 | 71.2 | 1.00 | |
| 2/15 | 33.6 | 30.7 | 0.91 | |
| | 32.7 | 42.1 | 1.28 | |
| 2/20 | 41.6 | 44.5 | 1.07 | 1.07 |
| 6/40 | 41.6 | 30.8 | 0.74 | |
| | 47.1 | 35.8 | 0.76 | |
| | 48.2 | 37.3 | 0.77 | 0.76 |
| 13/10 | 79.8 | 56.5 | 0.71 | |
| | 71.7 | 50.7 | 0.71 | |
| 14/7 | 80.8 | 50.6 | 0.63 | |
| | 79.1 | 57.3 | 0.72 | |
| 28/20 | 116.5 | 71.2 | 0.61 | |
| | 116.5 | 84.0 | 0.72 | |
| | 120.8 | 79.6 | 0.66 | 0.68 |

without significant change through the twenty-eighth hour from fertilization. The value 1.07 is not significantly greater than

1.00. This value is indicative of carbohydrate metabolism, or of protein metabolism, providing the protein is metabolized to ammonia rather than to urea. Since, as will be shown later, no protein catabolism occurs in these eggs, we may conclude that the egg combusts carbohydrates as its sole source of energy during the first hours after fertilization. The carbohydrate metabolism lasts for only a short time, however, and is soon displaced by fat metabolism, as the value 0.68 indicates. The value 0.76 which occurs at the seventh hour is the result of a mixed carbohydrate and fat metabolism, since protein metabolism has been ruled out.

It was attempted to measure the R.Q. of unfertilized eggs as well, in order to determine whether the short period of carbohydrate metabolism at the outset of development is simply a continuation of the pre-fertilization metabolism, or whether it is connected specifically with the early cleavage stages. As stated previously (p. 15) the unfertilized egg of Urechis shows considerable variability in its rate of oxygen consumption. It was believed that this might be associated, nevertheless, with a constant R.Q. This, however, turned out not to be the case. Only two sets of determinations were made on the unfertilized egg. The values agreed very well for a given batch of eggs, but the difference between the two batches was considerable. These results are shown in Table VIII. Average values of 0.69 and 0.89 were obtained, indicating a fat metabolism and a mixed fat-carbohydrate metabolism respectively. It is rather surprising that the unfertilized egg--generally regarded as a "resting"

cell--should be capable of such profound alterations in its metabolic processes.

Table VIII

Respiratory quotients of unfertilized Urechis eggs. Results expressed as $mm.^3$ gas consumed or produced per vessel during the experimental period. Temperature, 20° C.

| Experiment | $X_{O_2}$ | $X_{CO_2}$ | R.Q. | Ave. |
|---|---|---|---|---|
| A1 | 34.4 | 24.1 | 0.70 | |
| A2 | 36.8 | 25.6 | 0.70 | |
| A3 | 23.6 | 15.7 | 0.67 | 0.69 |
| B1 | 18.3 | 16.7 | 0.91 | |
| B2 | 34.4 | 29.6 | 0.86 | |
| B3 | 22.8 | 20.2 | 0.89 | 0.89 |

It is to be concluded from these data that the egg of Urechis is capable of metabolizing both carbohydrate and fat; that the carbohydrate phase lasts for only a short time in embryonic life; and that early in development--due possibly to the exhaustion of its carbohydrate supply, or for energetic reasons yet unknown--the organism changes over to a wholely fat metabolism. The data thus support Needham's view of a succession of energy sources in ontogeny. The succession proceeds in the direction predicted (carbohydrate to fat). As will be shown later, however, Urechis differs in one fundamental respect from the chick, upon which Needham's theory was originally founded, in not metabolizing protein at any stage in its embryonic life.

Stability of the R.Q.

It was of interest to know whether by artificial means the energy source at a given stage could be altered sufficiently to reflect in the developmental processes. For this purpose, embryos were reared in sea water containing 4 percent glucose. It seemed likely that with a plentiful supply of combustible carbohydrate available the period of carbohydrate metabolism might be extended over a longer period of time than is normal. Nothing of the sort occurred, however. As can be seen from Table IX, where a typical experiment is shown, the metabolism was unaffected by the additional supply of carbohydrate.

Table IX

Lack of effect of glucose on the R.Q. of Urechis embryos. Eggs placed in sea water containing 4 percent glucose 10 minutes after fertilization. Embryos 16 hours old at start of measurements. Results expressed as $mm.^3$ gas consumed or produced per vessel during the experimental period. Temperature, 20° C.

| | $X_{O_2}$ | $X_{CO_2}$ | R.Q. |
|---|---|---|---|
| Control | 250.0[1] | 166.0 | 0.66 |
| Glucose | 61.6 | 44.5 | 0.72 |
| Glucose | 55.0 | 39.9 | 0.73 |

[1] The difference between amount of gas exchange in controls and experimentals is due to the greater number of embryos used in the control vessel.

The apparent inability of the embryos to utilize glucose is surprising in view of the fact that the necessary enzyme systems for the combustion of carbohydrate are evidently present, as can be seen from the high R.Q. at the beginning of development.

Evidently, exhaustion of the available carbohydrate does not enter in here. A similar finding in the chick has been reported by Needham (1926). He showed that the injection of glucose into the chick egg does not prevent the combustion of protein which normally occurs in the middle of the developmental period. Needham has pursued the question further in his recent (1937) series of papers on the carbohydrate metabolism of the chick embryo. He has shown that two paths of carbohydrate breakdown exist in the chick, one proceeding through phosphorylation, as in yeast and muscle, and one taking the course of non-phosphorolytic glucolysis. The phosphorylating mechanism is incomplete in the early embryo, however, so that the main path is that of simple glucolysis. Whether the same holds true for marine eggs cannot be stated as yet. In any case, these findings bring us no nearer to a solution of the problem of the succession of energy sources. They do not explain the turning of the path of metabolism from carbohydrate to fat in the course of development.

## VI. Heat Production

### Introduction

From the oxygen consumption data and the respiratory quotient it is possible to calculate the heat production of the embryo. This calculation involves certain assumptions. These may be stated as follows:

(1). That all of the oxygen consumed during development is used in the combustion of carbohydrate, fat, or protein, or their derivatives.

(2). That all of the oxygen used in respiration is derived from the atmosphere.

(3). That the end products of the carbohydrate and fat metabolism are carbon dioxide and water.

(4). That no accumulation of the products of anaerobic metabolism occurs in the embryo.

The quantity of protein metabolized by the embryo is known to be zero.

The first assumption implies that oxygen is not used in the production of pigments or other oxygen-containing substances[1] which are not directly involved in the metabolism of the embryo. Although pigment granules are to be seen in Urechis embryos in association with the cilia, it is unlikely that they irreversibly combine with more than a small fraction of the respiratory oxygen, if at all. The second assumption implies that the embryo contains no "oxidizing reserve" to supplement

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[1] More properly, "oxidized substances", in the electronic sense.

the oxygen derived from the air. Such a reserve has been found in the frog's egg (Brachet, 1934a), but functions only when the embryo has been deprived of atmospheric oxygen; upon readmittance of air, the depleted reserve is restored, as indicated by the abnormally low R.Q. The third assumption is in agreement with the known facts of normal metabolism. The incomplete oxidation of fats and carbohydrates is known to occur only in pathological conditions--e.g., diabetes. There is no reason to believe that the present material is atypical in this respect. The fourth assumption involves the question as to what extent anaerobic oxidations occur in embryonic tissues. This point will be discussed in detail below. For the present it will suffice to point out that if heat is liberated in the present material by anaerobic processes it will in all probability be very slight in comparison with the aerobic heat production.

Results

In Table X the heat production and the corresponding amounts of fat and carbohydrate burned are shown for the stages indicated. The conversion of R.Q. and oxygen consumption into gram calories, fat, and carbohydrate was made with the aid of the table of Zuntz and Schumberg, modified by Lusk, modified by McClendon, to be found in Bodansky (1934, p. 509). The heat production (rate) is plotted in Figure 7.

Three investigations into the heat production of marine eggs have been made using the method of direct calorimetry. These studies (Meyerhof, 1911; Shearer, 1922; Rogers and Cole,

Table X

Heat production of developing Urechis eggs obtained by indirect calorimetry. All data expressed per hundred thousand eggs per hour. Temperature, 20° C. $\gamma$ = 0.001 mg.

| Hrs./min. from fertilization | mm.$^3$ $O_2$ consumed | mm.$^3$ $CO_2$ produced | R.Q. | $\gamma$'s carbohydrate burned | $\gamma$'s fat burned | Gram calories |
|---|---|---|---|---|---|---|
| 2/0 | 8.00 | 8.56 | 1.07 | 9.86 | 0 | 0.0404 |
| 6/40 | 13.50 | 10.26 | 0.76 | 2.88 | 5.63 | 0.0642 |
| 10/0 | 29.5 | 20.1[1] | 0.71 | 0 | 14.8 | 0.138 |
| 13/10 | 45.1 | 32.0 | 0.71 | 0 | 22.6 | 0.212 |
| 14/7 | 49.8 | 33.9 | 0.68 | 0 | 25.0 | 0.234 |
| 20/0 | 70.5 | 49.4[1] | 0.71 | 0 | 35.4 | 0.331 |
| 25/0 | 74.0 | 51.8[1] | 0.71 | 0 | 37.2 | 0.347 |
| 28/20 | 74.5 | 49.2 | 0.66 | 0 | 37.4 | 0.350 |

[1] Interpolated value

1925) were all made on the sea urchin egg. It is nevertheless interesting to compare their results, obtained directly, with the present ones obtained indirectly. For the second hour of development, the following values were found:

| | Gm. cals. per $10^5$ eggs per hour | |
|---|---|---|
| Meyerhof | 0.027 | (Paracentrotus) |
| Shearer | 0.040 | (Echinus) |
| Rogers and Cole | 0.052 | (Arbacia) |

(Partly from Needham, 1931)

The corresponding value obtained for Urechis, taken from Table X, is 0.040 gram calories per $10^5$ eggs per hour. The agreement is striking. It should be pointed out, however, that the values obtained by direct calorimetry represent the actual heat output of the eggs, whereas the values obtained by indirect calorimetry represent the total heat production irrespective of whether part of the heat (energy) is stored in the egg or not. The question of whether developmental processes involve the storage of heat as potential energy will have to be decided by parallel investigations by direct and indirect calorimetry made on the same eggs.

The close agreement here indicates, nevertheless, that the heat production of Urechis and sea urchin eggs are of the same order of magnitude, although the sizes of the eggs differ considerably (108 microns and 72 microns in diameter, respectively). This signifies that the metabolic rate per unit mass is much lower in Urechis than in sea urchin eggs. Whitaker (1933b) has found that the rate of respiration of the

fertilized eggs of a variety of marine invertebrates are closely the same. Urechis evidently falls outside the range of values found by Whitaker. As pointed out in a later (1935) communication by Whitaker, however, the method he employed for obtaining volumes of egg substance (centrifuging) is subject to large absolute errors, and no reliance can be placed on absolute rates referred to centrifuge volumes. If the respiration of Urechis and Strongylocentrotus eggs as measured in the present investigations are calculated in terms of volume of egg substance, it is found that Urechis eggs consume 1.31 cu. mm. of oxygen per ten cu. mm. of eggs per hour as against 3.47 cu. mm. of oxygen per hour for the same volume of Strongylocentrotus eggs, at the third hour after fertilization. On the basis of the rate per egg the values are 8.41 and 7.36 cu. mm. per hour per $10^5$ eggs, respectively. These values are much more alike. Taking Warburg's (1915) value of 0.9 for the R.Q. of sea urchin eggs, the heat production of Strongylocentrotus eggs comes out to be 0.036 gram calories per hour per $10^5$ eggs, in good agreement with the values obtained by direct calorimetry as well as with the heat production of Urechis eggs. It is difficult to decide at present whether any significance is to be attached to this resemblance in heat production of the eggs of two widely separated species. It would be of interest if comparable data were available for other forms to investigate this point further.

