STUDIES ON THE RESPIRATORY ENZYMES OF SEA URCHIN EGGS

Thesis by

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ABSTRACT

1. The literature pertaining to the respiratory metabolism of sea urchin eggs is reviewed.

2. Homogenates prepared from the unfertilized eggs of Lythechinus pictus and Strongylocentrotus purpuratus were found to possess enzymatic activity corresponding to the enzymes phosphoglucomutase, oxoisomerase, aldolase, triosephosphatedehydrogenase, lactic dehydrogenase and enolase. Lactic acid accumulates anaerobicaly in intact eggs. Respiration of homogenates is inhibited by fluoride.

3. Citric, alphaketoglutaric, succinic and malic acids are metabolized. Respiration is inhibited by malonate and increased by cytochrome c.

4. Spectroscopic evidence is presented for the existence of cytochromes a and b. A method of preparing an insoluble fraction containing these cytochromes is described.

5. It was found that the increased respiration obtainable with dinitrophenol increases faster than the normal respiration during the first eight hours of development.

6. It is concluded that the respiratory pathways of the sea urchin egg are similar to those known to exist in adult organisms.

7. The chemical basis for the similarity of the glycolytic mechanism in most forms of life is discussed.

Introduction

The amphibian embryo has proved the most fruitful material for the analysis of morphogenetic processes. On the other hand more information has been obtained about the very early events of development from studies of the eggs of marine invertebrates. Of these the eggs of sea urchins, for technical reasons have been the material of choice.

The study of earliest development encompasses several rather distinct fields. The maturation divisions have provided material for the cytologist and an opportunity of linking cytological and physiological phenomena. The earliest determinations often occur about the time of fertilization and are a field of study for the embryologist in the classical sense. Interacting substances in eggs and sperm have an interest in themselves and are possible convenient models for interactions of cells and tissues in general. Finally there are studies of a more biochemical and physiological nature seeking to link morphogenesis and differentiation with chemical phenomena.

It has been stated by Dalcq(1941) that a distinction must be made between true embryological research and physiological studies which use the egg and a convenient cell for the purpose of the investigation. There is no doubt that there is some truth in this point of view. It would, however, perhaps be better to state that there is no real distinction between any of the biological sciences, and that therefore,

no apologies need be offered for research if it does not fit into the strict frame of some well accepted discipline. In embryology particularly advances in related fields are likely to throw unexpected light on phenomena long not understood.

The biochemical phenomenon most intensively studied on embryological material has been respiration. The reason for such concentration on respiration has probably been that respiration, in the sense of gas exchange has been the easiest phenomenon to study. Its significance is only now becoming understood in detail and much of the older, as well as more modern work has failed to throw much light on problems of interest to the embryologist.

According to J. Needham (1931) the first investigator to study embryonic respiration was Spallanzani (1803), who found in the course of studies, during which he discovered tissue respiration, that eggs gave off and took in gas$es. A long period followed in which the only advance made was the substantiation of Spallanzani's results. The modern work on embryonic respiration can be said to begin with the investigations of the sea urchin egg by Warburg.

Using the Winkler technique, Warburg (1908) showed that there is a very great rise in the respiratory rate of eggs after fertilization. This result created great interest, as it supported the theory of J. Loeb (1906) that oxidations were intimately connected to the events of fertilization. Further papers of Warburg (1909, 1910 a, 1910 b, 1910 c) followed, in which he showed that parthenogenesis had the same

effects on respiration as fertilization with sperm. Another important result obtained was the demonstration that certain narcotics such as urethane could suppress cell divisions without significantly affecting respiration. The effect on respiration of various conditions, such as pH and osmotic pressure was also studied and observations of the influence of iron on the respiratory rate led Warburg to studies of the catalytic effects of heavy metals and to the ultimate discovery of the atmungsferment.

It was at first thought that the increase in respiration on fertilization was a general phenomenon, but the investigations of Loeb and Wasteneys (1912) showed that the eggs of the starfish Asterias forbesii showed no such phenomenon. A great deal of work has since been done on this question. The general result has been that eggs on fertilization may show any one of three types of respiratory behavior. The respiration may rise, remain unchanged or fall. This is partly a species characteristic, but Tyler and Humason (1937) have shown that even within the same species, in the case of Urechis caupo, eggs of different females may show any one of the three types of responses. The same phenomenon has been reported by Shapiro (1941) for Asterias forbesii. It is thus clear that no general significance can be attached to the direction of change of the respiratory rate upon fertilization.

Whitaker (1931) has attempted to fit the phenomena into a general scheme. He noticed that the respiratory rate of

the unfertilized eggs of marine invertebrates of different species was very different, but that after fertilization the rates per unit volume became more nearly the same. The unfertilized egg is therefore in some sense abnormal as far as its respiratory rate is concerned and fertilization "regularizes" the gas exchange. Most of the data fit this scheme rather well. However, it should be treated with caution if only because of the technical difficulties often encountered in measuring the respiration. The question of the temperature at which comparisons should be made and the influence of size and surface of eggs are factors to be taken into account.

The question of respiratory rise has been clarified to some extent by the recent work of Borei (1948). It has often been remarked that the behavior of unfertilized eggs of the sea urchin is apparently rather capricious. Borei, using the Cartesian diver technique has found that immediately after shedding the respiration of sea urchin eggs is almost equal to those fertilized, but that on standing in sea water the rate falls and asymptotically approaches a low figure. The rate after fertilization is the same regardless of when the eggs are fertilized, so that the actual rise on fertilization depends on the time after shedding that fertilization occurs. It had previously been shown by Lindahl and Holter (1941) that oocytes have a respiratory rate slightly higher than that of the fertilized egg. It therefore seems clear that the variation in the amount of rise of respiration of

eggs is a function of the fall in respiratory rate in the prefertilization period. The eggs of animals in which the respiratory rate falls on fertilization have not been investigated from this point of view. Neither has the problem of the causes and conditions of the fall in respiratory rate on shedding.

For a discussion of the relation of respiration to development the discussions of Needham (1942) and Tyler (1942) should be referred to. Since development frequently stops under anaerobic conditions it has some significance for development, and therefore the changes in respiratory rate must have some embryological significance. In any case the mechanism of the change of respiratory rate is an interesting problem in itself. The problem of the control of biochemical reactions is more complex than the establishment of biochemical pathways and the sea urchin egg has appeared to many to be a suitable object for such studies. The result is that a large though rather inconclusive literature has accumulated on the subject which can be mentioned here only rather briefly.

Efforts have been mainly concentrated toward determining what changes occur in the respiratory mechanism upon fertilization. The most detailed analysis has been made by J. Runnstrom and his school. (For review see Runnstrom (1949)) The early studies established the fact that like most tissues the respiration of fertilized sea urchin eggs is inhibited by cyanide and carbon monoxide. It was found that both

fertilized and unfertilized eggs are inhibited but in different degrees, the respiration of the fertilized being much more susceptible. In addition the eggs respond to the addition of dimethylparaphenylenediamine by a great increase in respiration. The maximum rate reached is identical for both fertilized and unfertilized eggs. From these results Runnstrom concluded that the cytochrome and cytochrome oxidase is equally active before and after fertilization, but that there is lacking a link to the dehydrogenase system. It would appear that the conclusion about the cytochromes is generally sound, in spite of more recent work which has uncovered some complications in the experiments with cyanide and carbon monoxide inhibition. It was found by Runnstrom (1935) himself, and then studied by Orstrom (1935) in more detail, that cyanide and carbon monoxide have considerable stimulatory effects on respiration. The effect of CO has recently been studied by Rothschild (1949) with interesting results. He found that carbon monoxide increases respiration, probably by being itself oxidized. This increase is inhibited by carbon monoxide and the inhibition is lifted by light. In addition, light inhibits the normal respiration. The effects are therefore very complicated and conclusions drawn from earlier work on carbon monoxide will probably have to be revised. With respect to the cyanide inhibition, Robbie (1946) recently demonstrated that the earlier work had frequently been vitiated by loss of cyanide from the reaction vessel into the center well,

and that if proper precautions are taken the respiration of unfertilized sea urchin eggs can be shown to be strongly inhibited by cyanide. The earlier conclusions of Runnstrom, however, still appear to be valid.

At one time Korr (1937) had suggested that the activation of cytochrome c resulted in the respiratory increase. Until recently the search for cytochromes in the sea urchin egg had not been successful. The work of Rothschild and the work reported in this thesis demonstrate that some at least of the cytochromes are present.

A great deal of discussion has centered around the question whether the dehydrogenases are more active in the fertilized egg. Runnstrom (1930) and Orstrom (1932) found that the reduction of methylene blue was no faster in the fertilized eggs than in the unfertilized. However, in what appears to be a more careful study Ballentine (1938) demonstrated that the reduction of both methylene blue and ferricyanide is much more rapid in the fertilized eggs, and furthermore that it parallels the changes in respiratory rate in other eggs, rising and falling as the respiratory rate rises or falls. Recently Lindahl (quoted in Runnstrom (1949)) has shown that the difference between fertilized and unfertilized eggs in rate of reduction of methylene blue is a function of the temperature, being greater at higher temperatures and slight at about 12° C.

As can be seen from the above brief summary, the question of the mechanism of the rise in respiratory rate of the egg

on fertilization remains obscure in spite of many efforts. The problem of fertilization has not been studied in this thesis, but a suggestion may perhaps be offered. It is now known, since the work of Meyerhoff (1937) and D. M. Needham and Pillai (1937), that certain dehydrogenations are obligatorally coupled to phosphate transfer. This is now believed to be a general phenomenon. Certain of the dyes used to study respiration act in an as yet unknown manner to uncouple the two phenomena. It may be that there is no activation of any respiratory enzyme on fertilization at all, but that respiration increases because of activation of some enzyme system that uses ATP and thus increases the availability of phosphate acceptor. Meyerhoff and Wilson (1947) have clearly demonstrated the effect of phosphate acceptor on the respiration of various tissues, especially brain. The main advantages of such an explanation, until decisive experimental evidence is produced is that it utilizes a known mechanism and further explains the difficulty hitherto experienced in locating the point of activation among the respiratory enzymes. Compatible also is the work demonstrating that phosphatase is more active in fertilized eggs, that labeled phosphate begins to exchange and that free SH groups appear on fertilization. All phosphokinases are SH enzymes.

Even if the above is the mechanism of the activation of respiration, the problem of the ultimate site of activation remains. The problem would seem to be the general one

of the nature of cell stimulation, as was suggested long ago by Lillie (1910). For a discussion the review of Runnstrom (1949) should be consulted. As can be seen from the review, little real knowledge exists and speculation is rife.

Besides the work on the physiological changes that occur in the respiration, a considerable volume of work exists on the biochemical pathways of this respiration. A summary of the pertinent data from the literature is given below.

There is apparently a considerable amount of carbohydrate material in the egg, but the nature of it is not completely clear. Ephrussi and Rapkine (1928) found a large amount of carbohydrate in the eggs of Paracentrotus lividus, almost all of it in the form of glycogen. Essentially the same results were obtained by Zielinski (1935) in the same species and also by Chaigne (1934). These results were confirmed by Stott (1931). On the other hand Perlzweig and Barron (1928) could find no glycogen in trichloracetic acid extracts of the eggs of Arbacia. On hydrolysis, however, a large amount of glucose was found. O. Meyerhof (1911) likewise found no glycogen in the eggs of Paracentrotus lividus at Naples. The most thorough investigation of glycogen was that made by Orstrom and Lindahl (1940). They were primarily interested in the substrate utilized immediately after fertilization. About 33 mg of glycogen per 100 mg egg nitrogen was found by them in the eggs of Paracentrotus lividus.

The exact nature of the substance in the egg called glycogen is not clear. It is well known that glycogen itself exists in two forms, the so called lyo and desmo glycogen. The latter is bound to some other material, probably protein and is not easily extractable. Brachet and Needham (1935) found the two forms of glycogen in the frog's egg and discovered that the desmoglycogen disappeared first, after which the lyoglycogen was metabolized. That the glycogen of the sea urchin egg is not identical with that from other sources is indicated by the work of Orstrom and Lindberg (1940). They added glycogen of unstated origin to egg brei and found that it was not attacked. They also quote Holter as saying that no appreciable amylase activity is to be found in the eggs of Paracentrotus lividus and Arbacia punctulata. Runnstrom (1949) states that Vasseur has pointed out that the usual tests for glycogen may give misleading results. An apparent glycogen content can be demonstrated in the jelly coat substance of sea urchin eggs, although no glycogen exists there.

The function of glycogen is now considered to be entirely that of carbohydrate storage. It has been pointed out by Needham (1931) that in the latter half of the nineteenth century there was a widespread opinion that glycogen was of some peculiar embryological and morphogenetic significance and a very great number of papers appeared on the distribution of glycogen during development. This belief gradually died out, but other substances have taken over the

position of glycogen as morphogenetic substances. With the discovery of new substances, vitamins, hormones, steroids, nucleic acids and what not have been successively hailed as the key to development and of course to the problem of cancer. Some have already been relegated to the status they deserve, but many are still going strong. No doubt the future still holds much in store.

In summary it can be stated that some polysaccharide resembling glycogen is present and available in the sea urchin egg. The work of Orstrom and Lindberg, quoted above, was designed to solve the problem of whether the carbohydrate is the substrate of respiration in the egg. They were able to show that the glycogen diminished very suddenly on fertilization and in the expected amount. For a more prolonged period Ephrussi and Rapkine (1928) found that the glycogen content diminished between fertilization and the pluteus stage. Some measurements of the respiratory quotient exist. According to Orstrom (1941) the quotient for unfertilized eggs is 1 and falls on fertilization to 0.89, or after correction for certain errors of measurement 0.73. This unfortunately does no more than possibly indicate that carbohydrate is not the sole substrate after fertilization.

As to the pathway of glycogen metabolism no very clear evidence exists. On the basis of what is known from other organisms, it would be expected that glycogen would be broken down phosphorolytically as found originally by Cori, Cori and Schmidt (1939). Amylitic breakdown apparently

does not occur according to the evidence of Holter, quoted above, that amylase activity is undetectable in sea urchin eggs. Orstrom and Lindberg examined the question and found that the breakdown of glycogen was inhibited by phloridzin and accelerated by addition of sperm extracts. Due presumably to active phosphatase activity no accumulation of esters was detectable and addition of cofactors of glycolysis did not influence the rate of glycogen breakdown. The evidence does point to a phosphorolytic cleavage of glycogen in the egg.

The further pathway of substrate in respiration has not been clarified. There is evidence that it takes the same course as is well known in other tissues and also evidence that the course is quite different.

Certain cofactors of glycolysis and respiration are known to be present in the egg. In 1933 Runnstrom demonstrated the presence of what was probably Coenzyme I in the eggs of Paracentrotus. Lindberg and Ernster (1948) found that addition of coenzyme I enhanced the respiration of brei in the presence of pyocyanine. Jandorf and Krahl (1942) estimated the amount of coenzyme I to be between 250 and 500 micrograms per gram wet weight of Arbacia eggs. They also found diphosphothiamine to be present.

The presence of adenosinetriphosphate has been established by the work of Needham and Needham (1930) and confirmed. For a recent review on phosphate esters in eggs see Whiteley (1949). There is also present an adenosinetriphosphatase which has been studied by Connors and

Scheer (1947). They found that the enzyme required calcium as a cofactor, a point that may be of interest in view of the importance of calcium in the fertilization process. The activity of the enzyme is also reported to increase in breis made from fertilized eggs over those of the unfertilized, but the authors express themselves with a certain amount of reserve on this point.

Attempts have been made to detect the presence of phosphorylated intermediates in the egg. The acid soluble phosphorus of the egg has been divided by Runnstrom (1949) into three fractions, one of which is orthophosphate (50%), easily hydrolyzable phosphate (25%) and the remainder an ester very stable to hydrolysis. The same results have been obtained by other investigators, Lindberg (1943), Zielinski (1935), and Orstrom and Lindberg (1940). The presence of the known intermediates of glycolysis was not detected. The methods of detecting such compounds are however rather poor and liable to interference from other materials in tissues and thus failure to detect them cannot be taken too seriously.