The relationship of oxygen consumption to the release of energy within the embryo

The possibility was suggested above that a small amount of heat may have been produced in the eggs by anaerobic processes which are not coupled with oxygen-consuming reactions. This point involves the question as to what extent oxygen consumption can be taken as a measure of the energy released within the egg for developmental processes. In living organisms energy is liberated by two general types of reactions: oxidative and fermentative. The first consists essentially in the indirect combination of oxygen derived from the atmosphere with foodstuffs within the cell, with the formation of carbon dioxide and water. The second consists in the anaerobic splitting of carbohydrates to smaller molecules of lower energy value. The work of recent years has shown that the processes of oxidation and fermentation are actually very similar in mechanism, and, in higher organisms, are closely linked metabolically. Oxidation differs essentially from fermentation only in having an additional hydrogen acceptor--molecular oxygen--at the end of the chain of reactions. Tissues which glycolyse rapidly under anaerobic conditions no longer do so, for the most part, in the presence of oxygen. It is also known that some of the most important reactions in biological systems are primarily anaerobic (e.g., muscle contraction), but are coupled in the recovery phase with aerobic reactions in such a way that the liberation of energy for the over-all transformation appears as an aerobic process.

How far these findings may be applied to the phenomenon of development is as yet an unsettled point. Loeb (1895) showed that development in the sea urchin ceases when the eggs are deprived of oxygen. At the same time, however, he demonstrated that the eggs of the minnow, Fundulus, are capable of dividing for 15 hours under anaerobic conditions. More recently, Brachet (1934a) has shown that frogs' eggs can develop as far as gastrulation in the absence of atmospheric oxygen or when poisoned by KCN. Brachet has investigated the problem further and has found that under such conditions an oxygen debt is built up within the eggs. This is indicated by the abnormally low R.Q. and the abnormally high rate of oxygen consumption exhibited by the eggs upon the readmittance of oxygen. From his data it cannot be decided conclusively whether the oxygen debt is equal to the oxygen which would have been consumed under normal conditions. Brachet concludes from his studies that cleavage is a fundamentally anaerobic process, similar to contraction in muscles and conduction in nerves. This implies that oxygen is concerned only in the restitution phase of the process. As pointed out above, this does not necessarily mean that oxygen consumption is unrelated to the release of energy. Only if it could be shown that the anaerobic reactions proceed independently of the oxygen supply, without involving oxygen even secondarily, would it be clear that the consumption of oxygen is not related to the release of energy. Such processes are well known in microorganisms, tumors, and isolated enzyme systems. The most important of these reactions is aerobic glycolysis.

Aerobic glycolysis has not generally been considered as occurring in embryonic tissues. That practically no aerobic glycolysis occurs in the chick embryo was first shown by Warburg, Posener, and Negelein (1924). Negelein (1925) demonstrated that no perceptible aerobic glycolysis occurs in rat embryos. Brachet (1934a) has shown the same thing for cleavage stages of the frog. He found evidence for aerobic glycolysis from gastrulation on, however, in confirmation of an earlier finding by Lennerstrand (1933). Trifonova (1937) has found that bursts of lactic acid production corresponding with periods of rapid growth occur in the developing perch; during periods of little growth and rapid differentiation the lactic acid is removed. The finding of Whitaker (1933b) that the rate of oxygen consumption decreases upon fertilization in the eggs of Chaetopterus and Cumingia has prompted the suggestion that "in an egg such as that of Chaetopterus which reduces its aerobic oxidation rate at fertilization, it is possible that anaerobic oxidations increase at fertilization so that the total oxidations may not decrease at this time when energy requirements would appear to be increased." (Whitaker, loc. cit.). The crucial point appears to be whether it is possible to cause an egg to attain a given stage of development with a lower consumption of oxygen than normally, without incurring an oxygen debt. Tyler (1936) has shown that this cannot be done by varying the temperature at which development occurs. Whether other physical or chemical agents are capable of producing this result cannot be stated at present.

## VII. The Partition of Nitrogen in Urechis Eggs

### Introduction and Historical

In connection with the studies on the respiratory metabolism of the developing egg, it was necessary to investigate the nitrogen metabolism. This was of interest from two points of view: first, to make possible a correct interpretation of the R.Q.; and, second, to determine whether the development of the egg involves a synthesis of protein.

Very little information concerning the nitrogen metabolism of eggs during the earliest stages of development exists in the literature. The extensive investigations of this subject in the hen's egg do not take into account the period of considerable development preceding the laying of the egg. Ephrussi and Rapkine (1928) have analysed the sea urchin (Paracentrotus lividus) egg for total nitrogen at three developmental stages: the unfertilized egg, the 12 hour embryo, and the 40 hour embryo. They found a decrease in total nitrogen during this period. These authors made no separate analysis for protein, but assumed that all the nitrogen was protein nitrogen. Hayes (1933) made analyses of sea urchin (Echinometra lucunter) eggs for total nitrogen and amino nitrogen during the first 25 hours of development. His finding are inexplicable in that they show an increase of total nitrogen during the first 4 hours after fertilization. Such a process, in the author's words, is extremely improbable. After the fourth hour, a decrease in total and amino nitrogen was found. Russo (1922), cited by Needham (1931), has analysed three stages of the developing

silkworm. The same author (1926) has made a detailed investigation of the nitrogen distribution in immature sea urchin (Paracentrotus lividus) eggs. His results will be discussed below.

Special methods

The embryos were reared as described previously. They were removed from the finger bowls at the desired stages, washed, counted, and concentrated to a small volume (5-10 cc.) by the methods outlined above. After concentrating, trichloracetic acid was added to the suspension to give a 2.5 percent solution. The trichloracetic acid killed the embryos immediately, precipitated the proteins, and prevented subsequent autolysis. The suspension was then frozen in a dry ice-ether mixture and ground in a mortar until all the embryos were pulverized, as observed under the microscope. This procedure was the only one found to give satisfactory results. Other methods that were tried were immersion in distilled water and addition of ether and saponin to the suspension; none of the agents were effective in breaking down the egg membrane. Nor was it possible to crush all of the eggs by grinding in an agate mortar with fine quartz, as can readily be done by the freezing and grinding method.

After thawing, the suspension was made up to volume and analysed by the micro-methods of Borsook and Dubnoff (unpublished), which allow complete analyses to be made on as little as 1 milligram of egg nitrogen. The eggs were analysed for

total, total non-protein, amino, peptide, amide, and ammonia nitrogen. Protein nitrogen was obtained by difference. A general outline of the methods employed follows:

(1). Total and non-protein nitrogen were determined by a micro-Kjeldahl method.

(2). Amino nitrogen was determined by a micro-formol titration.

(3). Peptide nitrogen was determined by micro-formol titration following hydrolysis by means of an active peptidase extract obtained from the mold, Aspergillus wentii.

(4). Amide nitrogen was determined by acid hydrolysis and subsequent distillation of the freed ammonia.

(5). Ammonia was determined by distillation from alkaline solution.

All titrations were electrometric (glass electrode).

Results and Discussion

Analyses were made on the unfertilized egg, the 6 hour blastula, the 14 hour gastrula, and the 24 and 30 hour trochophores. The analytical results are summarized in Table XI.

From the table it can be seen that there is no change in the total nitrogen per embryo up to the twenty-fourth hour after fertilization. The slight variations are within the limits of experimental error, the greatest part of which was in the counting; as shown on p. 14, the counting error was less than 5 percent. In the sea urchin, Ephrussi and Rapkine

(loc. cit.) found that the total nitrogen decreases from 10.7 percent of the dry weight in the unfertilized egg to 9.7 percent after 40 hours of development. They concluded from this a loss of nitrogen during development. Their own data, however, show an increase in the dry weight of 5.6 percent during the same period of time. This practically cancels out any loss of nitrogen, calculated as milligrams per embryo. It can be concluded that the combustion of protein does not occur in either Urechis or sea urchin eggs. This is in agreement with the R.Q. measurements on Urechis eggs, described above. These eggs thus differ from the chick egg, which passes through a period of protein combustion in the course of its development. This difference is possibly related to the relative amounts of protein in the eggs. As can be seen from the table, less than 60 percent of the nitrogen of the Urechis egg is in the form of protein. This is similar to Russo's (1926) value for the nearly-ripe ovarian eggs of the sea urchin, in which he found 62 percent protein nitrogen. In contrast, Needham (1927) found 95.6 percent of protein nitrogen in the chick egg at laying.

At the thirtieth hour a decrease in nitrogen amounting to 8 percent occurs. Although small, this difference is outside the experimental error and is probably real. This stage is identical with that that at which feeding would begin, as shown by the carmine experiment described on p. 23. Whatever significance there may be in this coincidence, it would have to be determined by experiments in which the food supply is carefully controlled. It should be noted that the loss of nitrogen at this stage

Table XI

Partition of nitrogen at various stages in the development of the egg of Urechis caupo. Values given are averages of several determinations.

| | Unfertilized | | 6 hours | | 14 hours | | 24 hours | | 30 hours | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mg/egg x $10^5$ | Per-cent | Mg/egg x $10^5$ | Per-cent | Mg/egg x $10^5$ | Per-cent | Mg/egg x $10^5$ | Per-cent | Mg/egg x $10^5$ | Per-cent |
| Protein N | 0.734 | 53.2 | 0.776 | 55.8 | 0.791 | 57.3 | 0.798 | 56.6 | 0.765 | 59.3 |
| N. P. N. | 0.646 | 46.8 | 0.614 | 44.2 | 0.589 | 42.7 | 0.612 | 43.4 | 0.525 | 40.7 |
| Amino N | 0.455 | 33.0 | 0.434 | 31.2 | | | 0.426 | 30.2 | 0.412 | 31.9 |
| Peptide N | 0.046 | 3.3 | 0.007 | 0.52 | | | 0.000 | 0.0 | 0.000 | 0.0 |
| Ammonia N | 0.014 | 1.0 | 0.012 | 0.86 | | | 0.041 | 2.9 | 0.039 | 3.0 |
| Amide N | 0.001 | 0.07 | 0.006 | 0.40 | | | | | 0.004 | 0.33 |
| Undet'd. | 0.122 | 9.43 | 0.155 | 11.22 | | | 0.145 | 10.3 | 0.070 | 5.47 |
| Total N | 1.38 | 100 | 1.39[1] | 100 | 1.38 | 100 | 1.41 | 100 | 1.29 | 100 |

[1] Assumed value. A percentage analysis for this stage was made, but the eggs were not counted.

occurs partly at the expense of protein, but to a larger extent at the expense of the undetermined fraction. This fraction includes, among others, the imino groups of arginine, histidine, proline, and hydroxy-proline, together with the cytoplasmic purines and pyrimidines.

While there is no change in total nitrogen during the first 24 hours of development there is, as Table XI shows, an increase in protein nitrogen amounting to 8.7 percent. This probably represents synthetic activity within the embryo. The increase in protein can be accounted for by the loss of amino and peptide nitrogen. An analysis for protein and non-protein nitrogen was made on the 50 hour trochophore (not shown in the table) to determine whether any change in the relative proportions of these occurred at a later stage. The results, in percentages of total nitrogen, were as follows:

| | |
|---|---|
| Protein N | 59.4 percent |
| N.P.N. | 40.6 percent |

There is no change over the 30 hour stage.

When one considers the large number of cell divisions and the considerable amount of morphological development undergone by the embryo during the first 24 hours after fertilization, it is surprising that the over-all protein synthesis is so slight. This does not imply, of course, the total absence of synthetic activity; for, on theoretical grounds, and by analogy from what is known of the metabolism of nitrogen in the chick (see Needham, 1931), it is probable that a continuous breakdown of yolk proteins and their resynthesis into the living proteins of the

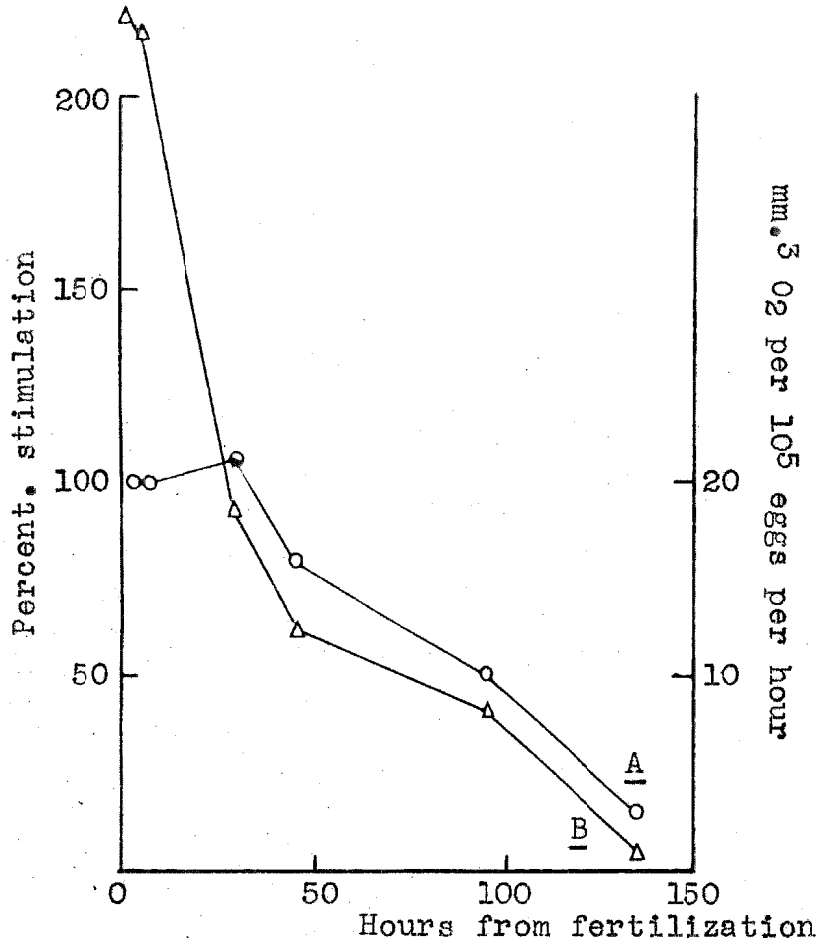


FIGURE 12

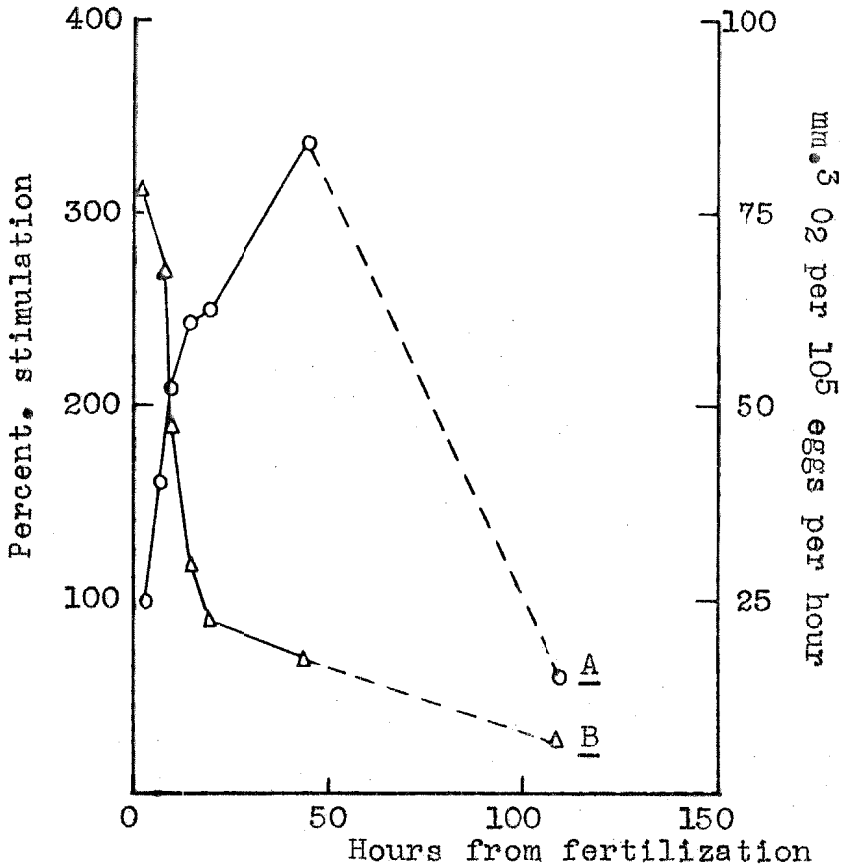


FIGURE 13

embryo occurs throughout development. Nevertheless, as can be seen from the table, the composition of the trochophore, as far as its nitrogenous constituents goes, is not very different from that of the unfertilized egg. No analyses of adult Urechis tissues were made, but it is very probable that the preponderant part of the nitrogen in the adult is in the form of protein. This is the case in all adult tissues which have been analysed (Terroine, 1933). Table XII, from Terroine, Hée, and Roche (1931), after Terroine (loc. cit.), shows the proportion of protein nitrogen in the muscle of two typical vertebrates and two typical invertebrates:

Table XII

| | Tortoise | Frog | Snail | Crayfish |
|---|---|---|---|---|
| Total N (percent wet weight) | 2.69 | 2.54 | 2.68 | 2.12 |
| Protein N (percent wet weight) | 2.57 | 2.41 | 2.55 | 2.03 |
| Protein N (percent of total N) | 96 | 95 | 95 | 96 |

Other tissues give similar values.

In the absence of any exact knowledge concerning the conditions for protein synthesis in vivo, one can only speculate as to the reasons for the apparent inability of the embryo to synthesize more of its amino nitrogen into protein. One possible explanation that suggests itself is a deficiency in one or more essential amino acids which must be supplied in the food before synthesis can occur. Teleologically, one may suppose that a certain advantage results to any organism, which like Urechis,

must undergo a certain degree of metamorphosis in transforming from the larval to the adult form, in keeping its nitrogen in a mobile state. In this way it perhaps avoids the necessity of a thorough-going histolysis, such as is found in insects, which would otherwise be imposed by the change into the adult type. That insect eggs differ markedly from Urechis eggs in the partition of nitrogen can be seen from Russo's (1926) figures for the silkworm egg, which show that 96 percent of the nitrogen of these eggs is bound as protein.

No attempt will be made here to interpret the observed variations in ammonia and amide nitrogen throughout development (Table XI). The amide fraction, in particular, was present in such small amounts as to approach the limits of sensitivity of the analytical method.

## VIII. The Respiration of Strongylocentrotus

### Introduction

Although a large body of knowledge exists concerning the respiration of sea urchin eggs, only two investigations have been reported in which the respiration was measured over an extended period of time: Warburg (1915) followed the oxygen consumption of Strongylocentrotus lividus eggs for 24 hours following fertilization; Rapkine (1927) extended the measurements to 40 hours. The rate curve published by Warburg is still rising at the 24th hour and shows no sign of attaining a plateau. Rapkine's curve levels off precisely at the 24th hour and continues without further rise to the 40th hour. Since the plateau is reached very sharply in Rapkine's curve, there is no contradiction, in this respect, with Warburg's work. Rapkine's curve disagrees with Warburg's, however, in the earlier stages, since it shows a drop in the rate of respiration during the first 4 hours after fertilization, whereas Warburg's curve rises in almost a straight line from the moment of fertilization on. Inasmuch as Warburg's technique was the more accurate (the manometric method, as opposed to the Winkler titration) Warburg's curve has been accepted in preference to Rapkine's. The correctness of Warburg's observations has been borne out by the repeated measurements of numerous workers. This leaves the question of the attainment of a plateau still open. In view of this fact, and inasmuch as it was necessary to know the course of the respiration in Strongylocentrotus for the purposes of the experiments to be described later, it was decided to reinves-

tigate this problem.

Normal development of Strongylocentrotus

Tyler (1936b) gives the following schedule of development for this form:

Table XIII

Summary of development of the eggs of Strongylocentrotus purpuratus. Temperature, 20° C. From Tyler (1936b).

| Hrs./min. from fertilization | Stage |
|---|---|
| 1/17 | 2-cell |
| 2/6 | 4-cell |
| 25/0 | 1/8 gastrulated |
| 28/48 | 1/2 gastrulated |
| 31/24 | 3/4 gastrulated |
| 49/0 | Prism with skeletal rods |

The change from the prism into the pluteus larva is very gradual. The pluteus becomes recognizable as such shortly after the 60th hour of development.

Results

The results of the oxygen consumption measurements are plotted in Figure 8. The curve represents cu. mm. of oxygen consumed per hundred thousand embryos per hour, plotted against time, the moment of fertilization being taken as zero. Each point is the average of several measurements. The curve for total oxygen consumption is shown in Figure 9. As with Urechis, the curves are composites of several determinations made at successive intervals along the abscissa.

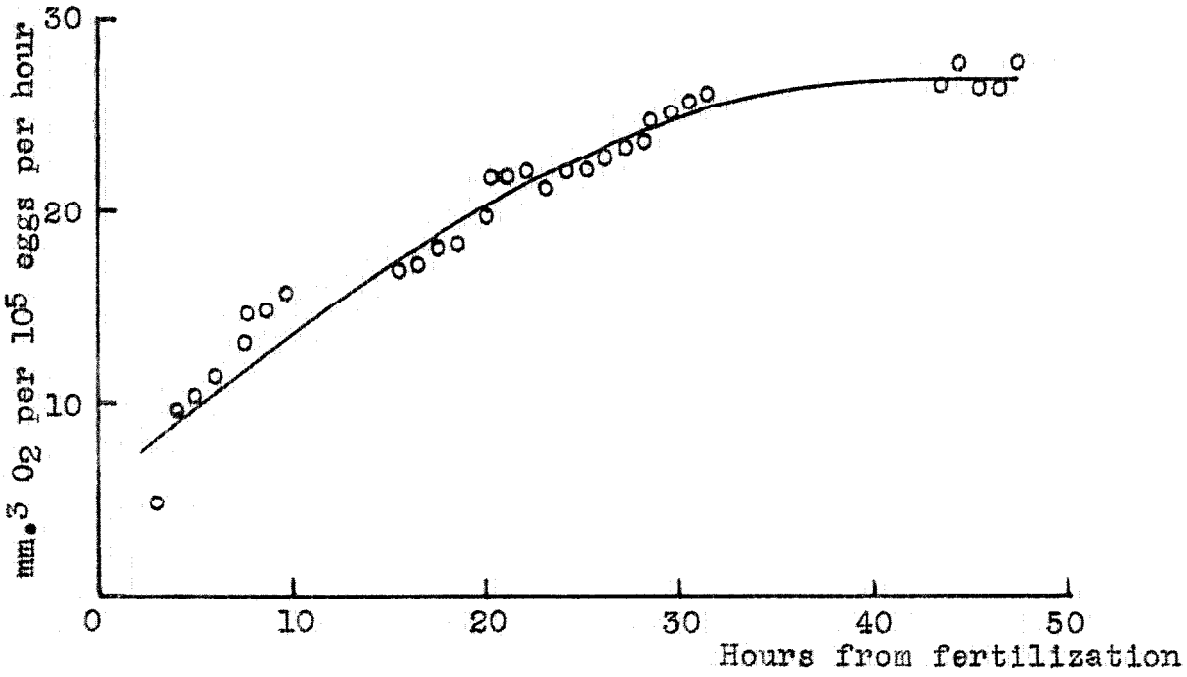


FIGURE 8

Rate of respiration of the developing eggs of Strongylocentrotus purpuratus.