Lindberg (1946) made an extensive investigation of the phosphate ester stable to hydrolysis. He found such an ester in the eggs of sea urchins, in sperm and in mammalian tissues excepting muscle. The ester was isolated from cow brain and characterized as a propanediol phosphate. The position of the phosphate was not established. It should be noted that the identification of propanediolphosphate

in eggs is based only on rate of hydrolysis. It appears that propanediolphosphate is the major constituent of the so called barium soluble alcohol soluble fraction from most tissues. That it may have some bearing on embryonic metabolism is indicated by its abundance in eggs. A large amount of a similiar material is found in the chick embryo and in tumors (Needham, 1942, p614).

Orstrom and Lindberg concluded from their studies that the pathway of substrate from glycogen on is different from that established in muscle. They state that such a conclusion is probable on comparative biochemical grounds, since the echinoderm is an animal relatively low phylogeneticaly and its egg could be expected to have a "simpler" type of metabolism. The factual arguments that they bring forward in support of such a conclusion are that they could detect no changes in phosphate esters during the disappearance of glycogen and no increase of lactic and pyruvic acids. They quote experiments by Runnstrom showing that the production of acid on cytolysis is not inhibited by iodoacetate and the work of Stott (1931) showing increases of reducing sugars. The metabolic scheme they favor is as follows. Glycogen is broken down phosphorolyticaly and the resulting esters are then hydrolyzed, the sugars are then metabolized in an unknown manner.

Lindberg and Ernster (1948) found that on the addition of pyocyanine and hexose, hexosemonophosphate and hexosediphosphate an esterification of ADP could be observed.

This is entirely to be expected on the basis of our knowledge of what happens in other organisms.

Much of the argument for the existence of a pathway different from ordinary glycolysis in the sea urchin egg stems from the inability of iodoacetate to inhibit the reduction of methylene blue by hexoses and their phosphoric esters. Thus Lindberg and Ernster (1948) found that iodoacetate had no influence on the oxygen uptake of their preparations. Tyler and Schultz (1932) found that iodoacetate does not inhibit the fertilization of the eggs of Urechis caupo. This was found to be the case for the sea urchin by Runnstrom (1933) and Ellis (1933) and also by Lillie (1931) and Pasteels (1935). This however does not necessarily have much bearing on the nature of carbohydrate breakdown. Hutchens, Keltch, Krahl and Clowes (1941) were unable to detect an triosephosphatedehydrogenase in Arbacia eggs.

Lindberg and Ernster (1948) found that in their homogenates, using glucose and phosphorylated hexoses as substrates methylene blue was decolorized rapidly, but that there was no accumulation of triosephosphates. Triosephosphate was tested for as phosphoric esters hydrolyzed in alkali.

The question whether lactic acid is formed by sea urchin eggs has been debated. In 1929 Ashbel showed that the eggs of Paracentrotus have a considerable anearobic glycolysis and incidentally that this increases almost

threefold on fertilization. For some reason her results are dismissed as technically faulty by Orstrom and Lindberg (1940). Perlzweig and Barron (1928) found that lactic acid accumulates in eggs in the presence of cyanide. It now seems clear that there is no significant rise of lactic acid on fertilization. Orstrom and Lindberg (1940) state that no accumulation of lactic acid occures in egg brei. Their experiments however do not seem to have been especially designed to promote such accumulation.

Runnstrom (1933,1949) believes that the dehydrogenase system of the egg is that described originally by Warburg and Christian (1932). This system has been described in more detail by Dickens (1938). Glucose-6-phosphate is oxidized to 6-phosphogluconic acid, oxidized again to 6-phospho-2-ketogluconic acid and then decarboxylated to a pentose. Dickens supposed that this process could continue in the same manner to yield an erythrose and even further, but as yet there is no evidence that such is indeed the case. The belief that the respiratory system of the egg is based on this series of reactions rests on several grounds. Egg homogenates readily oxidize phosphogluconic acid (Lindberg, 1946). Iodoacetate does not influence the oxygen uptake of homogenates (Lindberg and Ernster, 1948). No triose-phosphate can be detected, as stated above and triosephosphate dehydrogenase is absent. Formation of lactic acid is negligible. Lindberg (1943) has further shown that the propanediolphosphate isolated by him when added to tissues

produces an increase in pentoses and phosphorylated hexoses produce the same effect in egg brei. The increases however are scarely significant.

Henry (1943) claims that there are two distinct respiratory systems in the eggs of Arbacia. A basal respiration which maintains the egg's basal metabolism is not sensitive to sulfonamides. On fertilization another respiration functions, which is sensitive to sulfonamides and is concerned with providing energy for developmental processes. Perhaps differences in permeability could explain at least part of the phenomenon.

The metabolism of the acids of the tricarboxylic acid cycle has been little studied until recently. According to Barron and Goldinger (1941) and Krahl, Jandorf and Clowes (1941) fertilized eggs metabolize pyruvate at a greater rate than the unfertilized. The claim is made that the eggs are not able to oxidize alpha ketoglutaric and succinic acid. Runnstrom (1933) found that pyruvic and lactic acids were oxidized much more slowly by egg brei than hexose phosphates and that succinic and maleic acids were scarcely oxidized at all. Lindberg (1946) found that fumaric and malic acid rapidly reduce methylene blue in homogenates of Echinocardium eggs. Ball and Meyerhof (1940) during studies of the cytochrome system of eggs of various species found that succinic dehydrogenase was absent in sea urchin eggs. Recently Crane and Keltch (1949) reported that succinate,

alphaketoglutarate, glutamate and citrate are oxidized.

The metabolism of phosphate has been studied in some detail. Brooks and Chambers (1948) found that the uptake of labeled phosphate is very slow in the unfertilized eggs but rapid in the fertilized, and this has been confirmed by Lindberg (1948). What is probably more significant was the finding that only part of the phosphate in the unfertilized egg showed a turnover. The fertilized eggs, on the other hand, showed a continuously increasing activity throughout the period of the experiment.

Studies of the terminal oxidase of sea urchin eggs have continued since work on their respiration was initiated by Warburg. As mentioned previously, Warburg discovered that cyanide inhibited the respiration. The work of Runnstrom referred to above made it probably that the terminal oxidase is cytochrome oxidase as in so many other tissues. That this is indeed the case was established by Krahl, Keltch, Neubeck and Clowes (1941) who showed that the sea urchin egg contains an enzyme able to oxidize reduced cytochrome c. Borei believes this enzyme to be somewhat different from a typical cytochrome oxidase.

For a long time the function of cytochrome oxidase in the sea urchin egg was rather obscure since the presence of the other cytochromes could not be demonstrated. Brachet (1934), Lindahl (1936), Ball and Meyerhof (1940) and Krahl, Keltch and Clowes (1941) reported that no cytochromes were present in the egg. This was considered to be of some

importance in view of the hypothesis of Korr that the respiratory rise was due to the activation of cytochrome c. Rothschild (1949) has now reported that cytochromes a and b are indeed present in the sea urchin egg.

Many other aspects of the metabolism of sea urchin eggs have been studied. Since, however, they do not bear directly on the work reported in this thesis they will not be summarized here. All pertinent work except the most recent has been compiled in the two books of Needham (1931, 1942) and by Brachet (1947). A more recent review discussing metabolism to some extent is given by Runnstrom (1949).

Statement of the Problem

From the above review of the literature it is clear that the respiratory metabolism of the sea urchin egg has not been satisfactorily analyzed from the standpoint of present day knowledge of respiration in other animal or plant tissues. This was even more true at the time the thesis work was started; during the progress of this work the paper of Rothschild (1949) identifying the cytochromes and that of Crane and Keltch (1949) reporting the oxidation of the acids of the tricarboxylic acid cycle appeared.

There are several reasons for desiring more accurate information on the respiratory metabolism of the sea urchin egg. From the embryological point of view it would be of interest to know whether or not the egg has a different metabolism from that known in the adult organism. If such were the case then the usual respiratory system would be arising anew each time in ontogeny as, for example, the actomyosin system. It would, then, be a part of the processes of differentiation and being relatively well understood would be interesting to study from this point of view. Secondly, it is clear (Tyler, 1942) that the processes of growth and differentiation require some energy which is provided by the respiratory system. Observing changes that occur in respiration we can get a clue to events of perhaps deeper significance. At any rate we can suspect other changes if the respiration has changed. For example in the frog's egg on fertilization, Brachet (1934) observed that

on fertilization of the frog's egg the oxygen uptake remains the same, but that the respiratory quotient changes greatly. Thirdly, such studies are of biochemical interest. It is desirable to know how uniform is the respiratory mechanism of various organisms. If differences are established, then the detailed study of such systems is in order.

Investigations were therefore made of the presence or absence in the sea urchin egg of various respiratory enzymes whose properties have been reasonably well established in other animal tissues.

Materials and methods.

The studies were made using the eggs of Strongylocentrotus purpuratus and Lythechinus pictus. Since these two species are ripe at different times a supply of eggs is available throughout the year. The animals were collected in the vicinity of the William G.Kerckhoff Marine Laboratory of the California Institute of Technology at Corona del Mar. Animals were kept in aquaria with running sea water until needed. From time to time the required number of animals were brought to Pasadena. These were kept in jars of sea water in a refrigerator and air was bubbled through. Under these conditions the animals survived several days.

Eggs were removed from the animals in one of two ways. Either the animals were opened with scissors and the ovaries placed in sea water were they shed, or the animals were injected with isotonic (0.55 M) KCl (Tyler, 1949) which caused them to shed at once. The eggs were then allowed to settle in a beaker; the supernatant was removed, and fresh filtered sea water added. The washing process was always repeated three or four times.

It is clear that the study of intact eggs was not likely to give the information desired. The unfertilized sea urchin's egg is extremely impermeable to many substances that would be used in studies of respiratory metabolism. The fertilized is more permeable, but not to a degree sufficient to permit the introduction of cofactors and substrates into the intact eggs. Therefore homogenates of eggs

were used.

Homogenates were prepared in the following manner: the washed egg suspension was poured into a graduated centrifuge tube and then centrifuged for about ten minutes at 3000 rpm. The supernatant sea water was removed and the volume of the packed eggs noted. Buffer was added to the amount desired. The suspension of eggs was then homogenized in a test tube with a lucite pestle attached to an electric motor. After this treatment there were no intact eggs in the homogenate. The concentration of the homogenate was expressed as the per cent of packed eggs in the final volume of homogenate.

Some of the experiments were done on eggs that had been lyophilized and then stored in the deep freeze.

The findings of Lindberg andErnster (1948) indicated that the respiration of homogenates, unless kept very cold would soon cease. It was found, however, with the species of sea urchins used that the endogenousrespiration, that is respiration of a homogenate without added substrate was considerable. Using the Warburg technique it was found that the respiration at 22°C showed some decrease (Fig. 1) after two hours. Storage of eggs in the deep freeze for two days still yields preparations with a considerable endogenous respiration. Thus no special precautions are needed to study the respiration. The Warburg manometer bath temperature was set at 22°C which is a convenient temperature and within the range in which the eggs will develop. On warmer days a

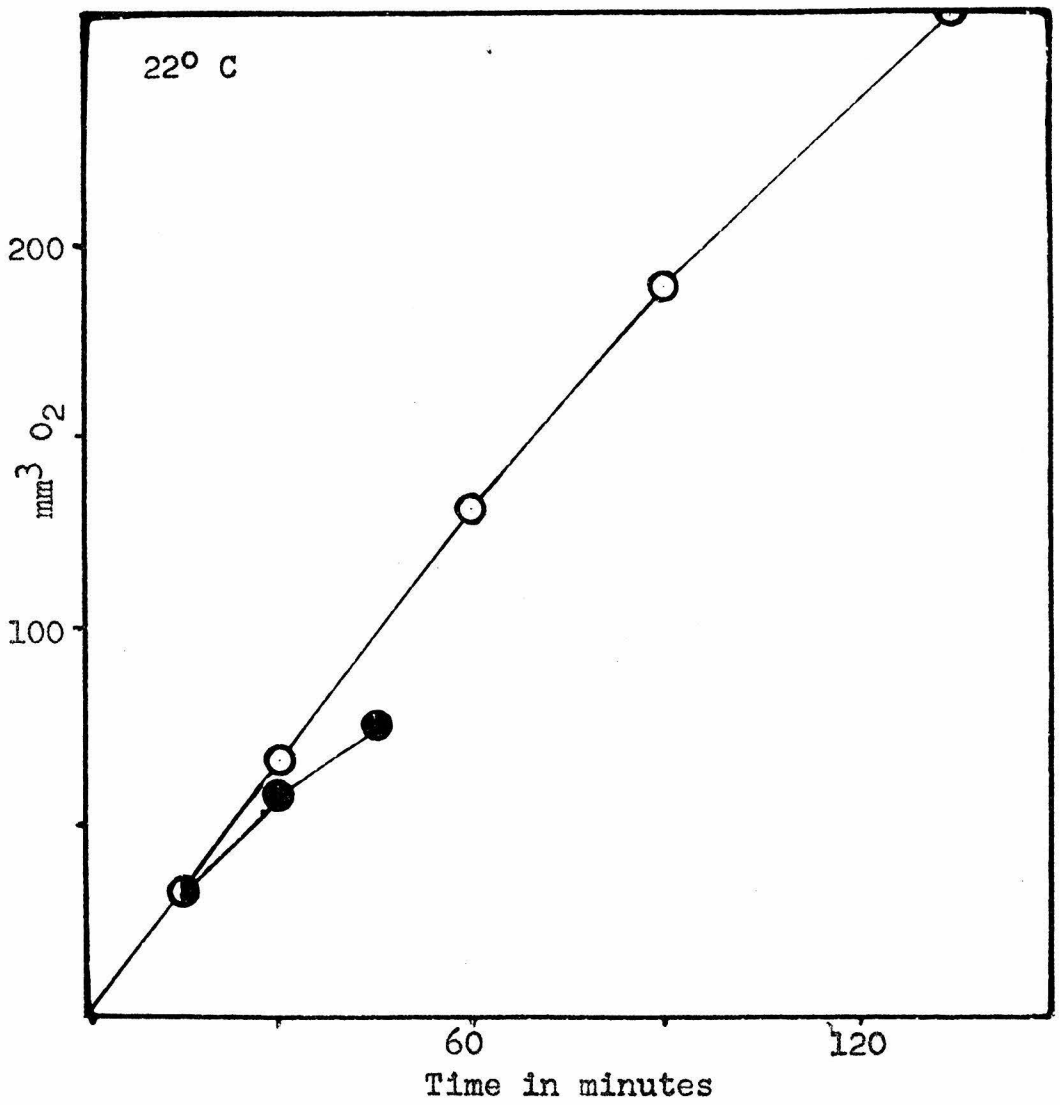

Fig. 1

Endogenous respiration of homogenates of eggs of S. purpuratus in phosphate buffer, 1/15 M, pH 7.1.

Dark circles - stored frozen two days.
Light circles - fresh.
Concentration of homogenates - 25%.

cooling system was needed for the bath.

The best approach seemed to be to take yeast and muscle as a model and attempt to demonstrate a similiar respiratory system in the eggs. The question of substrate has to some extent been clarified as can be seen from the literature survey and was therefore not studied.

Phosphoglucomutase

As shown by Cori and Cori (1936), the first product of the breakdown of glycogen is glucose-1-phosphate. Glucose-1-phosphate is then transformed into glucose-6-phosphate through the action of phosphoglucomutase. The mechanism of this transformation has recently been clarified by the work of Leloir et. al. (1948). A coenzyme is involved and has been identified as 1,6,glucosediphosphate. The reaction proceeds in such a manner that free phosphate is not formed.