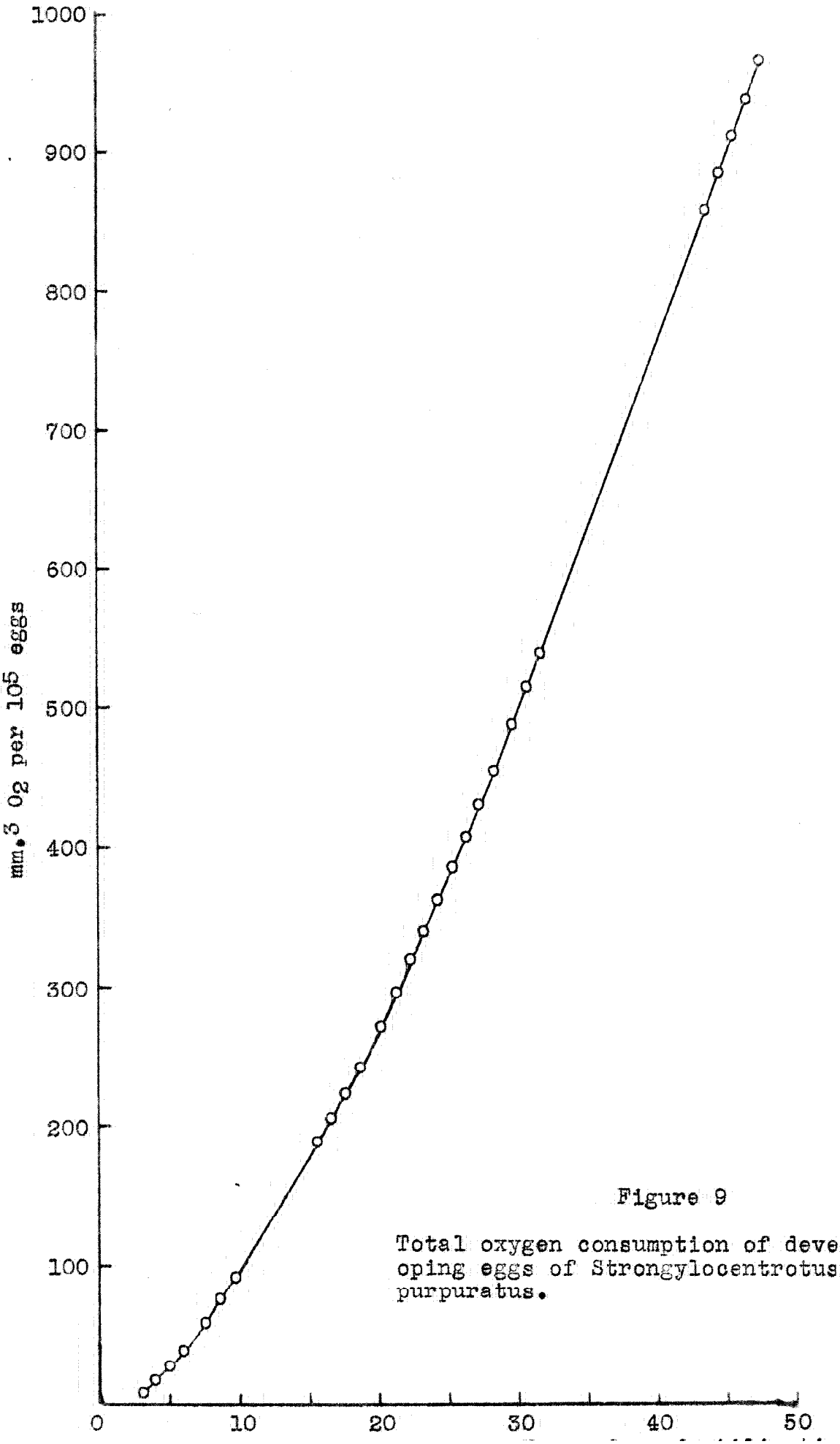


Figure 9

Total oxygen consumption of developing eggs of Strongylocentrotus purpuratus.

The curve of Figure 8 is similar to Warburg's for the first 24 hours. It levels off at later stages, but much more gradually than is indicated by the curve of Rapkine. In contrast with the curve obtained for Urechis (Figure 3), this one is not S-shaped; nor does the rate increase so rapidly, on the whole, as with Urechis. Whereas in Urechis the rate increases over 800 percent between the third and the twentieth hours, it increases by less than 300 percent during the same interval in Strongylocentrotus, although the initial rates are almost identical. It is interesting to note that the slower rise in Strongylocentrotus is correlated with a slower rate of development (compare Tables III and XIII).

Oxygen consumption of Dauerblastulae

Herbst (1895), in the course of his classical studies on the effects of inorganic salts on the development of marine eggs, discovered that the addition of potassium thiocyanate to the sea water suppressed gastrulation in the eggs of Asterias. This finding has been confirmed and extended by Runnstrom (1928) and by Lindahl (1936), working with the eggs of Paracentrotus. It was of interest in connection with the present investigation to measure the oxygen consumption of such "Dauerblastulae", since it seemed possible to demonstrate here a clear-cut connection between respiration and developmental processes.

Eggs treated with thiocyanate develop normally as regards cleavage rate and cleavage pattern. After the 24th hour of development, however, they become visibly different from the

controls: whereas the latter invaginate to form an archenteron, the treated embryos do not, but remain in the blastula form (see Figure 10). In typical cases, the subsequent formation of the skeleton is also inhibited. The mesenchyme remains as a bud within the blastocoel, near the vegetal pole. If the embryos are removed to ordinary sea water at sufficiently early stages they gastrulate, so that the effect is to a certain extent reversible. A detailed description of the development of these embryos is to be found in Lindahl's paper (loc. cit.).

To obtain Dauerblastulae, Strongylocentrotus eggs were reared in sea water containing 1.75 percent of an isotonic (0.55 M) NaSCN solution. The procedure followed in all subsequent operations was the same as that described previously (p. 6 ff.), except that the washing of the treated embryos was done in NaSCN-sea water instead of ordinary sea water. Three sets of measurements were made, at different intervals from fertilization, so that the curves obtained are composites, as previously. In all cases, 95-100 percent of the treated eggs developed into Dauerblastulae.

The rate of oxygen consumption of thiocyanate-treated eggs at various times from fertilization is shown in Figure 11, together with the oxygen consumption of controls run simultaneously. The curves contain several points of interest. First, the initial rate of respiration is the same in treated and control eggs, showing that NaSCN has no depressing action on the respiration as such. This important point was tested further in an experiment in which the respiration of untreated eggs was meas-

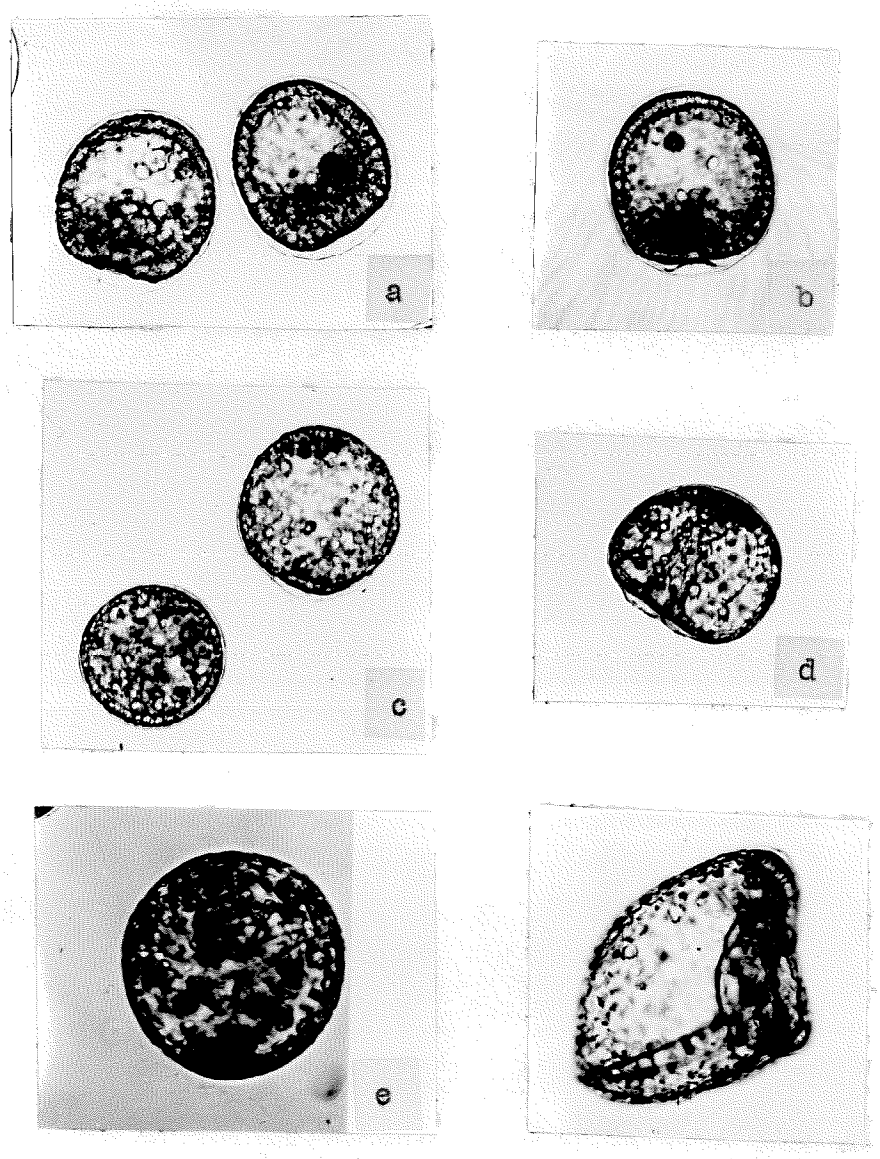


FIGURE 10

Normal embryos and NaSCN-Dauerblastulae of Strongylocentrotus purpuratus.

a. Dauerblastulae, 24 hours from fertilization.
b. Normal embryo, 24 hours from fertilization, showing beginning of gastrulation.
c. Dauerblastulae, 36 hours from fertilization.
d. Normal embryo, 36 hours from fertilization, showing archenteron.
e. Dauerblastula, 60 hours from fertilization.
f. Normal prism, 60 hours from fertilization, showing gut, skeleton, and beginning arm-buds.

Embryos reared in finger-bowls at 20° ± 1° C.

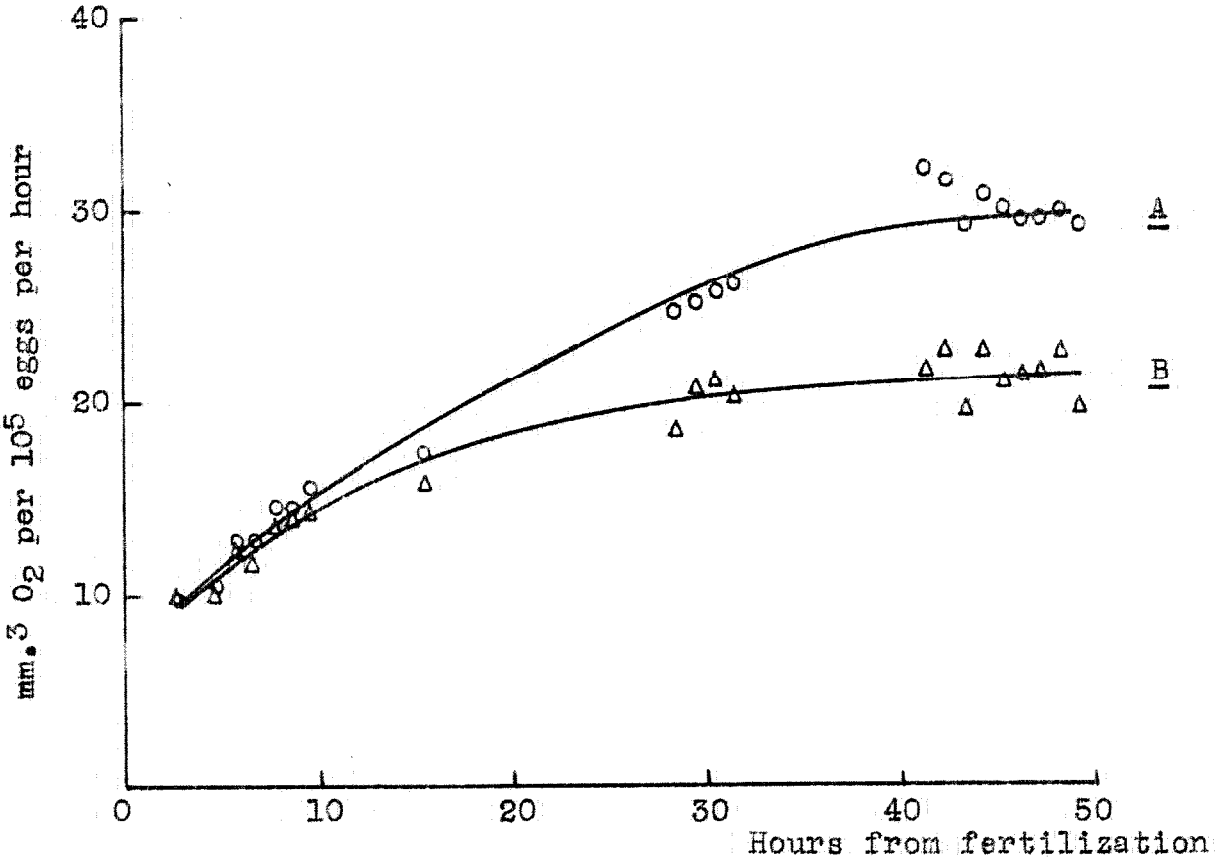


FIGURE 11

Comparison of respiration of Dauerblastulae of S. purpuratus with controls. Curve A: controls. Curve B: Dauerblastula

ured over a short period after which NaSCN was added to the suspension from a side-arm and the measurements continued. The NaSCN exerted no depressing action (Table XIV). Second,

Table XIV

Absence of inhibitory effect of NaSCN on the initial rate of respiration of fertilized eggs. Values expressed as mm.$^3$ $O_2$ consumed per vessel per hour. Each vessel contained the same number of eggs. Final concentration of NaSCN = 1.75 percent of a 0.55 M solution. Temperature, 20° C.

| Hrs./min. from fertilization | Vessel #22 | Vessel #23 |
|---|---|---|
| 2/50 | 26.5 | 27.4 |
| | NaSCN added | |
| 3/20 | 30.6 | 30.8 |
| 3/50 | 33.7 | 34.2 |
| 4/20 | 34.7 | 36.5 |
| | | NaSCN added |
| 4/50 | 36.7 | 36.5 |
| 5/20 | 39.8 | 41.1 |
| 5/50 | 40.8 | 41.1 |

the rate of respiration of the treated eggs does not increase to the same extent as that of the controls, but attains a plateau at a lower level. Third, the maximum rate of respiration attained by the Dauerblastulae is equal to the rate of the controls at 24 hours after fertilization, at which time gastrulation begins in the untreated eggs. Fourth, the rate of respiration of the treated eggs diverges from that of the controls long before gastrulation actually occurs.

If, as the evidence seems to indicate, we may regard NaSCN as acting specifically upon the gastrulation mechanism, then the above facts demonstrate a clear correlation between metabolic rate and morphological change in the embryo. Dauer-

blastulae are not only morphological blastulae, but they are metabolic blastulae as well. Of greater interest is the fact that the Dauerblastulae become metabolically different from the controls before they are morphologically distinguishable from them. The two curves of Figure 11 are clearly divergent after the 10th hour from fertilization; morphologically, however, the two types are practically identical until the 24th hour. This indicates that metabolic changes which originate in the egg as early as the 10th hour are responsible for transformations that lead to gastrulation at the 24th hour and the subsequent differentiation of the pluteus. This is essentially a statement of the concept of determination, long familiar to embryologists. It implies more than the spatial differentiation of parts usually associated with this concept, however; it implies not only configurational fixation, but metabolic fixation as well.

While experiments on the progress of determination with time have not been made on this sea urchin, Horstadius (1936) has carried out an exhaustive series of investigations with the eggs of the closely related form, Paracentrotus lividus. This form develops faster than does Strongylocentrotus purpuratus. According to Horstadius (loc. cit.), the blastula of Paracentrotus is swimming at the 10th hour from fertilization, and gastrulation begins at the 16th hour at 20° C. (compare Table XIII). By means of cutting and transplantation experiments, Horstadius was able to demonstrate the progressive determination in time of various parts of the embryo. As is well known from the work of Driesch (1891-92), the blastomeres of the sea urchin

egg, isolated at the 2- or the 4-cell stage, are able to regulate and form plutei of 1/2 or 1/4 normal size. Horstadius showed that meridional halves taken at later stages gradually lose their ability to regulate; if the embryo is cut in half at the 6th hour after fertilization, only slight differences are observed in the resulting plutei, but if cut at 8 hours, the plutei show marked complementary deficiencies, while if the cutting is deferred until gastrulation half-plutei result. Similar results were obtained with regard to the determination of particular organs and regions of the embryo. For example, an animal half isolated in early cleavage does not develop a normal apical tuft. If isolated at the 8th hour after fertilization, however, a normal tuft develops. Horstadius found the earliest indications of determination to occur at the 6th hour from fertilization, or 37.5 percent of the distance in time between fertilization and gastrulation. The first sign of metabolic differentiation in the present experiments occurred at 10 hours, or 41.7 percent of the distance in time between fertilization and gastrulation. In view of the difference in the rate of development between the two forms, it is unsafe to draw too many conclusions from these figures, especially since the time at which determination occurs may vary for different structures. Nevertheless, it seems clear that metabolic differentiation and morphological determination occur at approximately the same time in the echinoderm egg.

Several alternative hypotheses suggest themselves in explanation of the respiration curve of Dauerblastulae. Tyler and Horowitz (1938a) have shown that the slower than normal

increase in the rate of respiration of parthenogenetic and of phenylurethane-treated Urechis eggs is due to their slower development. Brachet (1938) has arrived at the same conclusion with regard to KCl-activated eggs of Chaetopterus. The retarded respiration of Dauerblastulae may thus possibly be explained by an inhibitory effect of NaSCN on the rate of development or the rate of cleavage after the 10th hour from fertilization. Since no comparative study of cleavage rates in late stages of Dauerblastulae and normal embryos has been made, this possibility cannot be completely excluded. This explanation seems unlikely, however, in view of the fact that the rate of cleavage in early stages is not affected. The possibility that NaSCN retards developmental processes as a whole at later stages also appears improbable, since on this basis one would expect the Dauerblastulae to eventually gastrulate, which they do not do. That the effect of NaSCN on development is an inhibitory one is undeniable. The position taken by the present author, however, is that NaSCN specifically inhibits that part of the metabolism which has to do with the determination of the gastrula. The mechanism of the inhibition is unknown. The results suggest, however, that one may assume as a working hypothesis the suppression of the energy-supplying reactions which make determination possible, by interference with the release of necessary substrate or with the production of necessary respiratory catalysts.

## IX. Experiments with 2,4-Dinitrophenol

### Historical

Clowes and Krahl (1936; also, Krahl and Clowes, 1936) were the first to show that nitro- and halophenols exert a stimulating effect on the respiration of marine (sea urchin) eggs. They also demonstrated that as the concentration of the substituted phenol is increased the respiratory stimulation passes through a maximum, and at the same time a reversible cleavage block occurs. These results were confirmed by Tyler and Horowitz (1937b; 1938b). Tyler and Horowitz further showed that the effectiveness of a given concentration of substituted phenol is dependent on the pH of the external medium, the internal pH of the egg, and the dissociation constant of the substance in question. They presented evidence to show that the substituted phenols penetrate the cell in the undissociated form and exert their effect in the dissociated form. Krahl and Clowes (1938, a,b) confirmed the finding that penetration occurs in the undissociated form and that respiratory stimulation is effected by the ion; they consider cleavage block and respiratory inhibition, however, to be due to the undissociated molecule.

The interest of the substituted phenols from the physiological standpoint lies in the fact that the effects they produce are completely reversible. Even in concentrations which block cleavage and inhibit respiration, permanent injury to the eggs does not result. This is in contrast to other respiratory stimulants, such as oxidation-reduction indicators (Barron, 1929; Runnstrom, 1930). The substituted phenols can thus be used to

investigate the nature of the respiratory mechanism in eggs without incurring the danger that the results may be artefacts resulting from injury.

In the experiments to be described, the action of 2,4-dinitrophenol on the respiration of Urechis and Strongylocentrotus eggs was studied in relation to the rising rate of oxygen consumption during development. It was desired to ascertain whether the stimulability of the respiration is independent of the initial rate of respiration or whether it is a function of the already existing respiration. It was shown by Clowes and Krahl (1936) that fertilized and unfertilized eggs of Arbacia differ in their susceptibility to stimulation by the substituted phenols. They found that a concentration of dinitro-o-cresol which produced a sixfold increase in the rate of oxygen uptake of unfertilized eggs produces only a threefold increase in the rate of oxygen uptake of fertilized eggs. Since the initial rate in fertilized eggs is four times that of unfertilized eggs, however, it can be seen that on the basis of absolute units the substituted phenol is 60 percent more effective in fertilized than in unfertilized eggs. These findings were extended in the present experiments to cover cleavage and later developmental stages. An investigation into the nature of the substrate burned by dinitrophenol-stimulated eggs was also made.

Effect of dinitrophenol on respiration of Strongylocentrotus eggs at various developmental stages.

Inasmuch as the respiration of dinitrophenol-stimulated eggs passes through a maximum with increasing concentrations, it is first necessary in working with these substances to determine the optimum concentration. The optimum concentration has been defined by Tyler and Horowitz (1938b) as that concentration of substituted phenol which produces the greatest increase in rate of respiration which does not diminish with time. This has been determined for the eggs of Strongylocentrotus as $6.8 \times 10^{-9}$ M in terms of undissociated molecules (Tyler and Horowitz, 1938b). It should be noted that the concentration of undissociated molecules is proportional to the concentration of anions, the active form of the molecule within the cell. Inasmuch as the concentration of the undissociated form may vary significantly with small changes in external pH, concentrations will always be given in terms of undissociated molecules, rather than as total concentration.

Using concentrations near that which was found to be the optimum, the relative and absolute stimulation at various intervals from fertilization was determined. For this purpose, eggs were reared as described previously until the desired stage had been reached. They were then washed and concentrated and placed in Warburg vessels of the one-side-arm type. The side-arm contained 2,4-dinitrophenol in a concentration which, when added to the main compartment, gave a concentration somewhat below the optimum; a sub-optimal concentration was used in order to avoid the cleavage

block which would otherwise have been incurred by a slight increase in the hydrogen ion concentration of the medium in the course of the run and the resulting increase in the concentration of undissociated dinitrophenol. The solutions in the vessels were buffered with glycylglycine at pH ca. 8.0, and the pH was again taken at the end of the experiment. The normal respiration of the eggs was first measured for a short time, after which the dinitrophenol was tipped in and the measurements continued.

Using a concentration of $3.0 \times 10^{-9}$M, the curves for relative and absolute stimulation shown in Figure 12 were obtained. The relative stimulation, or percentage stimulation, as used in the following paragraphs is defined as the quotient of the oxygen consumed during the hour following addition of dinitrophenol by the oxygen consumed during the hour preceding the addition of dinitrophenol, multiplied by one-hundred. The absolute stimulation is the difference in the rate of respiration during the hour following addition of dinitrophenol and the hour preceding the addition. Figure 12 shows that as development proceeds (i.e., as the normal rate of respiration increases), the percentage stimulation decreases. The absolute stimulation remains constant during the first 30 hours of development, after which it also falls off.

Lindahl and Öhman (1938), on the basis of investigations of the effects of lithium and pyocyanin on the respiration of sea urchin eggs, have suggested that two systems are responsible for the respiration of developing eggs, one of which increases

## EXPLANATION OF FIGURE 12

Stimulation of respiration of Strongylocentrotus eggs at various intervals from fertilization by $3.0 \times 10^{-9}$ M dinitrophenol. Curve A: absolute stimulation. Curve B: relative stimulation.