The property that most easily distinguishes glucose-1-phosphate from glucose-6-phosphate is the lability of the phosphate of glucose-1-phosphate to acid hydrolysis. This ester is completely hydrolized in 1 N HCl at 100° C in fifteen minutes, while under the same conditions glucose-6-phosphate is scarcely affected. This property was used to test for the presence of phosphoglucomutase in sea urchin eggs.

Unfertilized eggs of L. pictus were lyophilized. 0.4 g of the powder was added to 24 cc of distilled water and the suspension homogenized in a glass homogenizer. The homogenate was kept ice cold through the time of preparation. It was then dialyzed in the cold for five hours with three changes of dialyzing solution against a solution of 0.5% KCl and 0.26% Na_2CO_3, pH 8.0. This was used as the enzyme preparation.

Like many other enzymes concerned with phosphate metabolism phosphoglucomutase is rapidly inactivated by heavy metals (Najjar, 1948). It is also known to require Mg ions. Cysteine and Mg ions were therefore added to the incubation mixture. As substrate the potassium salt of glucose-1-phosphate was used. Incubation was at room temperature 23° C for 90 minutes and aliquots were pipetted into 4% trichloracetic acid at the beginning and end of the experiment. This was then filtered and the inorganic phosphate determined on aliquots after 15 minutes hydrolysis in a boiling water bath by the method of Fiske and Subbarow (1929). In addition, fructose was determined by the method of Roe (1943). Two separate experiments were done, both of which indicated activity corresponding to phosphoglucomutase. The results of one are presented in Table I with details of the composition of the incubation mixtures.

The amount of easily hydrolyzable phosphate decreases considerably in 90 minutes, a result to be expected if phosphoglucomutase is present. That the decrease is not greater is probably due to phosphatase activity.

There is a significant increase in the fructose value. This is to be expected if the next enzyme of glycolysis, oxoisomerase is present. A direct test was therefore made for this enzyme.

Table I

Time	Inorganic phosphate in mg P after 15 minutes hydrolysis in 1 N HCl at 100° C	
	#1	#2
0 min.	0.64	0.75
90 min.	0.38	0.82
	Fructose, as mg fructose-6-phosphate	
0 min.	0.56	0.50
90 min.	2.00	0.56

#1. 6 cc enzyme preparation, 1 cc 0.1 M cysteine hydrochloride, 1 cc 0.1 M $MgCl_2$, 1 cc glucose-1-phosphate, 8 mg of the potassium salt and 1 cc water

#2. Control. As above, substrate and water replaced by 1 cc of glucose, 4.4 mg and 1 cc of KH_2PO_4, containing 0.78 mg P.

Oxoisomerase

Lohman (1933) found that the equilibrium between glucose-6-phosphate and fructose-6-phosphate is established in a matter of seconds. A short incubation period was therefore used. The homogenate consisted of 0.5 g of lyophilized eggs of L. pictus, ground and suspended in 30 cc of distilled water. The suspension was then dialyzed against a solution of 0.5% KCl and 0.26% Na_2CO_3, pH 8.6 for six hours and stored in the deep freeze until used. Four cc of this suspension was taken. One cc of fructose-6-phosphate solution was added. This contained approximately 5 mg of the substrate. At 0 minutes 5 cc of 10% trichloracetic acid was pipetted into one reaction mixture and substrate added. At the same time substrate was added to the second reaction mixture and the reaction stopped seven minutes later with the same volume of trichloracetic acid. Activity was measured by determination of fructose by the method of Roe (1943) and by phosphate determination without hydrolysis and after hydrolysis in 1 N HCl for 180 minutes. Under these conditions fructose-6-phosphate is hydrolyzed completely while glucose-6-phosphate is little affected. Attempts to measure the glucose formed using the iodine titration method of Macleod and Robison (1929) gave excellent results using pure solutions of glucose, but unsatisfactory ones with trichloracetic acid extracts. The results of an experiment are presented below.

Time	Fructose-6-phosphate Found
0 min.	5.35 mg
7 min.	2.39 mg
Difference	2.96

Phosphate as mg KH_2PO_4

	0 min. Incubation	7 min. Incubation
No hydrolysis	0.24	0.72
180 min. hydrolysis	2.17	1.56
Difference	1.93	0.84

In seven minutes 1.1 mg of phosphate hydrolyzable in 180 minutes disappeared, which corresponds to 2.85 mg of fructose-6-phosphate as against 2.96 mg of fructose-6-phosphate as determined by the fructose method. The agreement is satisfactory. The presence of oxoisomerase is therefore indicated.

Attempts were made to demonstrate the presence of hexokinase and phosphohexokinase, ATP was added to dialyzed homogenates with and without glucose and easily hydrolyzable phosphate determined after varying periods of time. In some cases a small decrease of easily hydrolizable phosphate was noted, but it was small and not consistent. It has been pointed out by Broh-Kahn and Mirsky (1948) that the demonstration of hexokinase in homogenates, due to the complex nature of the phosphate metabolism, is difficult and purification of the enzyme is often neccessary. This was not

done and therefore the presence of hexokinase is indicated by only experiments showing that glucose is metabolized by sea urchin egg homogenates.

The attempts to demonstrate activity of phosphohexokinase were likewise not successful. No increases of 1,6,fructosediphosphate could be demonstrated after fractionation of trichloracetic acid extracts of homogenates incubated with fructose-6-phosphate, glucose and ATP by the barium method (LePage and Umbreit, 1945). This enzyme is reported to be very labile and so may be unstable during the dialysis periods that are needed to remove the coenzyme of triosephosphate dehydrogenase.

Aldolase

The next enzyme in the glycolytic scheme is aldolase which converts 1,6,fructosediphosphate into phosphoglyceraldehyde and phosphodihydroxyacetone. As mentioned in the literature survey, previous attempts to demonstrate the formation of triosephosphate in egg homogenates on addition of hexosediphosphate have not been successful. The method used was the determination of alkali labile phosphate. It would appear, however, that a method that would trap the products would be desirable for the demonstration of aldolase activity, Sibley and Lehninger (1949) have described a convenient and specific colorimetric method for aldolase. The principle is to trap the triose phosphates formed with hydrazine, add 2,4,dinitrophenylhydrazine and develop color with alkali. This method was used to detect aldolase activity in sea urchin eggs.

100 mg of lyophilized eggs of Lythechinus pictus were suspended in 6 cc glycylglycine buffer, 0.1 M, pH 8.6 and homogenized. This was used as the enzyme preparation. Substrate - fructose - 1,6,phosphate 20 mg/cc. Hydrazine 0.56 M. The reaction mixtures were incubated in test tubes for 30 minutes at room temperature of 26° C. The reaction was then stopped by adding 2 cc of 10% trichloracetic acid. The contents were filtered and 1 cc aliquots taken for analysis. 1 cc of 0.75 NaOH was added to the aliquots in colorimeter tubes and kept at room temperature for 10 minutes. 1 cc of 2,4,dinitrophenylhydrazine, concentration 1 g in

1 liter of 2N HCl, was added and tubes incubated in a water bath at 38° C for ten minutes. The contents were then made up to a volume of 10 cc with 0.75 N NaOH, kept at room temperature for 10 minutes and the color read with a green filter. The contents of the reaction mixtures and colorimeter readings are given in Table II. As can be seen, color development is initially proportional to enzyme concentration. Several repetitions of the experiment gave similar results. Since this method is reported to be specific, it can be concluded that aldolase is present in the sea urchin egg.

Sibley and Lehninger report that if the reaction of fructose-di-phosphate with aldolase is allowed to proceed in the absence of hydrazine and if phosphotriose isomerase is present a much stronger color development results, as the triose phosphate is then predominantly phosphodihydroyacetone which gives a stronger color than phosphoglyceraldehyde. Using undialyzed egg suspension, in one experiment enzyme incubated with hydrazine gave colorimeter reading of 72, with hydrazine added after incubation 40. Instead of an increase a decrease was observed. This is presumably due to further reactions of the triose phosphates formed.

Table II

Formation of Triosephosphate

Tube #	Buffer cc	HDP cc	Hydrazine cc	Enzyme cc	Water cc	Optical Density
1	1.0	0.3	0.2	0.1	0.9	25
2	1.0	0.3	0.2	0.1	0.9	27
3	1.0	0.3	0.2	0.2	0.8	50
4	1.0	0.3	0.2	0.2	0.8	52
5	1.0	0.3	0.2	0.4	0.6	96
6	1.0	0.3	0.2	0.4	0.6	91
7	1.0	0.3	0.2	0.8	0.2	145
8	1.0	0.3	0.2	0.8	0.2	145
9	1.0	(0.3)	0.2	0.8	0.2	standard

Concentration of reagents in text.

#9. HDP added after addition of trichloracetic acid.

Triosephosphate dehydrogenase

Since aldolase is present in the eggs of sea urchins, it is to be expected, in spite of the work mentioned in the introduction that triosephosphate dehydrogenase should be present. No triosephosphate as such was available, but addition of fructose-1,6-diphosphate should be equivalent to addition of triosephosphate to homogenates. Triosephosphate dehydrogenase in all cases known (Meyerhof and Kiessling, 1933) is strongly inhibited by iodoacetate and failure to detect such inhibition has been one of the main arguments against the existence of the usual glycolytic system in sea urchin eggs. However it is well known that the effect of iodoacetate is not instantaneous. Experiments were therefore done to see if firstly the endogenous respiration of egg homogenates is inhibited and also if such an inhibition takes time to develop. The methylene blue technique was used. Thunberg tubes were prepared, all components including homogenate were added and iodoacetate was added to the tubes 60, 30, 15, and 0 minutes before evacuation and tipping in of methylene blue. Fig. 2 shows clearly that the endogeneous respiration is inhibited by iodoacetate and that the effect is progressively stronger the longer the homogenate has been in contact with the inhibitor. This is some evidence that at least part of the respiration passes through the triosephosphate stage. Of course other enzymes are also inhibited by iodoacetate,

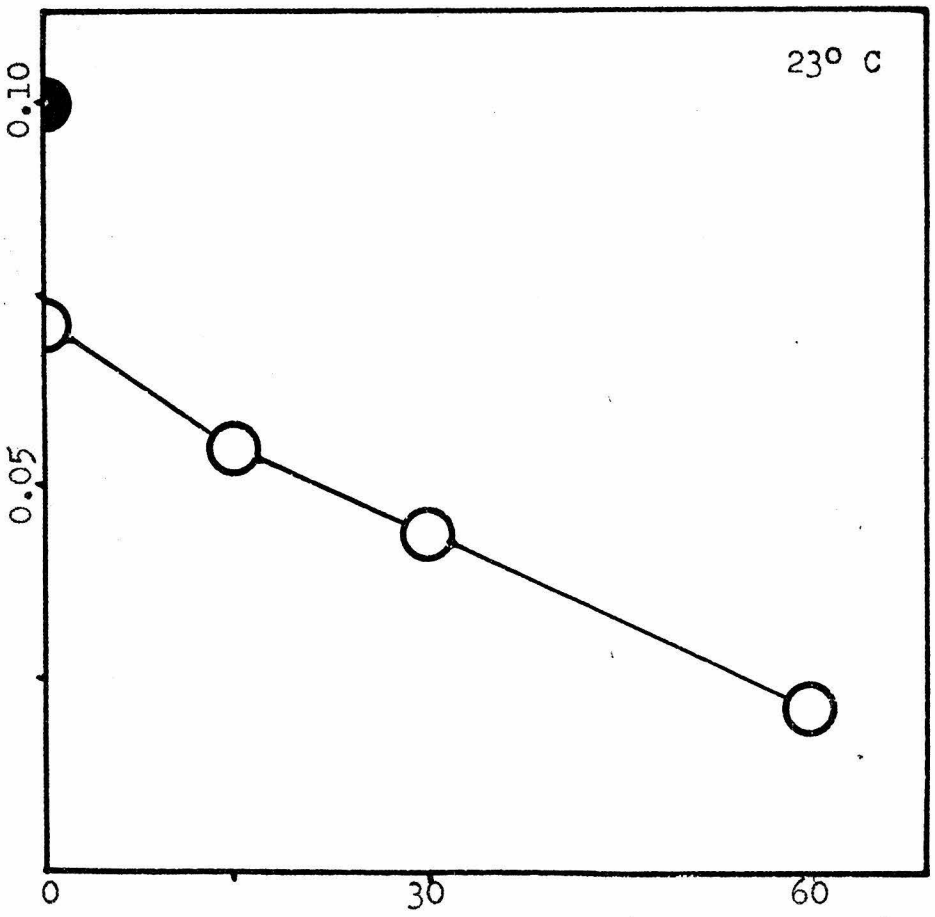

Fig. 2

Effect of iodoacetate on reduction of methylene blue by 20% homogenate of L. pictus eggs in 1/15 M phosphate buffer, pH 7.1 iodoacetate 0.03 M.

Ordinate - reciprocal of time in minutes till reduction.
Abscissa - length of time in minutes homogenate exposed to iodoacetate.
Black circle - control, no iodoacetate.

but not so strikingly.

It can be shown quite easily by the same technique that the dehydrogenation of hexose diphosphate is likewise inhibited by iodoacetate. It is however necessary to use a more dilute homogenate, of the order of 5%, as otherwise the reduction by the control is inconveniently rapid. A typical result is presented in Table III. Although the products of the dehydrogenation of 1,6,fructosediphosphate were not identified, it is to be expected that it would be phosphoglyceric acid. In that case it would be expected that the phosphoglyceric acid would lose water under the action of enolase and be transformed to phosphoenolpyruvic acid.

Table III

Reduction of Methylene Blue
by 1,6Fructosediphosphate and
Effect of Iodoacetate

Tube #	Tube contents	Side arm	Time decolor. min.
1.	2 cc homogenate 1 cc water	1 cc MB 1 cc water	100
2.	2 cc homogenate 1 cc iodoacetate	1 cc MB 1 cc water	>120
3.	2 cc homogenate 1 cc water	1 cc MB 1 cc HDP	52
4.	2 cc homogenate 1 cc iodoacetate	1 cc MB 1 cc HDP	>120

1-6-fructosediphosphate 0.05 M. The calcium salt (Schwartz) was used, calcium being removed by addition of sodium oxalate.

Iodoacetate 0.1 M. Iodoacetate was washed with petroleum ether, dried and the solution neutralized to pH 7 with NaOH.

Methylene Blue, a solution of 12 mg. in 100 cc water.

Homogenate. 5% S. purpuratus eggs in 1/15 M phosphate buffer, pH 7.1.

Iodoacetate was added and the tubes evacuated and allowed to stand one hour before tipping in the contents of the side arms.

Temperature 23° C.

Enolase

The presence of enolase can be suspected if respiration or glycolysis is inhibited by fluoride. Fig. 3 shows the course of respiration of a homogenate of Strongylocentrotus purpuratus eggs with no substrate added. As can be seen, the respiration is quite strongly inhibited by NaF at concentrations of 0.05 and 0.01 M in a phosphate buffer.

The inhibition of respiration by fluoride is not completely specific, as a variety of other enzymes, especially phosphatases are also inhibited, although not at as low a concentration. The presence of enolase was checked directly by the method of Lohman and Meyerhof (1934). If tissue is dialyzed ATP is removed. If phosphoglyceric acid is added and enolase is present phosphoenolpyruvic acid accumulates, since in the absence of phosphate acceptors it does not go to pyruvic acid. Iodine can be added to form iodoform and the excess iodine titrated with thiosulfate.

Lyophilized L. pictus eggs, weighing 0.5 g were suspended in 30 cc of distilled water and homogenized. The suspension was then dialyzed in the cold against 0.5% KCl and 0.26% Na_2CO_3. The barium salt of phosphoglyceric acid (Schwartz) was treated with excess of sodium sulfate to precipitate the barium and made up to a concentration of 0.02 M computed on the basis of the weight of the salt taken. Due to absorption of the barium sulfate the actual concentration was of course somewhat less.