## EXPLANATION OF FIGURE 13

Stimulation of respiration of Urechis eggs at various intervals from fertilization by $1.3 \times 10^{-8}$ M dinitrophenol. Curve A, solid line: absolute stimulation of normal embryos; dotted line: absolute stimulation of "starved" embryos. Curve B, solid line; relative stimulation of normal embryos; dotted line: relative stimulation of "starved"embryos.

in activity as development proceeds and the other of which remains at constant activity until a certain stage of development is attained (compare Gray's hypothesis, p. 22). They believe that pyocyanin stimulates only the constant fraction of the respiration. This scheme would fit the present data. The constant level of stimulability (absolute) during the first 30 hours would result from the action of dinitrophenol upon the constant fraction of the respiration. After the 30th hour, the heretofore constant fraction would begin to increase in activity, resulting in a decrease in the apparent stimulation due to dinitrophenol. Attractive as this hypothesis is, it must be remembered that there is no direct evidence for the existence of the postulated systems in the egg. It appears that the present results, at least, can be explained on a less hypothetical basis; namely, on the amount of available substrate within the egg. There is ample evidence to indicate that the substrate is not a limiting factor in the respiration of the cleaving egg. As development proceeds, however, and as the rate of oxidation rises, the substrate is depleted until it may well approach a limiting value unless supplemented by feeding. Experiments to determine the time at which feeding is normally initiated in the sea urchin, similar to that described for Urechis (p. 23) showed that the pluteus is unable to ingest carmine particles from the sea water before the 100th hour of development at the earliest. The fact that, molecule for molecule, dinitrophenol shows no loss in activity up to the 40th hour of development must indicate that the substrate is not limiting up to this stage. Beyond this time, however, the de-

pleted substrate is less able to support the added respiratory load, resulting in a gradually diminishing stimulability, until at the 135th hour of development the stimulation approximates zero.

Effect of dinitrophenol on respiration of Urechis eggs at various developmental stages.

Since the optimum concentration had not previously been determined for the eggs of Urechis, it was first necessary to investigate this. For this purpose, fertilized eggs were placed in a series of single-side-arm Warburg vessels. The side-arms contained 2,4-dinitrophenol in a graded series of concentrations. All solutions, as previously, were buffered with glycylglycine at pH ca. 8.0. The respiration was measured for a short time, after which the dinitrophenol was tipped in and the measurements continued for several hours. The optimum concentration was determined on the basis of the definition stated above.

Figure 14 shows the oxygen consumption curves of fertilized eggs for a number of different concentrations of dinitrophenol. It can be seen that the optimum concentration lies between 2.51 x $10^{-8}$ M and 5.02 x $10^{-8}$ M in undissociated molecules. The mean is 3.8 x $10^{-8}$ M, or five and one-half times the critical concentration in Strongylocentrotus. This difference can be explained by assuming a lower internal pH in Urechis eggs than obtains in the eggs of Strongylocentrotus, a shift of six-tenths of a pH unit being sufficient to account for the observed difference. The stimulability at various intervals from fertiliza-

## EXPLANATION OF FIGURE 14

Effect of different concentrations of 2,4-dinitrophenol on rate of respiration of fertilized Urechis eggs. Dinitrophenol added $3^h$ $15^m$ after fertilization in the following concentrations:

Curve A, $1.37 \times 10^{-8}$ M
Curve B, $2.51 \times 10^{-8}$ M
Curve C, $5.02 \times 10^{-8}$ M

in terms of undissociated molecules. The optimum concentration lies between B and C.

## EXPLANATION OF FIGURE 15

Effect of different concentrations of 2,4-dinitrophenol on rate of respiration of 43 hour old trochophores of Urechis. Dinitrophenol added $43^h$ $30^m$ after fertilization in the folowing concentrations:

Curve A, $1.33 \times 10^{-8}$ M
Curve B, $1.59 \times 10^{-8}$ M
Curve C, $3.31 \times 10^{-8}$ M

in terms of undissociated molecules. The optimum concentration lies between A and B.

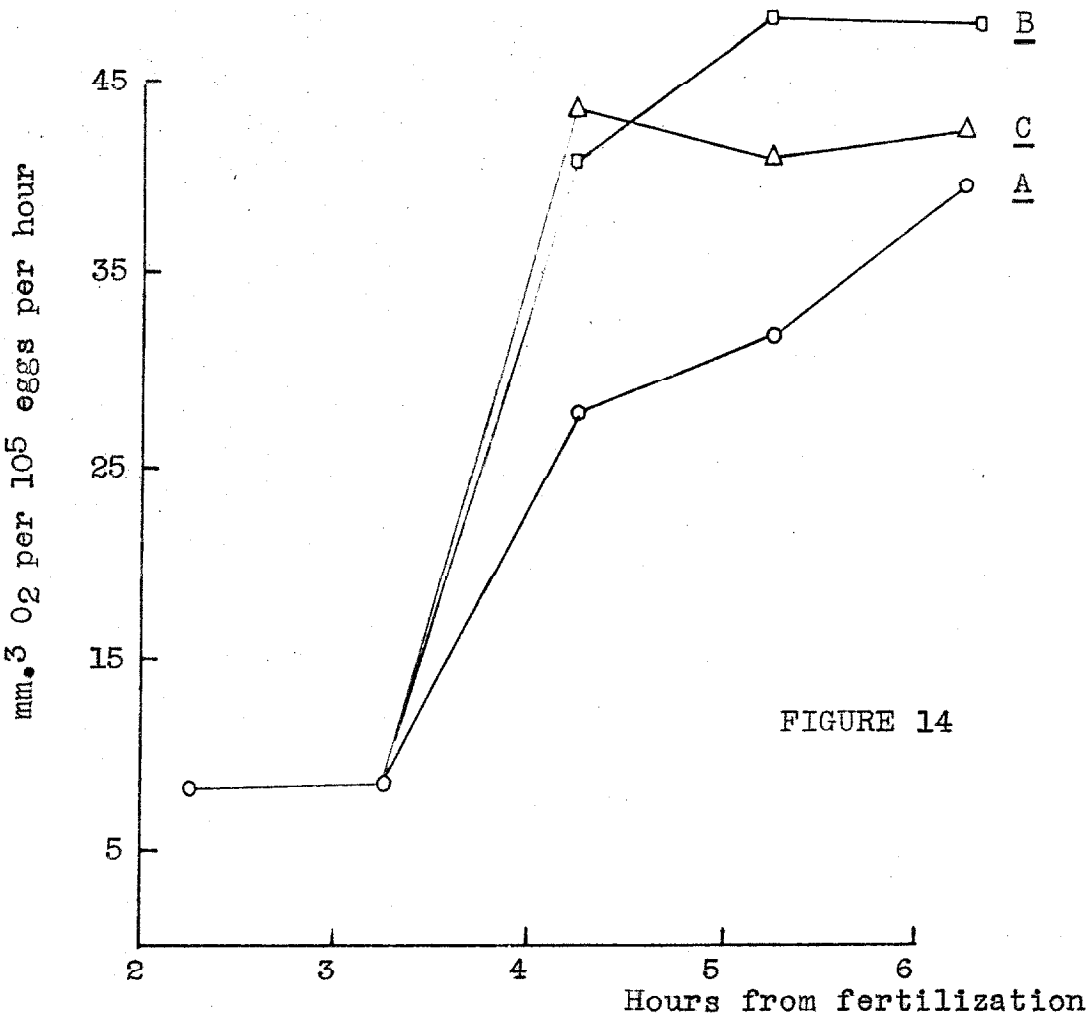


FIGURE 14

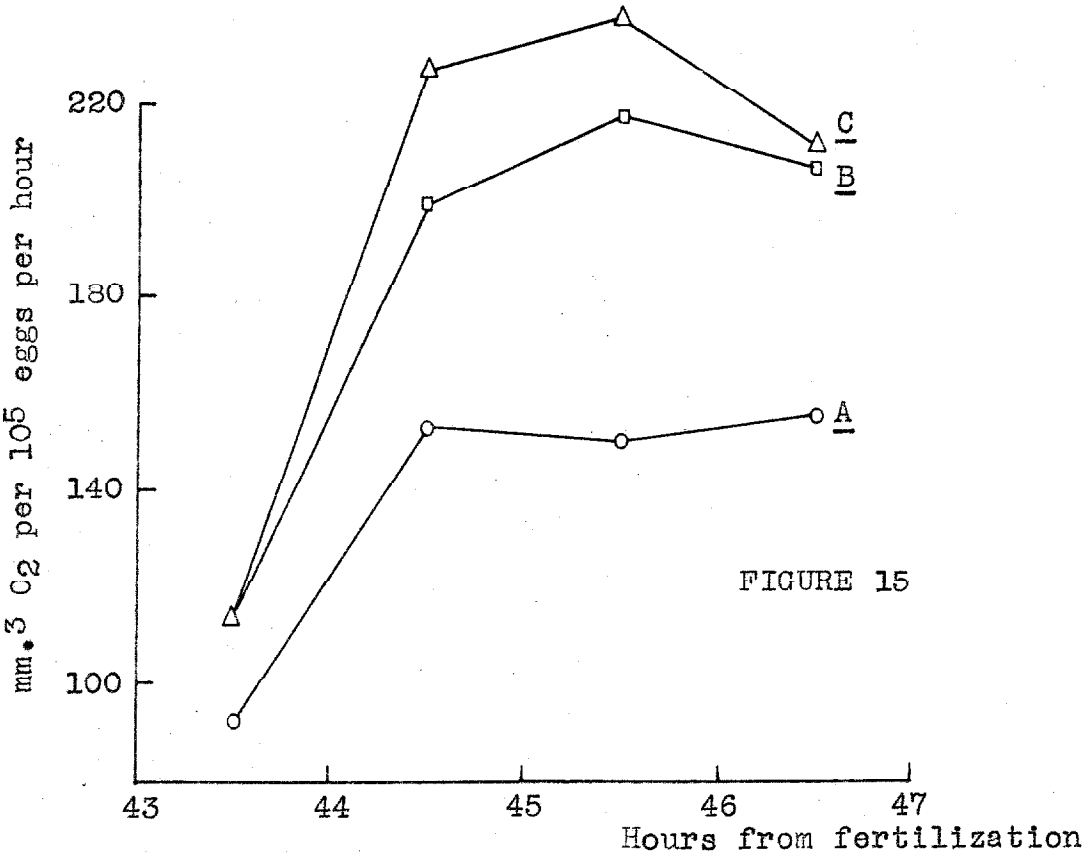


FIGURE 15

tion was then determined. Using a concentration of $1.3 \times 10^{-8}$ M, the curves for relative and absolute stimulation shown in Figure 13 (solid lines) were obtained. The curves show that as development proceeds (as the rate of respiration rises) the percentage stimulation decreases rapidly. The absolute stimulation, however, rises rapidly; in other words, molecule for molecule, dinitrophenol produces a greater response at later than at earlier stages of development.

This result at first appeared difficult to explain on the basis of any reasonable stimulation theory; for, as development proceeds and more and more of the respiratory enzymes of the egg come into play in normal respiration, and as the combustible reserve of the egg grows less and less, one would expect the absolute stimulability to decrease--as in Strongylocentrotus eggs--rather than increase. The best explanation seemed to lie in a shift of the optimum concentration toward lower values as development proceeds. Thus, a concentration of undissociated dinitrophenol which produces a sub- maximal effect at the 3rd hour of development would produce a maximal, or nearly maximal, effect at the 50th hour. Such a shift was demonstrated and is shown in Figure 15, where the stimulation of 43 hour old trochophores is plotted. It will be seen that the optimum concentration drops from $3.8 \times 10^{-8}$ M at the 3rd hour of development (Figure 14) to $1.5 \times 10^{-8}$ M at the 43rd hour. The shift is possibly due to increases in pH in micro-regions of the cell during the course of development, resulting in an increased concentration of dissociated dinitrophenol at the respiratory centers. Such changes

in pH have been described for the eggs of another annelid, Nereis, by Spek (1930). Experiments with sea urchin eggs showed no variation in the optimum concentration during development.

The effect of a limiting supply of oxidizable substrate can be shown at late stages of Urechis development, using the "starved" embryos described on p. 24. These embryos, as stated previously, exhibit a very low rate of oxygen consumption, comparable to 13 hour old blastulae. But whereas the respiration of 13 hour blastulae can be stimulated 120 percent by the concentration of dinitrophenol used, the respiration of "starved" embryos is stimulated less than 60 percent with the same concentration. This is shown by the dotted lines of Figure 15. The effect of feeding on the stimulability at late stages was tested, but neither the addition of the extract of sea urchin eggs used previously (p. 25) nor glucose produced any significant increase in the stimulability. This probably means that even in these cases the substrate was still a limiting factor.

Effect of dinitrophenol on the R.Q. and nitrogen content of Urechis eggs.

In view of the enormous stimulation of the oxidative processes of eggs produced by dinitrophenol, it would not be surprising if part, or even all, of the excess respiration involved the combustion of reserves not ordinarily utilized in development. To provide an answer to this problem, the R.Q. of dinitrophenol-stimulated eggs was measured. Clowes and Krahl (1936) found no significant change of the R.Q. in Arbacia eggs due to dinitro-

o-cresol. The conclusion to be drawn from their finding is that the stimulation represents simply an increase in the activity of the normal respiratory mechanism. Bodine and Boell (1938), on the other hand, found a significant increase in the R.Q. of dinitrophenol-stimulated grasshopper embryos--the R.Q. rising from a value of 0.75 to 0.91. By measuring the ammonia production, they showed that the increase could be accounted for by the combustion of protein to ammonia as the end product. This finding indicates that dinitrophenol initiates the breakdown of tissue proteins--a process foreign to the normal embryo.

For the present experiments, the two-side-arm vessels previously described in connection with R.Q. measurements were used. Dinitrophenol in the proper concentration was added to the egg suspension immediately before pipetting the eggs into the vessels. Measurements were made both at early and late stages of development. The results obtained are shown in Table XV.

Table XV

Effect of 2,4-dinitrophenol on the R.Q. of developing Urechis eggs. Concentration of undissociated dinitrophenol = $5.0 \times 10^{-9}$ M. Values expressed as $mm.^3$ gas consumed or produced per vessel per hour. Temperature, 20° C.

| Hrs./min. from fertilization | Controls | | | Dinitrophenol | | |
|---|---|---|---|---|---|---|
| | $X_{O_2}$ | $X_{CO_2}$ | R.Q. | $X_{O_2}$ | $X_{CO_2}$ | R.Q. |
| 1/29 | 16.7 | 20.2 | 1.21 | 75.3 | 66.6 | 0.89 |
| | | | | 66.3 | 52.4 | 0.79 |
| 2/5 | 35.0 | 40.6 | 1.16 | 250.0 | 229.0 | 0.92 |
| | | | | 222.0 | 197.0 | 0.89 |
| 14/42 | 118.5 | 84.0 | 0.71 | 225.0 | 176.0 | 0.78 |
| | | | | 200.0 | 151.0 | 0.76 |
| 16/35 | 113.0 | 80.0 | 0.71 | 245.0 | 186.5 | 0.76 |
| | | | | 214.0 | 161.5 | 0.76 |

As shown in the table, dinitrophenol tends to lower the R.Q. at early stages, when it is high, and to raise it at later stages when it is low. The time of the experiments at early stages was very short in order to avoid the using up of the carbohydrate reserve, which might result in a premature shift to fat metabolism and an apparent lowering of the R.Q. The results seem best interpreted on the basis of the combustion of the protein reserve within the egg. Since the R.Q. of protein combustion (0.80) is intermediate between those of carbohydrate and fat, this would explain the effects observed. This is in agreement with the observations of Bodine and Boell (loc. cit.) who worked with relatively later stages. The observed R.Q.'s may, on the other hand, be interpreted as signifying mixed carbohydrate-fat metabolism. This implies that stimulation at early stages causes the egg to draw on its fat reserve, and stimulation at later stages causes it to draw on its carbohydrate reserve. Against this view it can be pointed out that the existence of a carbohydrate reserve is questionable and that the embryo is unable to utilize carbohydrate at late stages even when supplied it in excess (p. 40).

In order to clarify this point, an investigation of the effect of dinitrophenol on the nitrogen content of the embryos was carried out. Fertilized eggs were placed in sea water containing undissociated dinitrophenol in a concentration of $5.0 \times 10^{-9}$ M. After 6 hours they were removed and washed three times in filtered sea water in order to remove the dinitrophenol. Making ample allowance for any dinitrophenol which may not have

been removed from the eggs by washing, calculation showed that the extra nitrogen due to dinitrophenol was too slight to be detected by the analytical method and so could not interfere with the results. After washing, the eggs were concentrated, counted, and analysed by the method of Borsook and Dubnoff (see p. 52).

The analytical results are shown in Table XVI. There is significantly less nitrogen in the treated than in the control eggs. The decrease in total nitrogen is 5.6 percent.

Table XVI

Effect of dinitrophenol on the partition of nitrogen in Urechis eggs. Eggs in dinitrophenol during first 6 hours of development. Fixed at 7 hours after fertilization.

| | Controls | | Dinitrophenol | |
|---|---|---|---|---|
| | Mg/egg x $10^5$ | Percent | Mg/egg x $10^5$ | Percent |
| Protein N | 0.787 | 55.0 | 0.805 | 59.6 |
| N. P. N. | 0.643 | 45.0 | 0.545 | 40.4 |
| Amino N | 0.441 | 30.9 | 0.453 | 33.6 |
| Ammonia | 0.011 | 0.79 | 0.009 | 0.65 |
| Undet'd. | 0.191 | 13.31 | 0.083 | 6.15 |
| Total N | 1.43 | 100 | 1.35 | 100 |

The error of sampling was 3.7 percent. That the loss of nitrogen is real is supported by the fact that it appears to occur at the expense of the undetermined fraction. In this it is similar to the loss previously noted in 30 hour trochophores (see p. 53). There is no significant change in the protein, amino, or ammonia fractions. The ratio of protein to amino nitrogen is the same in treated and control eggs. The present

evidence, combined with the fact that the loss of nitrogen in 30 hour embryos occurs chiefly at the expense of the undetermined nitrogen, suggests that this fraction contains the most readily combustible reserve nitrogen. The most probable constituents of this fraction are listed on p. 55. It cannot be stated at present which of the constituents named, if any, are metabolized. The fact that no loss occurs in the amino fraction indicates that arginine and histidine, if combusted, are not broken down completely. However, the growing evidence of the importance of arginine phosphate in the metabolism of invertebrates (see review of Kutscher and Ackermann, 1933) suggests that arginine may be involved here.

These results indicate that the normal substrates--fat and carbohydrate--are not supplied in sufficient quantities to keep pace with the additional respiratory load imposed by dinitrophenol; other substrate must be provided. It is to be expected that if the substrates of the embryo are depleted by the normal metabolic activity and are not replenished, then the substrate will limit the stimulation produced by dinitrophenol. This was the result obtained with "starved" embryos. In conjunction with the previous experiments on the results of feeding (p. 25), these findings lead to the conclusion that substrate limits the rate of respiration at late stages. At early stages, however, there is no such limitation. The stimulability does not begin to decrease until development has proceeded a considerable distance. The factor which limits the respiration at early stages cannot be the substrate, but must reside with

the availability of respiratory catalysts. The stimulation experiments show, however, that catalysts are present at early stages in sufficient amounts to carry a much greater respiratory load than is normally imposed upon them at this time. It must be concluded that the respiratory catalysts are present at early stages in excess of the requirement, but are for the most part inactive. Runnstrom (1930) has arrived at a similar conclusion. The nature of the "inactivation" of the respiratory catalysts during early development will be considered in the next section.

## X. Discussion

A number of investigators in the past have attempted to relate the oxygen consumption curves of developing eggs to the visible changes of development. There has been, on the whole, little consistency or agreement in the results obtained. With regard to cleavage, it has been recognized since the early work of Warburg that no simple relationship exists between the rate of respiration and the increase in the number of cells. The exponential increase in cell number during cleavage is not reflected in a proportional increase in respiration (Warburg, 1908). Nor is the rising rate related to the increasing surface of the developing embryo. Warburg (loc. cit.) showed that the rate of respiration was independent of the partial pressure of oxygen down to concentrations 1/4 that of the atmosphere. This means that the rate of transfer of oxygen across the cell surface does not limit the rate of respiration. On the other hand, there are two lines of evidence which indicate an intimate connection between division rate and respiratory rate during early cleavage. The first of these is to be found in the recent work of Brachet (1938) and Tyler and Horowitz (1938a) in the respiration of activated and of phenylurethane-treated eggs. Although the initial rate of respiration in these cases was not altered by the experimental treatment, its subsequent rise was retarded; this was correlated with retarded cleavage rates in the treated eggs. The second line of evidence is from temperature coefficient data. Tyler (1936b) has shown that the temperature coefficients of cleavage and oxygen consumption are the same in the

eggs of four marine invertebrates. Although this is in contradiction to the results of earlier workers (e.g., Loeb and Wasteneys, 1911) there are several reasons for preferring the more recent data. Tyler's finding has been confirmed by Atlas (1938) on the eggs of the frog. The temperature coefficient evidence is not conclusive, but it supports the hypothesis that respiration and cleavage, if not mutually interdependent, are related through a third reaction.

Attempts to relate respiration to the increase in nuclear material, rather than in the number of cells, have not been entirely satisfying from the experimental side (see results of polyspermy experiments, p. 26 ff.). On the analytical side some indication of the situation may be obtained by comparison of the oxygen consumption curve of Paracentrotus obtained by Rapkine (1927) with the curve showing the increase in nucleic acid during development obtained by Brachet (1933) in the same animal. Rapkine's curve shows no increase in rate of respiration from the 24th to 40th hours of development. Brachet's curve, however, shows a steady increase in the nucleic acid content of the embryos over the same period. Information of a similar nature was obtained in the present investigation by observing the number of mitotic figures in embryos fixed and stained at different developmental stages. In Urechis it was found that although the rate of oxygen consumption (Figure 3) is fairly constant between the 20th and 30th hours of development, cell divisions occur frequently, although with a decreasing rate, over the same period; mitosis does not cease, in fact, until the

90th hour after fertilization. In Strongylocentrotus, mitotic figures are fairly numerous up to the 60th hour from fertilization, after which they appear less frequently; mitoses are still to be seen, however, at the 105th hour after fertilization. It is clear in both cases that the attainment of a plateau in the rate of oxygen consumption does not correspond with the cessation of cell divisions. It is not to be expected, however, that respiration and cell division will bear the same relation to one another at late as at early stages of development, since as development proceeds the embryo becomes the site of many new oxygen-consuming activities. As was shown in the case of Dauerblastulae, the oxygen consumption is influenced as early as the 10th hour from fertilization by factors in the embryo which do not manifest themselves morphologically until many hours later.

Parnas and Krasinska (1921), in their study of the respiratory metabolism of the frog's egg, found that the rate of oxygen consumption was accelerated at three points in development: gastrulation, formation of the medullary plate, and formation of the external gills. Gray (1927) observed a sudden increase in the rate of respiration of sea urchin eggs at the time when the blastulae began to swim. Atlas (1938) observed a discontinuity in the rate of respiration of frogs' eggs at the time of the initiation of tail growth. Other workers have not found such clear evidence of the interaction of developmental processes and respiration. Thus Brachet (1934a) working with frogs' eggs and Fischer and Hartwig (1938) working with Amblystoma eggs found no abrupt changes in the rate such as described by Parnas and

Krasinska and by Atlas. The present results tend to support the former workers. Reference to the respiration curve of Urechis (Figure 3) shows that it has three points of interest: (a) the time at which rapid acceleration of the rate begins, (b) the point of inflection, and (c) the time at which the plateau is reached. During the first few hours following fertilization the rate of oxygen consumption rises only very slowly. Between the fourth and fifth hours there occurs a sudden increase in the rate. This continues for about 8 hours, at which time the curve inflects. Finally, at the 20th hour following fertilization, the rate of respiration becomes constant. There has been an increase of 900 percent in the rate of oxygen consumption over the whole period. None of these peculiarities of the curve, except perhaps the point of inflection, can be readily related to visible, concurrent developmental changes. The point at which the curve begins to rise rapidly (4-5 hours) is marked by no great developmental change, except possibly the development of cilia; but swimming does not begin until several hours later. This region of the curve does correspond, however, with the change from a carbohydrate to a fat metabolism by the embryo. It is not surprising that this event is reflected in the oxygen consumption rate of the egg. Assuming that the maintenance and development of the egg require the expenditure of a certain number of calories per hour, one would predict an increase in the rate of oxygen consumption at this point if development is to continue, since more oxygen is required for the production of a given amount of heat when fat is burned than when carbohydrate

is burned. (This cannot account for the whole increase, however, as can be seen from the heat production curve [Figure 7] which also rises in this region). The point of inflection of Figure 3 corresponds with the beginning of gastrulation, but it is difficult to see what significance, if any, lies in this coincidence. The attainment of the plateau in the Urechis curve corresponds with no particular morphological change. In view of the sharp ascent from the plateau which occurs when feeding starts, however, it seems reasonable to regard the plateau as resulting from a relative substrate deficiency preventing further rise, rather than to developmental necessity.