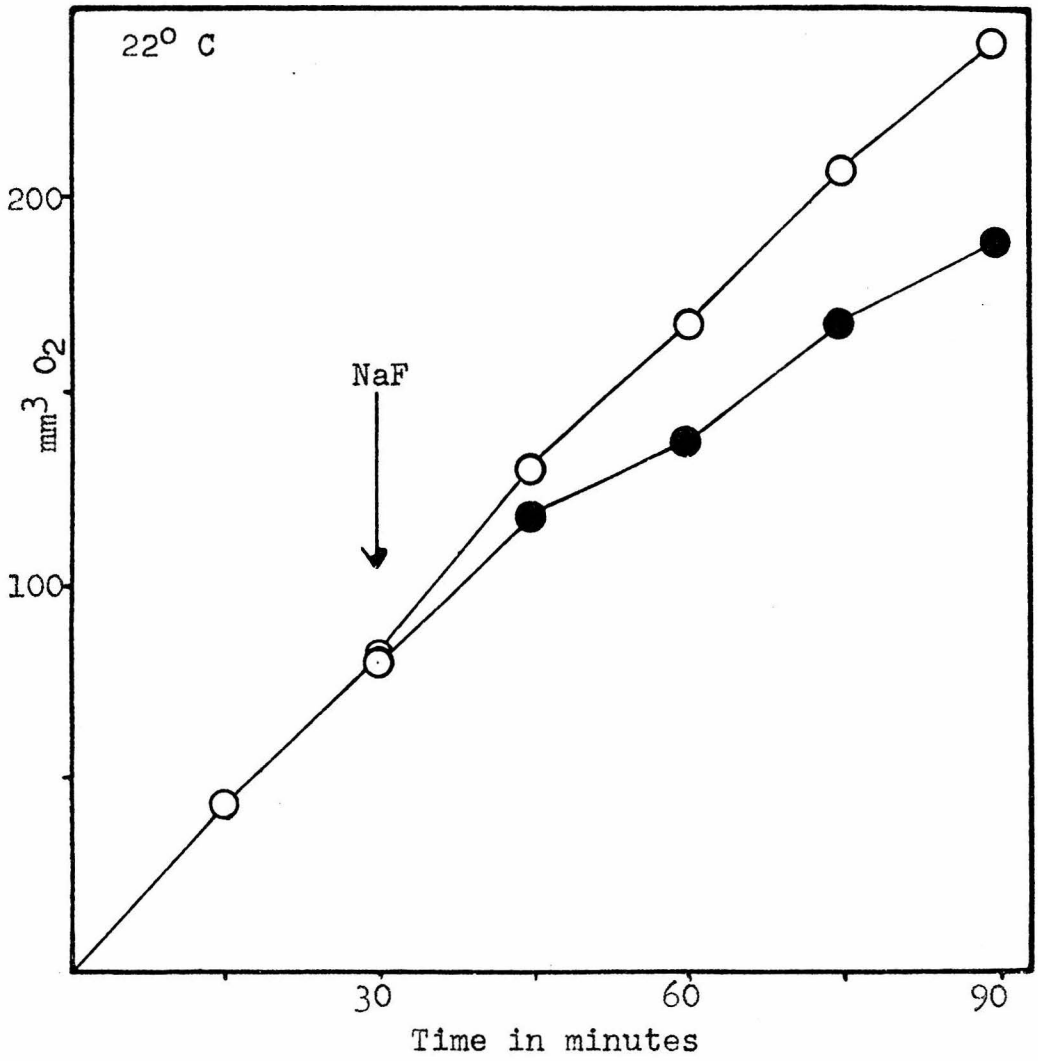

Fig. 3

Effect of NaF on the endogenous respiration of a 25% homogenate of S. purpuratus eggs in 1/15 M phosphate buffer, pH 7.1 volume of homogenate 2.0 cc.

Light circles - control.
Dark circles - with 0.05 and 0.01 M NaF.

Three series of test tubes were used. The reaction was run at room temperature 23° for 10 minutes, and stopped by addition of 8 cc of 4% trichloracetic acid, filtered and a 2 cc aliquot treated with iodine in the usual manner and titrated with 0.01 N thiosulfate. Enzyme was added to the blank after addition of trichloracetic acid. About fifteen per cent of the theoretical amount of phosphoglyceric acid added was converted to phosphoenolpyruvic. The equilibrium amount reported for this temperature is about 30%. Activity is completely abolished by fluoride. The above results together with the inhibition of the endogenous respirations of homogenates indicates that enolase is present and functions in the respiration.

It is to be expected that if the normal glycolytic mechanism is operative in the intact egg then under the proper conditions certain intermediates could be shown to accumulate. As discussed in the literature survey the accumulation of lactic acid has been questioned. An attempt was therefore made to see if under anaerobic conditions lactic acid would accumulate in intact eggs. 3 cc suspensions of unfertilized eggs of S. purpuratus, containing 0.1 cc of packed eggs were pipetted into Thunberg tubes to one series 3 cc of 10% trichloracetic acid was added at once, another series was evacuated and allowed to stand at room temperature for three hours, then opened and trichloracetic acid added. Lactic acid was determined on aliquots by the method of Barker and Summerson (1941).

Table IV

Formation of Phosphoenol Pyruvic
from Phosphoglyceric Acid

Vessel #	Substrate	H_2O	NaF	Enzyme	Thiosulphate	Pyruvate Formed
Blank 1	1 cc	1cc	---	1cc	8.07	---
Blank 2	1 cc	1cc	---	1cc	7.93	---
				average	8.00	
3	1 cc	---	1cc	1cc	7.97	
4	1 cc	---	1cc	1cc	7.94	
				average	7.95	0.03 mg
5	1cc	1cc	---	1cc	7.60	
6	1cc	1cc	---	1cc	7.61	
7	1cc	1cc	---	1cc	7.68	
				average	7.63	0.30 mg

The tubes in which the reaction was stopped at the start showed only a trace of lactic acid while those incubated anaerobically showed a total accumulation of 0.086 mg of lactic acid, or 0.86 mg per cc of eggs. This can be compared with the value given by Perlzweig and Barron (1928) who found about 0.1 mg of lactic acid per cc of unfertilized eggs of Arbacia incubated with KCN for varying periods of time. The other values in the literature vary a great deal, Zielinski reporting about 0.5 and Runnstrom 0.3 mg per cc of eggs. The point made here is that there is an accumulation of lactic acid in anaerobiosis. This would indicate that some anaerobic glycolysis occurs with lactic acid as the end product. This conforms with the early findings of Ashbel (1929) that acid is produced by sea urchin eggs under anaerobic conditions.

Runnstrom (1949) reports that lactic acid is metabolized to a small extent by egg homogenates. This was confirmed by Thunberg tube experiments, but as in the experiments of Runnstrom the increases of rate are not large. One experiment, for example, showed time of methylene blue decolorization of control 19 minutes, with 0.03 M lactate 14 minutes. Lactic acid can thus be metabolized by egg homogenates.

Runnstrom (1933) was the first to show that on fertilization of sea urchin eggs there is a production of acid which can be detected by evolution of carbon dioxide from

the bicarbonate of the solution. The amount of acid produced is rather small, about 20 microequivalents per 100 mg of egg nitrogen. The production of acid was also observed by Rothschild (1939) if eggs were injured or cytolyzed by various means. Runnstrom (reported in Orstrom and Lindberg, 1940) attempted to analyze eggs before and after fertilization for lactic acid and found no change. The eggs however were washed exhaustively before analysis and the lactic acid, if given off into the medium would not be found. That no great change occurs in the lactic acid content on fertilization was shown by Perlzweig and Barron (1928). By direct attempts to obtain a precipitate with ketonic reagents Orstrom concluded that the acid was not pyruvic. It is however doubtful if pyruvic could have been detected at such concentrations.

An attempt was made to study the nature of this acid. Eggs were cytolyzed by distilled water and after periods of five minutes trichloracetic acid was added and the filtrate tested for lactic acid by the method of Barker and Summerson (1941). The difference in lactic acid content between eggs cytolyzed and the controls was found to be negligible.

The amount of acid produced is so small that the problem of its identification is a difficult one. The most obvious approach is to attempt extraction with ether. This method was tried.

A continuous ether extractor was used. When isolating such minute quantities of substance the danger of artifacts

from the ether or from impurities is considerable. The entire apparatus was carefully cleaned with dichromate and rinsed with glass distilled water. The ether was CP grade, redistilled from ferous sulfate and kept over anhydrous calcium chloride for twelve hours. A large quantity, about fifty cc of the eggs of S. purpuratus was used. The eggs were washed four times and suspended in filtered sea water. They were then fertilized, allowed to stand for ten minutes and then centrifuged. The supernatant was removed, filtered through filter paper and then through a Seitz filter. A similar batch of eggs was used as a control. These eggs were not fertilized, and an equivalent quantity of sperm was added to the supernatant just before filtration through the Seitz filter. A blank of filtered sea water served as a second control.

The filtered solutions were acidified with sulfuric acid and extracted with ether for three hours.

The ether extracts were then evaporated in a stream of air. It was found that the residue of all three extracts was acidic to indicators. An attempt was made to see if the acidic substance would move on paper chromatography. Using the method of Lugg and Overell (1947), one dimensional chromatograms were run with a solvent of water saturated butanol in an atmosphere saturated with formic acid. After chromatography overnight, the paper was dried and developed by spraying with pH indicator. One spot was found common to all three extracts which had moved slightly. In

addition the extract from the fertilized eggs showed another spot that had not moved at all. A similar spot, but extremely faint was found with the unfertilized extract and was absent in the blank.

The amount of material after evaporation of the ether was so small, about 0.2 cc, that regular proceedures of fractionation were difficult. Tests with 2,4,dinitrophenylhydrazine indicated absence of active carbonyl groups. A direct test for lactic acid by the method of Barker and Summerson (1941), which is sensitive to quantities of the order of 0.5 gamma gave a negligible color. Further work on this extract has not been completed.

The above work confirms earlier reports that the acid of fertilization is neither lactic nor pyruvic. In addition it does not appear to be any of the acids of the tricarboxylic acid cycle, all of which move on paper with very appreciable R.F. Its nature is not known, but the determination of its identity would be of great interest, as it might give a clue to the events occurring immediately on fertilization.

Since enolase activity can be demonstrated in egg homogenates and the endogenous respiration, as shown in Figure 3 is inhibited by fluoride it would be expected that if intact eggs are incubated with fluoride phosphoglyceric acid should accumulate. A negative result of course would not be significant, since fluoride might not be able to penetrate into the intact egg.

Twenty cc of a suspension S. purpuratus eggs were

pipetted into small Erlenmeyer flasks. The nitrogen content of the suspension was determined by the Kjeldahl method and found to be 6.6 mg. To two flasks 2 cc of 0.55 M NaF in distilled water was added, to the controls 2 cc of sea water. The flasks were incubated in a water bath at 23° C for 4 hours. Ten cc of 10% trichloracetic acid was then added to each flask. The suspensions were filtered and the filtrate treated with barium to precipitate phosphosphoglyceric acid according to the method of LePage and Umbreit (1945). An attempt to estimate the phosphoglyceric acid by the method of Meyerhoff and Schultz (1938) based on the enhancement of optical rotation in the presence of molybdate did not prove possible due to interfering substances present. The less specific method of Rappoport (1937), based on the development of color with alpha naphthol in concentrated sulfuric acid was therefore used. The results were as follows:

mg Barium Phosphoglycerate	Control	With NaF
	0.984	1.279

A rather unexpected phenomenon was noted on adding fluoride to egg suspensions. The eggs would immediately agglutinate. Because of the possibility that this phenomenon could be related in some way to the agglutinations produced by interacting substances of eggs and sperm some tests were made to determine its nature. It was found that the sperm of sea urchins were likewise agglutinated in large sheets.

On suspending eggs in isotonic sodium chloride solutions agglutination did not occur. On addition of calcium agglutination would occur at once. The calcium and magnesium salts of fluorine are very insoluble. The addition of fluoride to sea water therefore results in the precipitation of these salts. Under the microscope the precipitate was found to be in the form of a gell. A suspension of carbon black particles was observed to agglutinate in a manner similar to that of eggs, although not in so striking a manner. It was therefore concluded that the agglutination of the eggs and sperm observed has no bearing on the specific interaction of their surfaces. The stickiness of both eggs and sperm to the calcium fluoride gell is nevertheless rather striking.

Although the increase of color in the test for phosphoglyceric acid could indicate an increase in phosphoglyceric acid this is actually doubtful. The precipitation of fluoride by magnesium and calcium in the sea water lowers the concentration of fluoride. The method of Rappoport is not very specific and is reported by LePage and Umbreit (1945) to give at times too high values due to reasons that are not understood. In the test reported above interfering materials were present that gave a brown coloration and undoubtedly interfered with the accuracy of the test. Therefore the accumulation of phosphoglyceric acid should not be regarded as demonstrated.

Metabolism of certain acids of the tricarboxylic acid cycle

If the metabolism of the sea urchin egg is similar to that known in other organisms it is to be expected that it would be able to metabolize the various intermediates of the tricarboxylic acid cycle.

The utilization of intermediates was determined by measuring the oxygen uptake of egg homogenates prepared from unfertilized eggs of S. purpuratus. As will be shown later the addition of cytochrome c was found to stimulate the respiration very considerably. Phosphate buffer, 1/15 M pH 7.1 was used in preparing the homogenates.

Malonate is known to be a specific inhibitor of succinic dehydrogenase and if respiration is inhibited by it, it is probable that the respiration is proceeding through the tricarboxylic acid cycle. It was found that the endogenous respiration of homogenates is quite strongly inhibited by malonate. The inhibition is not instantaneous but develops gradually. Fig. 4 shows the course of respiration in the presence and absence of malonate. The inhibition occurs both in the presence and absence of cytochrome c.