The respiration curve for Strongylocentrotus (Figure 8) presents no significant discontinuities. The slow leveling-off of the curve cannot be ascribed to any particular developmental stage. It can be said, however, that the rate of respiration does not increase so rapidly after gastrulation (24 hours) as before gastrulation. This was also found in the case of Urechis (see above). It is important to note that although the respiration of Strongylocentrotus has attained a plateau, or practically so, at the 50th hour from fertilization, the definitive pluteus does not develop until after the 60th hour. It would appear that the change from the prism to the pluteus occurs without any accompanying increase in the rate of oxygen consumption.

It has been shown that no sudden breaks occur in the oxygen consumption curves of either Urechis or Strongylocentrotus eggs at the time when important developmental changes such as

swimming, gastrulation, and differentiation of various parts are manifested. It can be concluded from this that no immediate relationship exists between morphological changes and the respiration of the embryo. What, then, is the relationship between the rising rate of respiration and morphogenesis? The view of the present author has already been outlined in part in the discussion of the respiration of Dauerblastulae (p. 60 ff.). In these experiments, development was stopped by an agent (NaSCN) which prevents gastrulation in an apparently specific manner. The resulting embryos remain permanently in the blastula stage. When the respiration of these embryos was measured, it was found that their rate of oxygen consumption never rose beyond that of normal embryos at the same stage of morphological development. If respiration were totally independent of morphological changes, it would be expected that the rate of respiration would rise in the normal fashion regardless of the stopping of morphological development. In addition, it was found that differences in the respiration of treated and control embryos became evident long before any morphological distinction appeared. It was concluded from this that thiocyanate inhibited transformations in the egg which, if allowed to proceed normally, would result in gastrulation. It was suggested that the transformations in question are those which are known collectively as "determination". In order to make the relationship clearer it will be necessary to consider the mechanism of determination.

Experimental evidence indicates that we may regard the unfertilized egg as an anisotropic system consisting of an ag-

gregate of qualitatively different, morphologically significant elements. The determination of parts, on this view, would consist in the directed redistribution of elements, as development proceeds, into circumscribed regions of the embryo, where their subsequent changes produce the visible differentiation of parts. This theory, which has been called the "mosaic" theory of development, has been considered in detail by Wilson (1928, Chap. XIV). Wilson shows that the theory is applicable not only to mosaic-type eggs, but to regulative eggs as well, the differences between the two types being due, for the most part, to the way in which the ooplasmic materials are separated in early cleavage. In principle, the development of both types is the same.

Following the above line of argument, it is clear that the same principle which applies to the determination of the morphology of the embryo will apply to its metabolism as well. The present evidence, together with that of other authors, shows that each stage of development and each part of the embryo and of the adult has a characteristic metabolism. The succession of energy sources in ontogeny and the reproducibility of respiration measurements at any given stage of development, as well as the respiration of Dauerblastulae, indicate that each morphological stage is characterized by a particular metabolic level. Recent work on the respiration of the various regions of the amphibian embryo (e.g., Fischer and Hartwig, 1938; Brachet, 1939; Boell, Needham, and Rogers, 1939) clearly show that metabolic differentiation proceeds simultaneously with morphological differentiation. In echinoderms, Tyler (1933) has shown that dwarf

and normal embryos develop at the same rate and consume the same amount of oxygen per mg. egg nitrogen as far as gastrulation. After this point, the development of the dwarfs becomes slower than that of the controls. If the view presented above is correct, one would expect the rising rate of oxygen consumption to be retarded in the dwarfs at this time. Tyler reports that this is the case. We may assume that the differentiation of the morphology and of the metabolism of the embryo come about through the same fundamental mechanism--i.e., the spatial redistribution of the elements of the egg. On this basis we can relate the rising rate of respiration with developmental processes.

In the dinitrophenol experiments it was shown that the rate of respiration in early development is limited by the availability of respiratory catalysts. These experiments showed that it was possible to raise the rate of respiration in eggs at early stages of development by several hundred percent, indicating that the respiratory systems of the egg are capable of much greater activity at these stages than is normally exhibited. At late developmental stages, the stimulability of the respiration decreased. It was concluded that respiratory catalysts are present in early development in excess of the normal requirement, but are inactive. The rising rate of respiration during development would then consist in the activation of these catalysts. The inactivity of part of the respiratory system in early development may be regarded as resulting from breaks in the chain of hydrogen carriers linking the substrate on one hand with molecular oxygen on the other (compare Runnstrom, 1930). The redis-

tribution of elements in the course of development results in the coming together of enzyme-substrate complexes in the egg, with a consequent increase in the rate of respiration as development proceeds. The respiration continues to rise as long as the substrate concentration is high. When the latter is depleted the respiratory rate ceases to increase; if substrate is then not provided, the rate will eventually decrease. These results were indicated in the feeding experiments with Urechis embryos, in the observations on "starved" embryos, and in the dinitrophenol experiments.

The concept outlined above brings into relation with one another several of the most important facts established in the preceding investigations. It relates the rising rate of oxygen consumption with morphological changes, not immediately, but through a process common to both; it indicates how each stage in morphogenesis is characterized by a peculiar metabolic state and clarifies the connection between respiration and determination shown by the Dauerblastulae experiments. It offers a mechanism which explains how it is possible to stimulate the respiration (short-circuiting of the respiratory chain), as with dinitrophenol, without affecting the rate of development, which can proceed no faster than the distribution of morphogenetic elements; but why it is impossible to stop development (as in Dauerblastulae) without at the same time stopping the respiratory increase.

## XI. Summary

1. The rates of oxygen consumption of the fertilized eggs of Urechis caupo and of Strongylocentrotus purpuratus have been measured to the 29th and 48th hours of development respectively. In both forms, the rate rises as development proceeds and finally attains a plateau at the 20th and 40th hours respectively.

2. It has been shown that after the 30th hour of development, the respiration of Urechis larvae is dependent on the food supply.

3. No increase in the rate of respiration of polyspermic Urechis eggs over that of normal eggs could be demonstrated.

4. The R.Q. of Urechis embryos was measured at various intervals from fertilization. The results show that the embryos subsist on a carbohydrate metabolism during the first hours of development and on a fat metabolism from the 6th hour onward. The addition of glucose after the 6th hour does not restore the carbohydrate metabolism. These findings are discussed in relation to the succession of energy sources in ontogeny.

5. The heat production of Urechis embryos has been calculated.

6. Analyses of total, total non-protein, amino, peptide, amide, and ammonia nitrogen were made on Urechis embryos at different developmental stages. No nitrogen is lost during early development. Only a slight amount of protein synthesis occurs.

7. The rate of consumption of artificial Dauerblastulae of Strongylocentrotus has been measured. It does not attain the same level as that of normal embryos. The difference in rate of respiration of Dauerblastulae and controls appears earlier than do observable morphological differences. This is discussed in relation to the problem of determination.

8. The effect of 2,4-dinitrophenol on the respiration of embryos at different stages of development has been tested. The relative stimulability of the respiration decreases as development proceeds. The absolute stimulability of Urechis eggs first increases and then decreases as development proceeds. The absolute stimulability of Strongylocentrotus eggs decreases after a period during which it remains at a constant level. Dinitrophenol lowers the R.Q. at early developmental stages of Urechis and raises it at later stages. Analysis shows that a loss of nitrogen is incurred. These findings are discussed in relation to the availability of substrate and of respiratory catalysts during development.

9. The relationship of oxygen consumption to developmental processes is considered, and a hypothesis to account for the relationship is proposed.

# APPENDIX

A. The respiration of developing Urechis eggs. Plotted in Figures 3 and 4. Each value is an average of several measurements.

| Hrs./min. after fertilization | $mm.^3$ $O_2$ per $10^5$ eggs per hour | Total $O_2$ per $10^5$ eggs |
|---|---|---|
| 2/15 | 8.22 | 8.22 |
| 3/10 | 8.41 | 16.63 |
| 4/10 | 8.63 | 25.26 |
| 5/10 | 9.63 | 34.89 |
| 6/10 | 11.30 | 46.19 |
| 7/10 | 15.67 | 61.86 |
| 8/10 | 19.64 | 81.50 |
| 9/10 | 25.65 | 107.15 |
| 9/40 | 27.80 | 121.10 |
| 15/2 | 54.3 | 326.6 |
| 15/32 | 55.5 | 349.4 |
| 16/32 | 58.8 | 408.2 |
| 17/32 | 64.6 | 472.8 |
| 18/32 | 67.2 | 540.0 |
| 19/32 | 69.6 | 609.6 |
| 20/32 | 71.5 | 681.1 |
| 21/32 | 72.0 | 753.1 |
| 23/45 | 72.6 | 896.3 |
| 25/0 | 74.5 | 989.2 |
| 26/0 | 75.1 | 1064.3 |
| 27/0 | 74.7 | 1139.0 |

B. The respiration of developing Strongylocentrotus eggs. Plotted in Figures 8 and 9. Each value is an average of several measurements.

| Hrs./min. from fertilization | $mm.^3$ $O_2$ per $10^5$ eggs per hour | Total $O_2$ per $10^5$ eggs |
|---|---|---|
| 3/0 | 7.36 | 7.4 |
| 4/0 | 9.62 | 17.0 |
| 5/0 | 10.44 | 27.4 |
| 6/0 | 11.40 | 38.8 |
| 7/30 | 13.07 | 58.0 |
| 7/40 | 14.63 | 60.4 |
| 8/40 | 14.69 | 75.1 |
| 9/40 | 15.68 | 90.8 |
| 15/30 | 16.92 | 188.6 |
| 16/30 | 17.14 | 205.7 |
| 17/30 | 17.99 | 223.7 |
| 18/30 | 18.21 | 241.1 |
| 20/0 | 19.60 | 271.0 |
| 20/10 | 21.9 | 274.6 |
| 21/10 | 21.8 | 296.4 |
| 22/10 | 22.0 | 318.4 |
| 23/10 | 21.2 | 339.6 |
| 24/10 | 22.2 | 361.8 |
| 25/10 | 22.2 | 384.0 |
| 26/10 | 22.8 | 406.8 |
| 27/10 | 23.2 | 430.0 |
| 28/10 | 23.6 | 453.6 |
| 28/30 | 24.8 | 461.9 |
| 29/30 | 25.2 | 487.1 |
| 30/30 | 25.8 | 512.9 |
| 31/30 | 26.2 | 539.1 |
| 43/25 | 26.5 | 855.9 |
| 44/25 | 27.8 | 883.7 |
| 45/25 | 26.5 | 910.2 |
| 46/25 | 26.5 | 936.7 |
| 47/25 | 27.8 | 964.5 |

C. The respiration of Dauerblastulae of Strongylocentrotus. Values expressed as $mm.^3 O_2$ per $10^5$ eggs per hour. Each value is an average of several measurements.

| Hrs./min. from fertilization | Dauerblastulae | Controls |
|---|---|---|
| 2/40 | 10.29 | 10.24 |
| 4/40 | 9.95 | 10.61 |
| 5/40 | 12.62 | 12.86 |
| 6/40 | 11.69 | 12.90 |
| 7/40 | 13.56 | 14.63 |
| 8/40 | 13.80 | 14.69 |
| 9/40 | 14.26 | 15.68 |
| 15/30 | 15.65 | 17.50 |
| 28/30 | 18.5 | 24.8 |
| 29/30 | 20.8 | 25.2 |
| 30/30 | 21.2 | 25.8 |
| 31/30 | 20.3 | 26.2 |
| 41/20 | 21.9 | 32.4 |
| 42/20 | 22.6 | 31.6 |
| 43/20 | 19.6 | 29.2 |
| 44/20 | 22 .6 | 30.8 |
| 45/20 | 21.0 | 30.0 |
| 46/20 | 21.4 | 29.5 |
| 47/20 | 21.7 | 29.5 |
| 48/20 | 22.6 | 30.0 |
| 49/20 | 19.6 | 29.2 |

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