Fig. 5 shows the effect of varying concentrations of malonate on the respiration. The respiratory rate is calculated for a period starting about an hour after the addition of the inhibitor when the inhibition had reached an approximately constant value. As can be seen from the figure the inhibition is almost maximal at a malonate concentration of 0.002 M. This is a low figure and probably

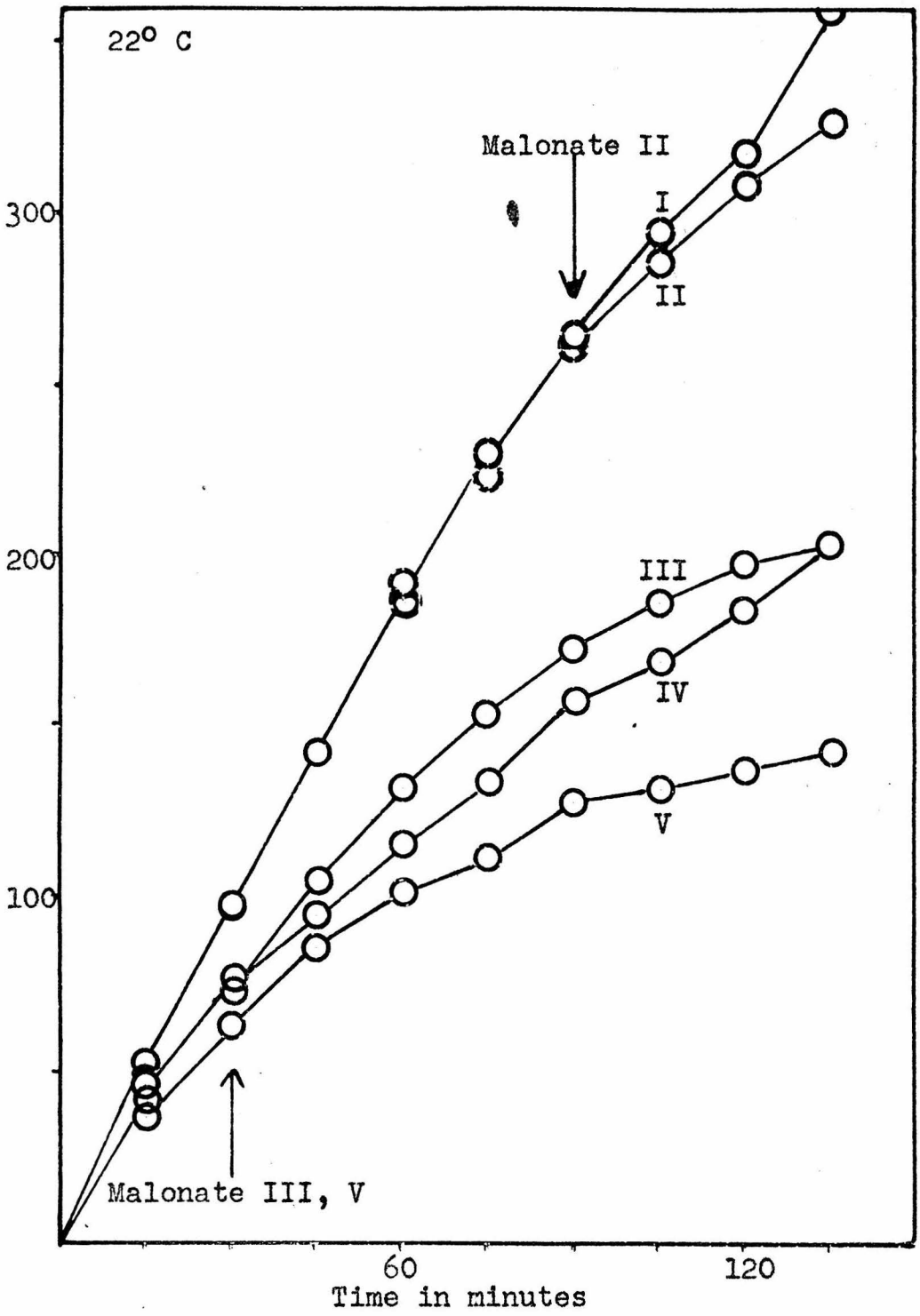

Fig. 4

Effect of malonate respiration of a 2 cc homogenate, 25%, of S. purpuratus eggs in 1/15 M phosphate buffer pH 7.1

Malonate 0.044 M.
Cytochrome c 5×10^{-5} M.
I, II, III cytochrome c added.

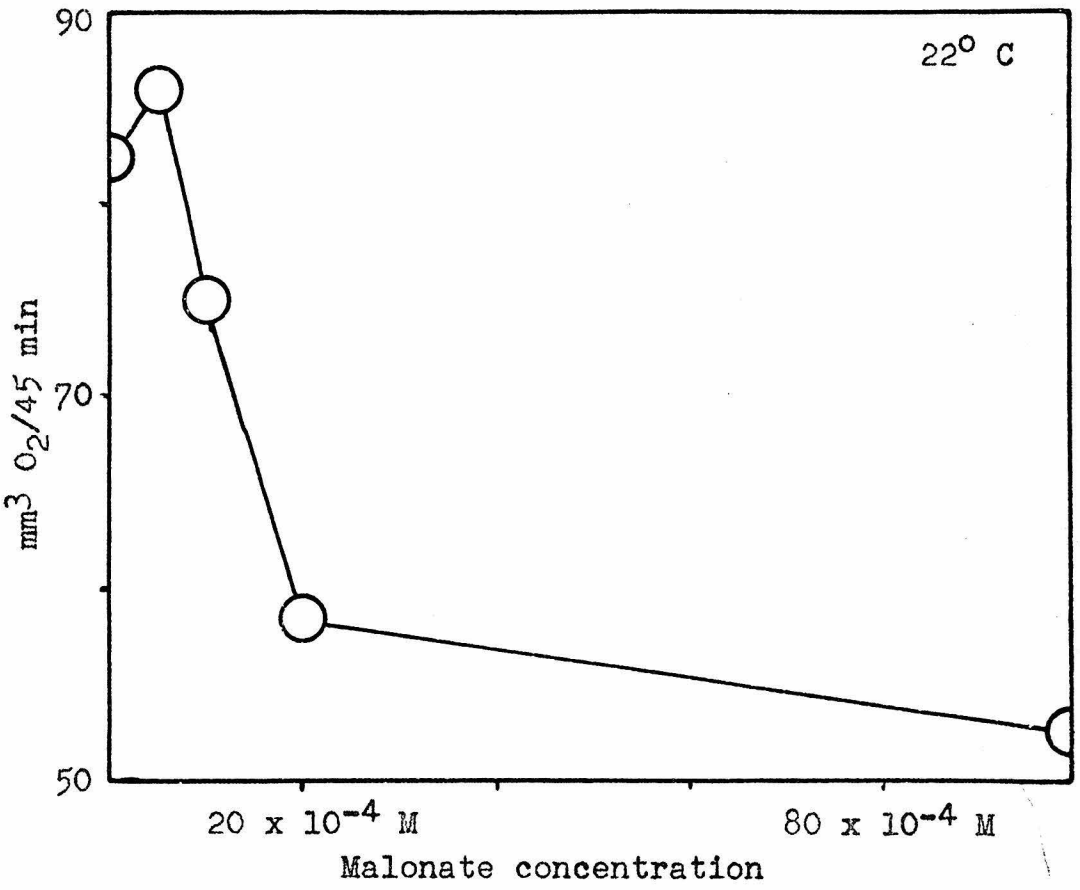

Fig. 5

Effect of malonate on respiration of 2 cc of 25% homogenate of S. purpuratus eggs in 1/15 M phosphate buffer, pH 7.1.

indicates a low concentration of succinate in the homogenate.

Since the endogenous respiration at least in homogenates is malonate sensitive it is to be expected that the respiration passes through succinic dehydrogenase. Succinate should then be metabolized by the homogenate.

Fig. 6 shows that the addition of both succinate and cytochrome singly and together results in a greatly increased respiration. The increase above that of the control on the addition of both substances together is greater than that due to each singly. This indicates that the electron transfer from succinic dehydrogenase passes through the cytochrome system, which is of course the case in most other organisms studied.

The typical succinic dehydrogenase does not require a dialyzable coenzyme. That this is the case also for the sea urchin egg is shown in Fig. 7. A short period of dialysis greatly reduces the endogenous respiration of the homogenate but the ability to oxidize succinate is unimpaired.

It was originally noticed by Keilin and Hartree (1940) that the activity of their preparations of succinic dehydrogenase made from washed heart muscle was inhibited by coenzyme I. This was shown to be due to the formation of oxaloacetic acid from succinic. Coenzyme I is required for the dehydrogenation of malic to oxaloacetic. To see if such an effect would occur in egg homogenates a homogenate

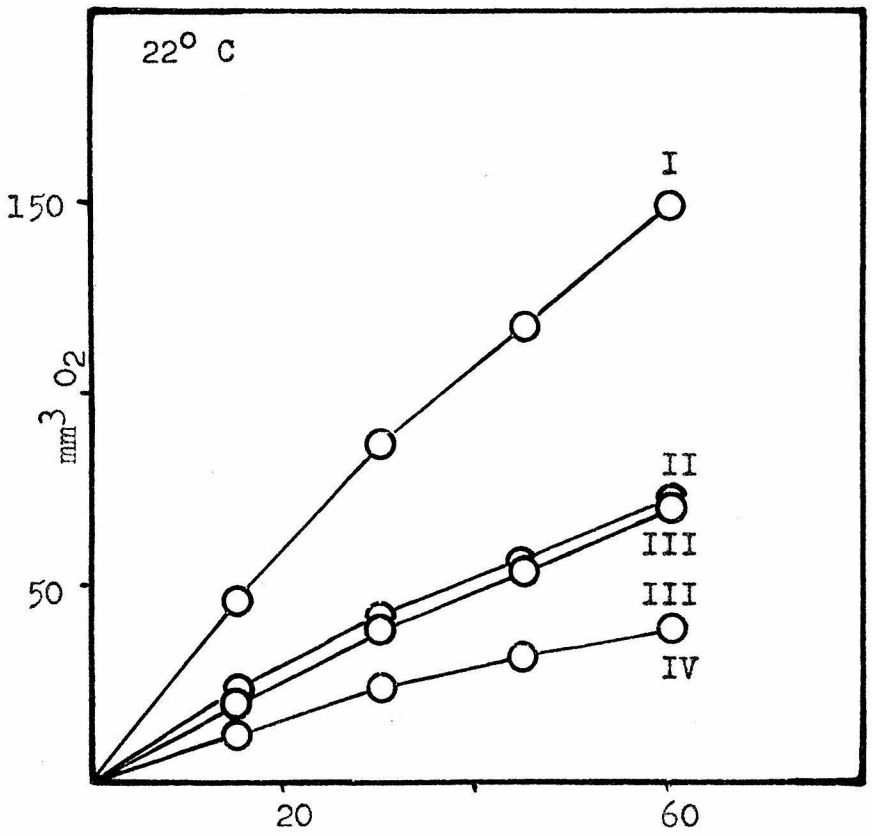

Fig. 6

Effect of cytochrome c and succinate on the respiration of a 25% homogenate of S. purpuratus eggs in 1/15 M phosphate buffer, pH 7.1.

I - succinate, 0.02 M cytochrome 5×10^{-5} M.
II - succinate 0.02 M.
III - cytochrome c 5×10^{-5} M.
IV - no additions.

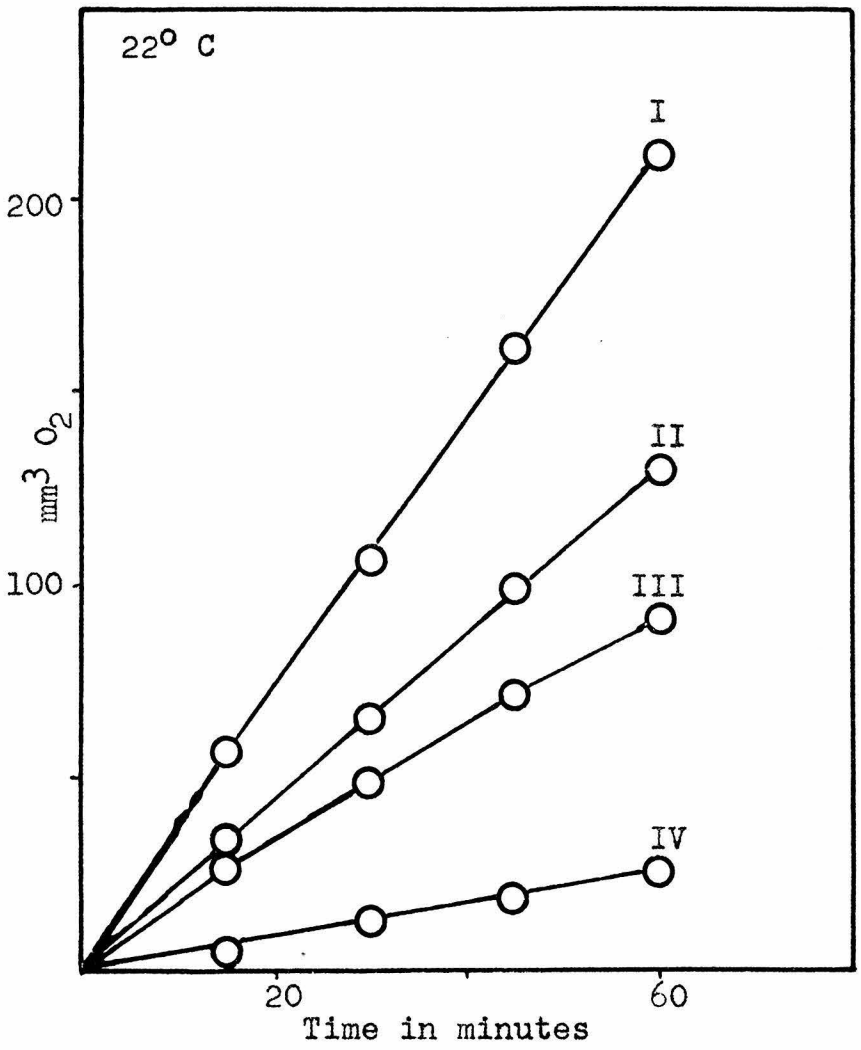

Fig. 7

Effect of dialysis on succinic dehydrogenase. Dialyzed against 1/15 M phosphate buffer, pH 7.1 2 hours in cold. Controls kept cold for same period. Homogenate of S. purpurlatus eggs, 25%. Cytochrome c added to all vessels, 5×10^{-5} M.

I, III succinate 0.005 M.
II, IV no additions.
III, IV dialyzed, I, II controls.

was dialyzed in the cold for 20 hours. Succinate and cytochrome c were then added and the effect of coenzyme I on the respiration observed. As can be seen from Fig. 8 there is no inhibition. Since, as shown below, malic dehydrogenase exists in the homogenate the lack of inhibition is presumably due to inactivation of some steps leading to oxaloacetic acid by dialysis or to a rapid destruction of any oxaloacetate formed.

The addition of citrate or of alpha ketoglutarate causes an increase in the oxygen uptake. The magnitude of the increase varies from one preparation to another. Besides increasing the oxygen uptake the respiration proceeds without much fall in the rate. In the controls there is a tendency for the respiration to fall over the period of the experiments. This fall varies with different homogenates even if they are prepared in what seems to be an identical manner. Fig. 9 shows the respiration in the presence of added citrate and alpha ketoglutarate.

Two different types of alpha ketoglutaric oxidases have been described. Ochoa (1944) prepared an enzyme from cat heart muscle that required orthophosphate, ATP and Mg ions for activity. On the other hand Stumpf, Zarudnaya and Green (1947) made a similar preparation from pigeon breast muscle and found that it did not require any of the above substances. Whether such differences are an intrinsic property of the enzymes or depend on such things as the physical state of the protein is not clear. At any rate the enzyme in sea

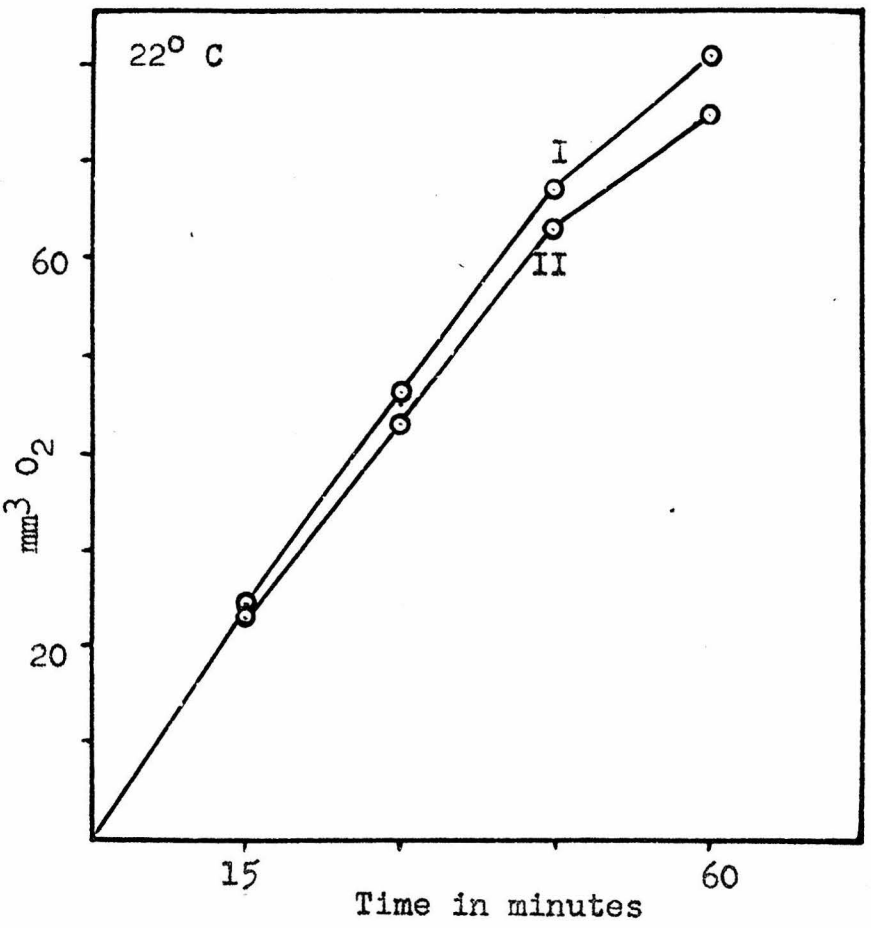

Fig. 8

Effect of Co I on succinic dehydrogenase
2.0 cc of 25% homogenate of S. purpuratus egg, dialyzed 20 hours against 1/15 M phosphate buffer, pH 7.1.

Succinate - 0.01 M
Cytochrome c - 2×10^{-5}

I 0.5 mg Co I.
II No addition.

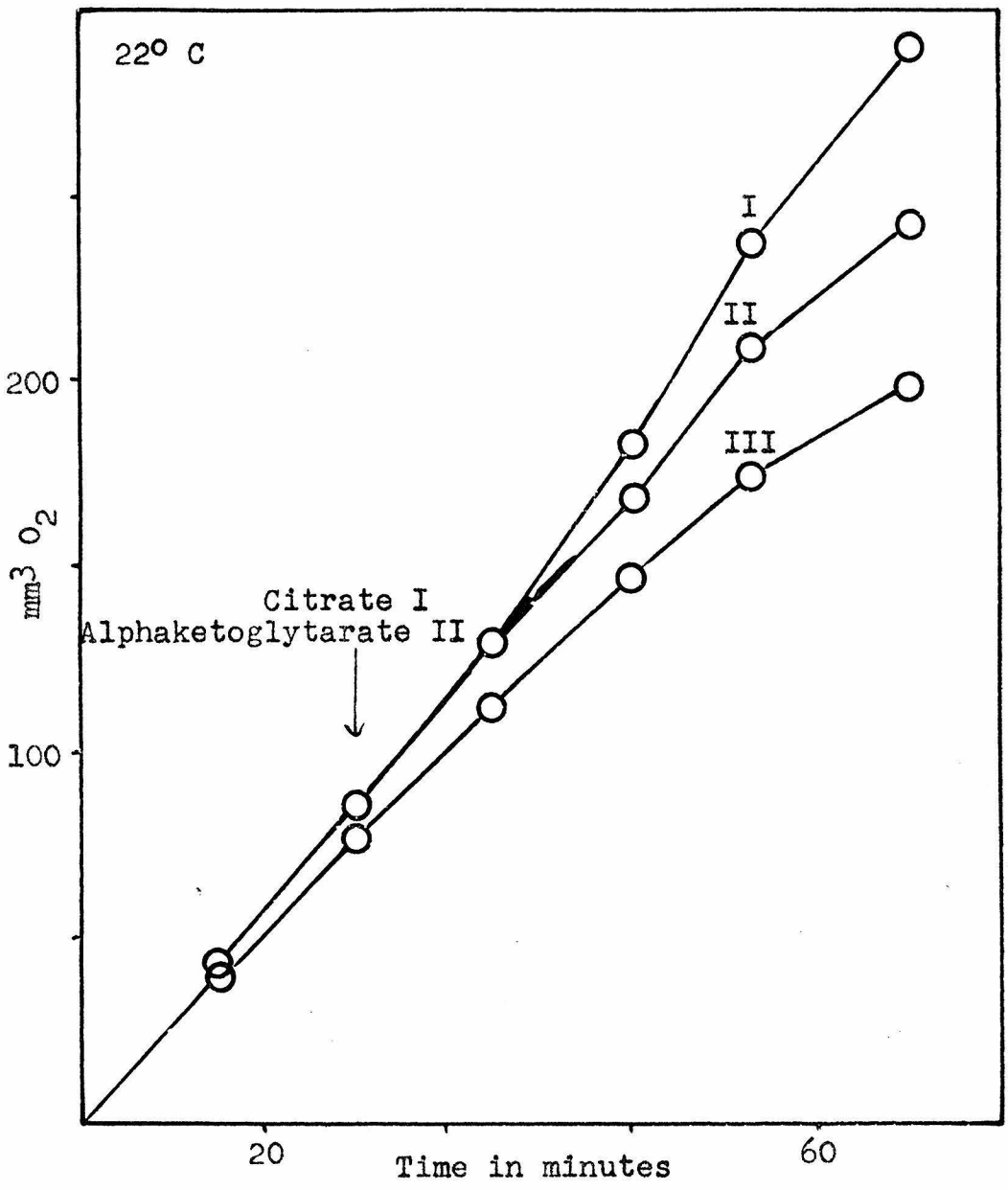

Fig. 9

Effect of citrate and alphaketoglutarate on the respiration of 2.0 cc of a 20% homogenate of S. purpuratus eggs in 1/15 M phosphate buffer, pH 7.1.

Cytochrome c 5×10^{-5} M.
Citrate - 0.015 M.
Alphaketoglutarate - 0.023 M.

urchin eggs resembles pigeon breast muscle more than cat heart. Fig. 10 shows that the addition of Mg and of a boiled muscle extract prepared from rats does not stimulate the oxidation of alphaketoglutarate but rather inhibits it. These effects have not been studied further.

Using the same homogenates and the Thunberg technique it was found that malic dehydrogenase is present. An example of such an experiment is presented below.

Cyanide was added to trap the oxaloacetate formed. The composition of the tubes was as follows: 1 cc of 25% homogenate, 1 cc phosphate buffer, 0.5 cc of 1/4000 methylene blue solution and 0.5 cc of 0.1 M KCN. In the side arms 1 cc of 0.08 M malate. Incubation at 24° C.
The time for decolorization was

Control	With malate
> 120 min.	40 min.

As will be shown later in the discussion of the cytochrome system it is possible to precipitate the particles carrying cytochromes a and b by the simple proceedure of bringing the pH of a homogenized suspension of egg material down to 6.0. Since the tricarboxylic acid cycle enzymes occur in the insoluble material such precipitates were resuspended in buffer and tested for citric and succinic dehydrogenase activity. Even with addition of cytochrome c and boiled muscle extract the preparations were completely inactive. Likewise there was no activity toward methylene blue.

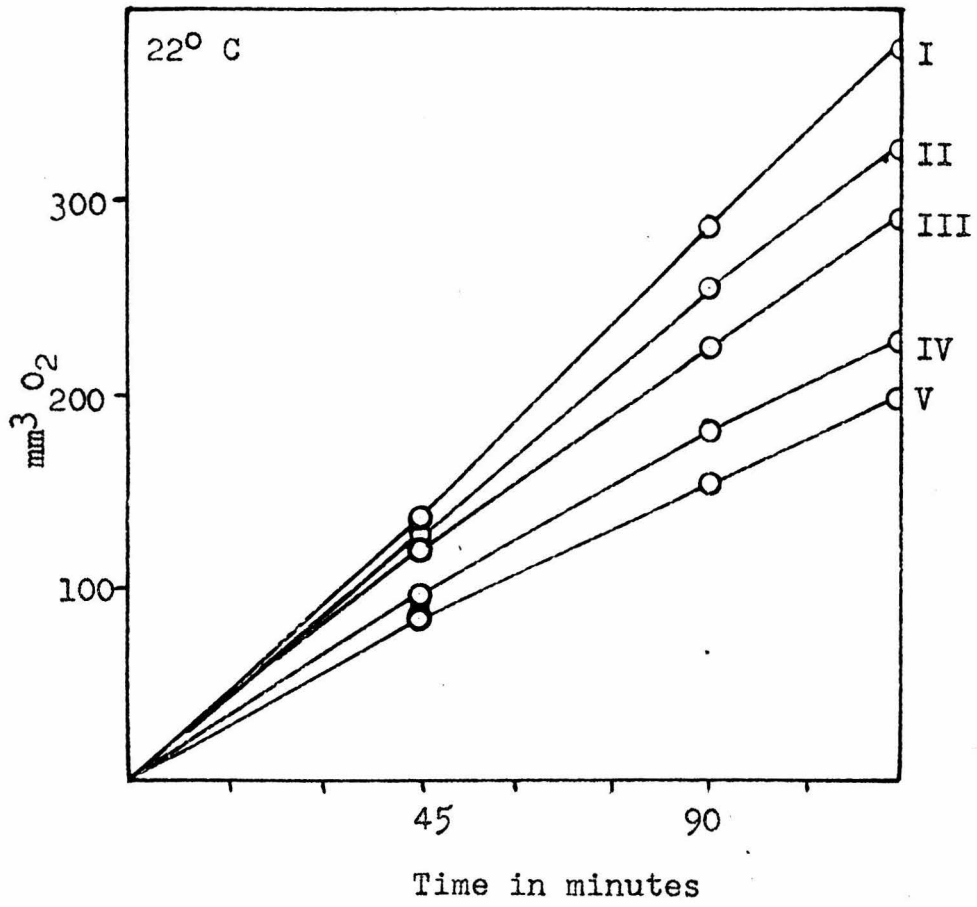

Fig. 10

20% homogenate, 2.8 cc of S. purpuratus eggs in 1/15 M phosphate buffer, pH 7.1.

I 2 x 10^{-5} cytochrome c, 0.007 M alphaketoglutarate

II as I, and boiled muscle extract.

III as II and 0.02 M $MgCl_2$
IV 2 x 10^{-5} M cytochrome c.
V 2 x 10^{-5} M cytochrome c, 0.02 M $MgCl_2$ and boiled muscle extract

While this work was in progress the report of Crane and Keltch (1949) on the metabolism of intermediates of the tricarboxylic acid cycle appeared. Lindberg (1943) had previously noted that fumaric and malic acids are metabolized by egg homogenates. Together with the inhibition of respiration by malonate reported here this constitutes an indication that the tricarboxylic acid cycle functions in sea urchin eggs.

The Cytochrome system

Spectroscopic examination of thick suspensions of eggs of both S. purpuratus and L. pictus using a hand spectroscope reveal rather weak absorption bands at approximately 565 and 695 millimicrons. This corresponds to the bands of cytochrome a and b. In view of the disagreement that has existed as to the presence or absence of cytochromes in sea urchin eggs a more detailed study of the cytochrome system was attempted.

Cytochrome c is a well defined substance of known properties, but the other cytochromes are for practical purposes merely names for absorption bands in the spectra of tissues. An attempt was therefore made to extract cytochrome c from eggs; this however was not successful. No spectroscopic evidence for cytochrome c could be discovered. This, however, cannot be taken as evidence against its existence for two reasons. In many tissues the concentration of cytochrome c is very low (Drabkin, 1950), this being especially striking in embryonic tissue and tumors. Its existence there has been demonstrated by extraction proceedures. It has been shown by Keilin and Hartree (1940) that even very low concentrations of cytochrome c may be sufficient, as the turnover number of the enzyme in vivo is greater than that of the same substance added in vitro. This is presumably due to the organization of the particles on which the other iron porphyrin enzymes and presumably cytochrome c are located. The high turnover rate of cytochrome c is perhaps interpretable in the light

of the work on its structure by Theorell(1941), and Horecker and Kornberg (1946) who give reason to believe that the iron atom is surrounded by a "cage" of amino acid molecules which are sufficient to prevent access of CO and CN to the iron atom. Thus presumably cytochrome c does not act by actual contact of substrate with the iron atom, but the iron atom undergoes change of valence state, gaining and losing electrons through the peptide chain. This probable fact has been made the basis of a new hypothesis of enzyme action recently (Geissman, 1949). The rate of turnover of cytochrome c could thus be high and a considerable respiration could exist without much of the enzyme being present. The amount of cytochrome c varies greatly in different tissues but the significance of this is at present completely obscure (Drabkin, 1950).

The absorption band of cytochrome c is at about 550 millimicrons. In sea urchin eggs strong general absorption begins at just about this point. It is therefore possible that it could exist in fair concentration and still not be visible spectroscopically.

Attempts to extract cytochrome c were made in several different ways, starting with the standard proceedure of Keilin and Hartree (1937) used in preparing cytochrome c on a large scale. This method is essentially based on precipitations with trichloracetic acid and ammonium sulfate. A large volume of eggs of S. purpuratus (460 cc) was used as starting material. These eggs had previously been treated

with acid to remove the jelly coat and stored in the deep freeze. No trace of cytochrome c could be detected in the fraction in which it should have precipitated.

An attempt was made to use the method of Rosenthal and Drabkin (1943) especially adapted for extraction of cytochrome c in small amounts of tissue. 170 cc of frozen stored eggs were used. Again no yield was obtained. The authors of this method point out however that mucoproteins, in which the eggs are rich, interfere with the separation.

Since it was found that commercial preparations of cytochrome c are absorbed strongly by alumina, an egg extract was prepared by heating eggs with dilute acetic acid for one hour at 80° C. This method of extraction has also been used by Keilin and Hartree (1937). The extract was filtered and dialyzed against phosphate buffer, pH 7 and then run through an alumina column. No cytochrome was obtained in this way. Various methods based on absorption on $BaSO_4$ likewise yielded no cytochrome c. Cytochrome c cannot, then, be present in large amounts in the eggs. Preparations frozen in liquid air failed to show the expected absorption band of cytochrome c.

While no direct evidence for the presence of cytochrome c in eggs was obtained, it is nevertheless probably present. The presence of a cytochrome oxidase, perhaps identical with the cytochrome a (or a3) here demonstrated to be present in the egg, has been known for a considerable time. Fig. 11 confirms earlier results of Krahl et.al. (1941) that an

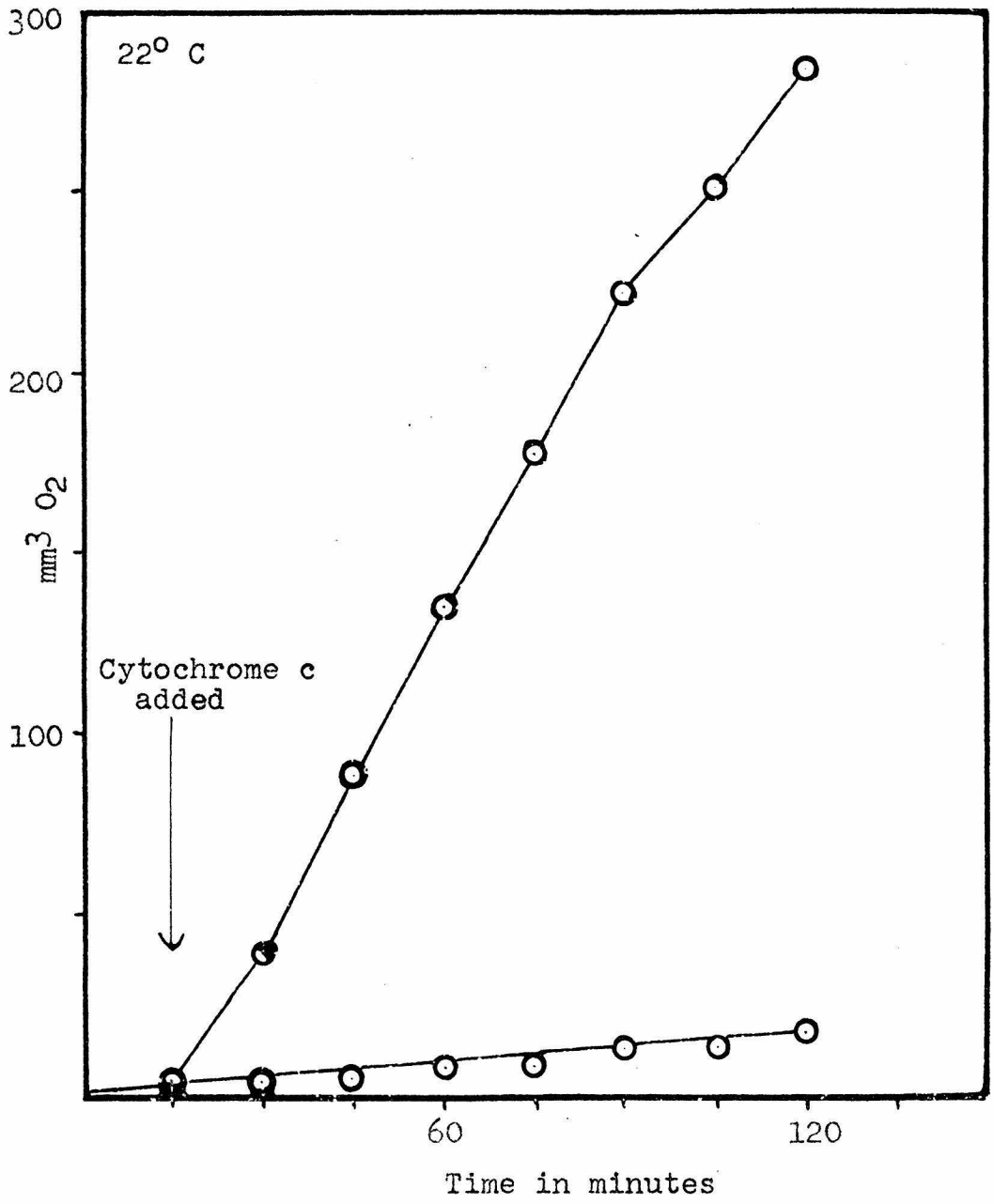

Fig. 11

Oxidation of cytochrome c by 1 cc of 15% homogenate of S. purpuratus eggs in 1/15 M phosphate buffer, pH 7.1

Top curve - with 1.3×10^{-4} M cytochrome c and 0.02 M paraphenylenediamine.
Bottom curve - with 0.02 M paraphenylenediamine.

enzyme capable of oxidizing cytochrome c is present in eggs. That the respiration of fertilized eggs is inhibited by cyanide has been known since the early work of Warburg (1909). Robbie (1946) has recently shown that previous reports that unfertilized eggs are cyanide insensitive were based on technical errors. The only known substrate for cytochrome oxidase is cytochrome c. Probably therefore the normal respiration proceeds in part at least through cytochrome c. Dimethylparaphenylenediamine is oxidized by living systems and its oxidation is believed to proceed through cytochrome c. Runnstrom (1932) has shown, as mentioned in the literature survey that the respiration of intact eggs increases greatly on addition of dimethylparaphenylenediamine.

Besides possessing a system for oxidizing reduced cytochrome c, eggs are also able to reduce it. The endogenous respiration of an egg brei is considerably increased upon addition of cytochrome c. (Fig. 12). A system that specifically requires cytochrome c is the succinic oxidase system. Fig. 6 shows that addition of cytochrome c greatly accelerates the rate of oxidation of succinate.

As mentioned above, cytochromes a and b are characterized chiefly by the positions of their absorption bands. Bands corresponding to such components are observable in suspensions and breis of the eggs of both S. purpuratus and L. pictus after addition of hydrosulfite. The b component is clearly visible, but the a is very faint. On freezing the preparation in liquid air both bands, but especially a

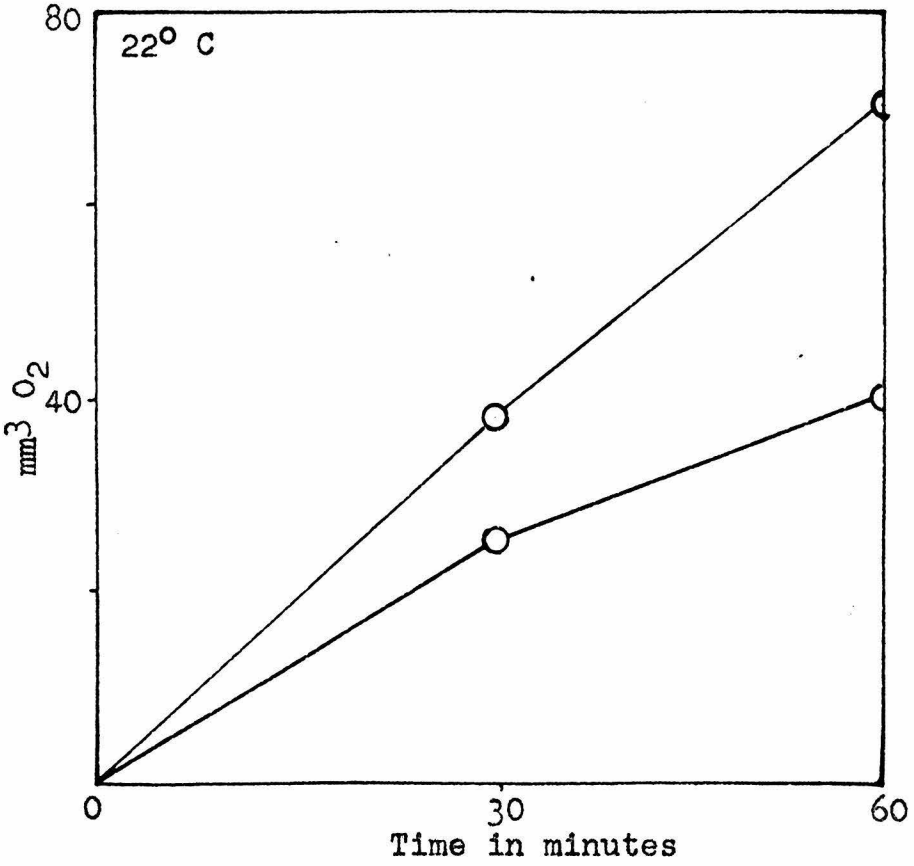

Fig. 12

Effect of cytochrome c (4×10^{-5} M) on endogenous respiration of a 25% homogenate of S. purpuratus egg.

Bottom curve - no additions.
Top curve - with cytochrome c.

becomes much more distinct. A major difficulty in their study is that the solutions are turbid and therefore cannot be studied quantitatively with instruments such as the Beckman spectrophotometer. Attempts to clarify suspensions by the use of detergents or desoxycholeate did materially decrease the turbidity, but not to a sufficient degree. It was therefore necessary to estimate the strength of the absorption bands visually, a proceedure that is particularly unfortunate here, as the faintness of the bands makes even estimates of the order of magnitude doubtful. So far as it was possible to judge, fertilization, anaerobiosis, CO and KCN in intact eggs or addition of succinate to homogenates did not cause any increase in strength of the absorption bands.

Attempts to concentrate the cytochrome fraction of breis was made in the hope that it would be possible to obtain a solution with sufficiently low turbidity for instrumental study. In all cases thus far described in the literature cytochromes a and b are present on small particles containing numerous other enzymes, whose separation without inactivation has proved impossible. A possible exception is the recent work of Eichel et. al. (1950).

A concentration of this particulate fraction was attempted using the method of Battelli and Stern (1912) for preparing cytochrome oxidase from heart muscle.

Eggs of L. pictus were washed several times in sea water and their jelly coats removed by bringing the pH of the sea water to 3.5. They were then centrifuged and the volume found to be 3.2 cc. Throughout the rest of the treatment the preparation was kept ice cold. 20 cc of M/15 phosphate buffer, pH7.15 was added and the eggs broken up in a Waring blender. The preparation was then centrifuged for ten minutes at 3000 rpm and 1 cc of sediment came down. This fraction on reduction with hydrosulfite did not show any absorption bands. The cloudy yellow supernatant was adjusted to pH 6 with dilute acetic acid. A precipitate formed at once. This was centrifuged again for ten minutes at 3000 rpm. yielding 1 cc of precipitate. On reduction this precipitate clearly showed bands corresponding to cytochromes a and b. Further acidification of the supernatant to pH 5 gave another precipitate, which however showed only a very faint b band. A considerable concentration of cytochromes a and b thus appears feasible.

That the cytochromes are really on particles is shown by a centrifugation experiment. Seven cc of S. purpuratus eggs, washed and jelly coats removed at pH 3.5 were suspended in 13 cc of M/15 phosphate buffer pH 7.15 and homogenized. The very viscous preparation thus obtained was centrifuged in a refrigerated centrifuge with a high speed head at 10,000 g., the sediment resuspended and recentrifuged. The sediment shows strong a and b bands. The preparation can be considerably clarified by addition of taurocholeate.

This same preparation, acidified to pH 6 precipitates at once. When tested with methelene blue, however, it shows no succinic dehydrogenase activity. The bands indicate that the cytochromes are in a reduced state, even after shaking in air, and addition of succinate does not seem to intensify them.

Although the physiological reduction and oxidation of the a and b bands could not be demonstrated, evidence was obtained that they are probably iron porphyrins linked to proteins. Cytochrome c is relatively heat stable. The a and b bands disappear when the homogenate is subjected to conditions that denature proteins. One minute at 100° C destroys the bands at once. Addition of organic solvents such as methanol causes the bands to fade out gradually. If after such treatment pyridine is added to the preparation, a very strong absorption band at 555 due to the pyridine hemochromogen appears. This indicates that treatment has liberated the prosthetic groups from protein.

Small amounts of hydrogen peroxide destroy the cytochromes irreversibly.

The b and a cytochromes seem to be autooxidizable at a low pH. If a lyophilized preparation of the eggs of L. pictus is suspended in water, the a and b bands can be observed at least partially reduced. If the pH is brought down to 2.6 the bands disappear and do not reappear if the pH is readjusted to 6.0. That the cytochromes are not destroyed, but only oxidized, is shown by adding hydrosulfite to the preparation which causes the bands to become clearly visible again.

The Rise in the Respiratory Rate during Early Development

It is a well known fact that after fertilization the respiration of sea urchin embryos rises during cleavage, the rate following an S-shaped curve until hatching. At 22° C this takes about eight to nine hours in the case of Strongylocentrotus purpuratus. The course of the respiration is shown in Fig. 13. The rise has been frequently commented on and various explanations advanced; it is usually stated that enzymes are being synthesized during this period. On the other hand, there is a concept that cell division and differentiation which may include synthesis of new cell material, are mutually exclusive. This is obviously not true in the case of desoxyribonucleic acid, which has been clearly shown first by Brachet (1933) and then by other workers to increase linearly during early development in the case of the sea urchin. At least some type of synthesis of cell constituents is thus possible during the cleavage period. Of course such syntheses may be limited to the intercleavage periods, but as yet the problem has been scarcely touched. There are several possible interpretations of the increase in the rate of respiration during cleavage. First, it might be related in some as yet undefined way to the increase in desoxyribonucleic acid. It is clearly not directly proportional to the number of nuclei, as was first shown by Warburg (1910a) and discussed at length by Needham (1931). As Brachet (1938) showed, KCl treated embryos which undergo a certain amount

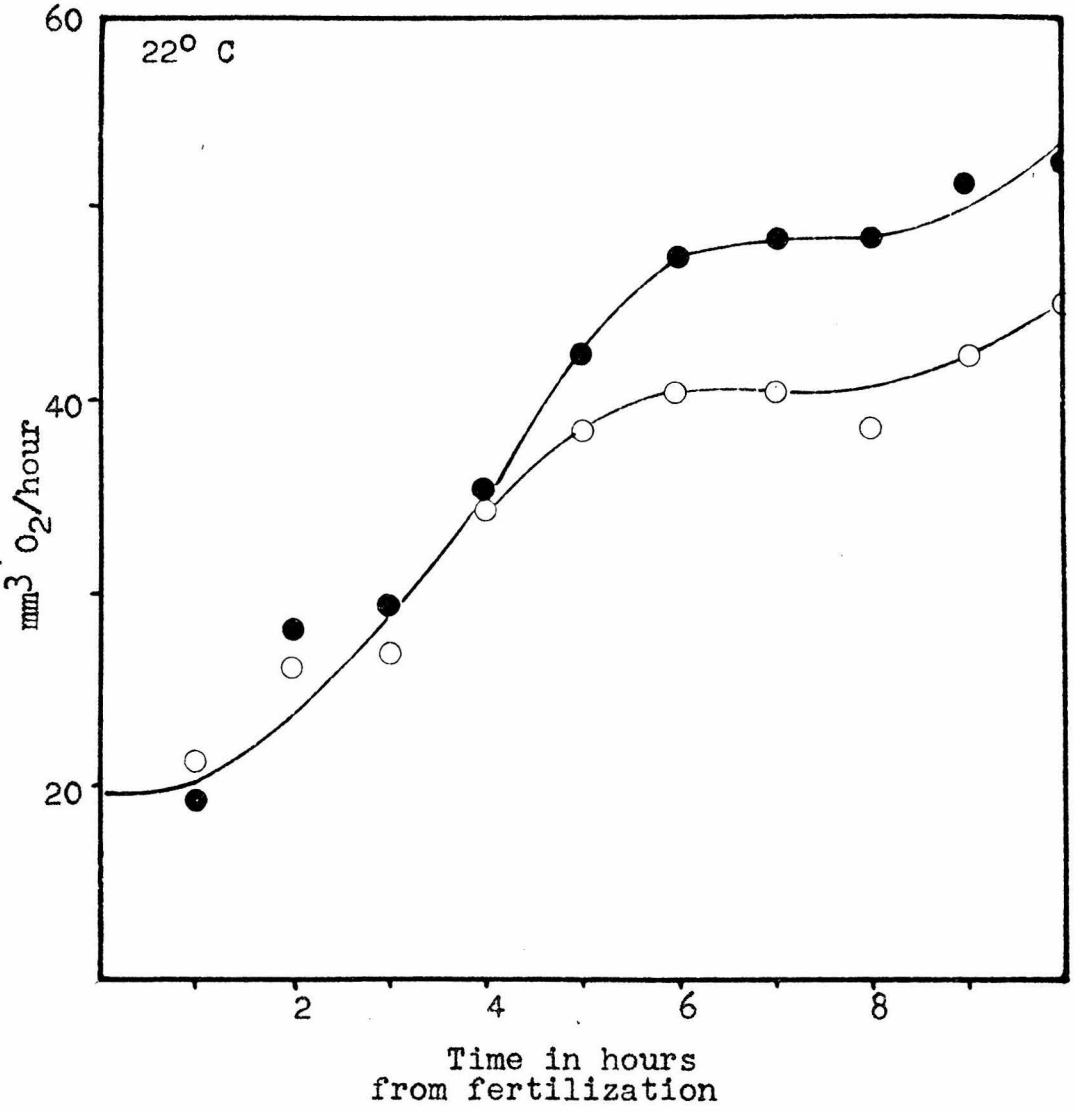

Fig. 13

Respiratory rate during early development of L. pictus.

Light circles - in sea water.
Dark circles - in sea water with 0.03 M glyclyglycine buffer, pH 8.1.

of differentiation without cleavage (Lillie, 1902) show a slower rise in respiratory rate. Such embryos also show a slower rise in content of desoxyribonucleic acid (Brachet, 1938), correlated no doubt with the abnormal and rather obscure nuclear divisions that occur. It is also true that since respiratory rate rises as an S-shaped curve while desoxyribonucleic acid increases linearly, the respiratory rate is also not a linear function of desoxyribonucleic acid content. It is of course possible that the two are linked even if the respiratory rate is not linearly related to desoxyribonucleic acid content.

A second possibility, prefered by Needham (1942) is that respiratory enzymes increase in amount during this period. Even if this is true, it by no means follows that an increase in enzyme concentration would of itself produce an increase in metabolism. Most tissues of the body have amounts of enzymes greatly in excess of that needed to maintain the metabolism which is normally observed. The classical example is of course muscle, but secretory cells, nerves and almost any other tissues could serve as examples. There exist mechanisms which control the metabolic rate independent of enzyme concentrations, providing of course that these are adequate. This will be discussed shortly.

It is possible that substrate could be becoming more available. This does not seem very plausible on general grounds but is difficult to disprove.

An interesting possibility has been suggested by Szent-Gyorgyi (1948). He points out that at least some of the energy producing reactions are obligatorally coupled to phosphorylations and therefore their rate depends on the presence of phosphate acceptors. It is very probable that this is a general phenomenon, the dissociation of respiration and phosphorylation that can be observed in homogenates being an artifact. Since the so called high energy phosphates are now believed on rather plausible grounds to be the only methods of transfering energy from the energy yielding to the energy using reactions, it is clear that activation in any manner of energy requiring reactions increases the concentration of phosphate acceptor and automatically activates the respiratory mechanisms. This hypothesis appears very attractive because it invokes only known mechanisms and because of its simplicity. In a sense, of course, it only pushes the problem a step further back, the reason for the activation of phosphate acceptor being now the problem to be explained. Here, however, there is a model provided by actomyosin, which is an adenosintriphosphatase in the contracted, but not in the extended state.

There are several possible consequences of this hypothesis that are in principle subject to experimental test, all however involving certain assumptions. One is through a study of the Pasteur effect. It is now becoming more widely believed that the Pasteur effect is due to the fact that the ratio of ATP/ADP is much greater in aerobic

respiration than in the glycolysis (Johnson, M. J., 1949). Oxygen therefore suppresses glycolysis by decreasing the concentration of ADP in the glycolytic cycle. The evidence for such a mechanism, while indirect, is rather convincing. If there is indeed an increase of phosphate acceptors during cleavage, there should be a decrease of the Pasteur effect, that is, aerobic glycolysis should increase. This idea occured too late to be tested, but there are several papers showing in the case of the frog a significant increase of aerobic glycolysis during development. (For discussion see Brachet, 1947.)

Another possibility is to study the effects of arsenate, a competitive analogue of phosphate. If the respiration is indeed controlled by the concentration of phosphate acceptor, a sufficient replacement of phosphate by arsenate should bring the respiration at all stages of cleavage to a common level. An attempt was therefore made to study the effect of arsenate on respiration.

Attempts were made to introduce arsenate into the intact eggs. Whether the arsenate penetrated into the eggs was not directly determined, but at any rate its influence on the respiration of the eggs proved slight. Experiments were done on the eggs of L. pictus. First an attempt was made to introduce arsenate into unfertilized eggs by soaking them in an arsenate solution, removing them to sea water and observing the effects of the treatment on the respiratory rise on fertilization. Fertilization in arsenate

solutions is not possible due to the precipitation of the calcium. The details of the procedure are given below.

To washed unfertilized eggs one fourth of the volume of isotonic arsenate solution, 0.37 M pH 8.1 was added to give a 0.09 M solution of arsenate in sea water. Actually, of course, the molarity was less due to the low solubility of calcium and magnesium arsenates which caused a precipitate to form. The eggs were allowed to stand in test tubes in this solution, the controls in ordinary sea water for 45 minutes, with occasional shaking. The eggs were then transfered to manometer vessels, the appropriate samples fertilized by the addition of a drop of sperm into the vessels and the respiration measured. Fig. 14 shows the course of the respiration. At the end of the run the eggs were examined. About 50% of the eggs were fertilized and had reached the 2-4 cell stage. The arsenate apparently had no significant influence on the unfertilized or fertilized eggs. Repetition of the experiment gave essentially the same result.

Experiments were also done suspending fertilized eggs for periods up to four hours in arsenate solutions (0.05 M) and measuring their respiration. No effect on the respiration was found. More significant is the fact that the experiments showed that the eggs went through 4 to 5 cleavages both in the arsenate and in the controls. Since it is known, as will be mentioned shortly, that agents uncoupling

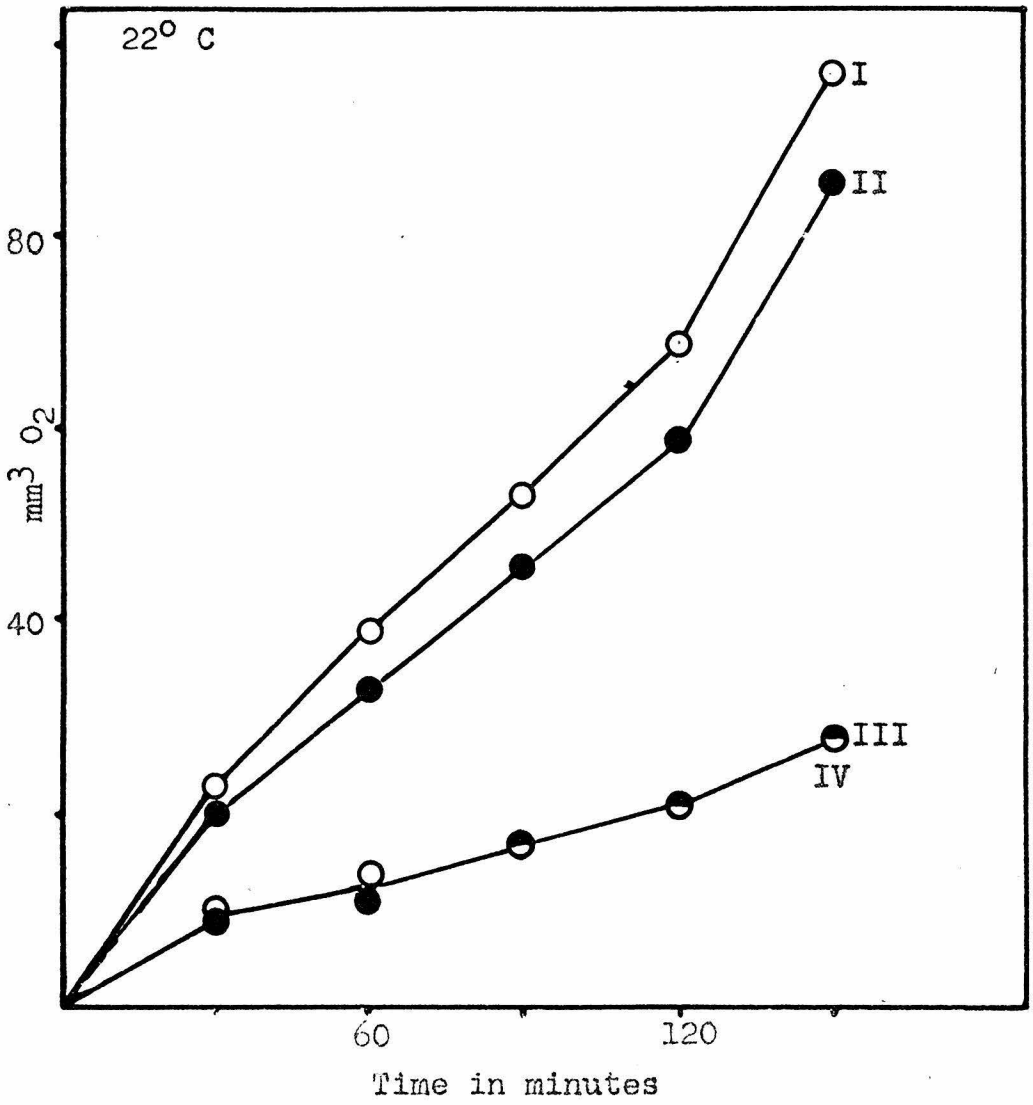

Fig. 14

Effect of treating eggs with 0.09 M arsenate before fertilization. Details in text.

Dark circles - arsenate.
Light circles - no arsenate.

I, II - Fertilized.
III, IV - Unfertilized.

respiration from phosphorylation stop cell division, it was concluded that the arsenate was not acting, most probably due to permeability considerations.

Another method of studying the effect of phosphorylations in controlling the respiratory rate is to use agents that "uncouple" respiration from phosphorylation. Several such agents are known, the most spectacular probably being the dinitrophenols and related compounds. A very considerable amount of work has been done on these compounds. Clowes and Krahl (1934) have studied the effects of substituents on physiological activity, Tyler and Horowitz (1937) have discussed the physiologically active form. It is now established very clearly that there are two distinct effects of these compounds. Respiration in many cases is greatly increased and simultaneously the normal esterification of phosphate is suppressed. An explanation of these two effects has been advanced by Loomis and Lipmann (1948). According to their hypothesis, the dinitrophenols in some fashion replace either phosphate itself or serve as unstable phosphate acceptors, so that respiration is never limited by phosphate requirements and can proceed at a rate determined only by the substrate and enzyme concentration.

If such is indeed the case, dinitrophenol becomes a valuable tool for the study of the nature of the rise in respiratory rate during the cleavage period. A series of determinations were therefore done to measure the effect

of DNP on the respiratory rate shortly after fertilization and at about eight hours at which time the normal rate has approximately doubled.

The experiments were conducted at the Kerckhoff Marine Laboratory, using the eggs of L. pictus. Since it has been shown that the effect of pH on the physiological activity of dinitrophenols is very great, it was neccessary to use a buffer in the sea water. The most suitable appeared to be glycylglycine (Tyler and Horowitz, 1937). Fig. 13 shows that the respiration of developing embryos is not adversly affected by the presence of glycylglycine. The embryos in fact develop somewhat better in it than do the controls. This is probably not a specific effect of glycylglycine but rather due to the maintanance of a constant pH in the manometers. This is especially important since the main buffer of sea water is bicarbonate and that of course, is removed by the alkali in the center well.

Another important consideration is the concentration of dinitrophenol. Since it was desired to measure the maximum capacity of the respiratory system of the embryos, it was neccessary to use the amount giving the maximum stimulation. On the other hand, too large a concentration produces inhibition, and lesser amounts, while raising the respiration to a high level, causes a rapid decline in the respiratory rate after a short time. After some preliminary experiments, it was decided to use two different concentrations and compare the results.

The experimental proceedings were the following. Eggs were removed from the animals and washed several times. They were then fertilized and placed in manometers. The composition in the manometers was as follows: 3.06 cc of 0.05 M glycylglycine buffer, pH 8.1 in sea water, 1.86 cc egg suspension. NaOH 0.3 cc in center well, 5%. Either 1.0 or 0.5 cc of 0.001 M dinitrophenol in sea water was added to the side arm. The temperature of the manometer bath was 22° C. Since this was slightly below room temperature, a cooling arrangement was needed. The manometers were shaken at the rate of 84 strokes per minute, with an amplitude of 7.5 cm. A separate experiment showed that the oxygen uptake was not limited by the rate of shaking. Readings were begun about an hour after fertilization. Dinitrophenol was tipped in at the beginning of the readings and also into other vessels seven hours later to determine the rate of respiration after eight hours of development. The contents of the vessels were examined at the end of the runs. It was found that the development was very good. The stage reached was that just before hatching. The concentrations of dinitrophenol used was such as to stop the development of most of the eggs. A few did, however, go through one or two cleavages after the addition of the reagent.

The results are shown in Table V. In spite of all precautions there is some variation, but on the whole the results appear to be reasonably reproducible. They show quite

Table V

Experiment		I	II	III	IV
1		100	193	362	495
2		100	155	297	356
	mean	100	174	329	425
3		100	200	434	618
4		100	216	477	591
5		100	195	451	578
6		100	174	394	622
7		100	217	412	587
	mean	100	200	434	599

Effect of 2,4,dinitrophenol on the oxygen uptake of embryos of S. purpuratus at one hour and eight hours after fertilization. Rate at one hour expressed as 100. Temperature 22° C. Experiments 1,2 - dinitrophenol concentration 1.1×10^{-4} M. Experiments 3-7 - dinitrophenol concentration 2×10^{-4} M. Columns: I - rate at one hour. II - rate at eight hours. III - rate at one hour with dinitrophenol. IV - rate at eight hours with dinitrophenol.

clearly that not only does the unstimulated respiration about double through the period of eight hours, but that the dinitrophenol stimulated respiration also rises. The absolute rise of the latter is somewhat greater than the normal increase of respiration.

The interpretation of this phenomenon is difficult. Taking the results at face value, one can say that the total capacity of the respiratory system of the embryo increases somewhat more rapidly than the rise in respiration. This should be the interpretation if the Lipmann and Loomis (1948) explanation of the dinitrophenol effect is correct.

Unfortunately the real mode of action of dinitrophenol is not entirely clear. Quite recently Lardy (1950) reports that in the presence of dinitrophenol a very labile phosphate ester accumulates. The nature of this ester is not yet known. There are reasons to suppose that the action of dinitrophenol may be complex.

At any rate the data presented above demonstrate that in some sense there is a considerable increase in the capacity of the respiratory system during the first eight hours of development, and that this increase in capacity parallels the increase in respiration. This could be, as mentioned above, because of an increase in the availability of substrate or it could be because of enzyme synthesis in some sense. To test the possibility of enzyme synthesis eggs and eight hour embryos of L. pictus were lyophilized and stored

in the deep freeze. An assay for aldolase was then done by the method previously described. The activity of eight hour embryos in this enzyme was found to be so much lower than that of the eggs that it was concluded that this was either due to a lesser stability of their enzyme or else through inadvertence the lyophilized powder had been inactivated in some way.

The attempt to measure activity of enzymes is a difficult approach. It is neccessary to test a large number of enzyme systems, to determine their optimum conditions for assay and to check on all possible sources of error, which are many. It would appear better to start such work by determining the grosser changes in the protein composition by some method such as electrophoresis or salting out, and after determining this to attempt a correlation with enzymatic activity.

The problem of the rise of respiration is of very real interest, since it is a general phenomenon, unlike the rise on fertilization, and because many embryologically important processes are then taking place. An understanding of this phenomenon might throw light on several problems now obscure.

Discussion

A. The Enzymatic Pathway of Respiration of Eggs Compared with Adult Tissues

The results reported above make it probable that the respiration of the sea urchin egg proceeds through the same pathway as that known in adult organisms. As the survey of the literature shows, this has been doubted by a large number of embryologists. It may be worth while to examine some of the evidence which has been gathered in support of the latter view.

A large part of it is of a purely negative kind, based on the inability to demonstrate some enzymatic process. Such evidence should be treated with caution. For example the absence of the cytochromes has been frequently reported and some significance has been attached to this supposed fact. The work of Rothschild (1949) and the work reported in this thesis demonstrate the existence of the b and a components. The c component has not as yet been found, but obviously the conclusion cannot be drawn that therefore it does not exist.

Lindberg and Ernster (1948) reported that no triose-phosphate is formed by homogenates on addition of hexose-diphosphate. It was also found that there was no inhibition of respiration by iodoacetate. That aldolase is present in eggs has been clearly shown in this thesis by the use of a method superior to the one available to Lindberg and Ernster.

The question of the inhibition by iodoacetate has been clarified to some extent by the demonstration that inhibition develops rather slowly. This must have been even more true in the experiments of Lindberg and Ernster, since they worked at a temperature of only 6° C.

Differences in penetration and rate of reaction with proteins and glutathione can probably explain most of the anomolous results obtained by some investigators with iodoacetate.

Great stress is laid by the Swedish school on the similarities between the decarboxylative breakdown of glucose demonstrated by Dickens in 1938 and the respiratory mechanisms found in sea urchin eggs. The evidence bearing on this point has been referred to in the literature survey. (For a recent expression of opinion see Runnstrom, 1949.) The following points however should be noted. Part of the evidence rests on failure to demonstrate certain reactions. Some of these reactions have now been shown to take place. The proposed decarboxylative breakdown of sugar has not actually been demonstrated in the sea urchin egg. Even if it were, it is well known that many tissues which can oxidize glucose-6-phosphate to 6-phosphogluconic acid utilize the Meyerhof glycolytic mechanism. Therefore it is reasonable to conclude that there is no real evidence that oxidative decarboxylation is the main respiratory pathway of the sea urchin egg.

The decarboxylation of 6-phosphogluconic acid leads ultimately to the production of a pentose. Since pentoses

are components of nucleic acids and nucleic acids have some as yet not clearly defined role in growth it is very conceivable that the egg may have a well developed system for the production of pentoses. This however is not a subject to be decided by speculation but by experiment. The two points to determine are first how pentoses are produced and incorporated into nucleic acids and second how well developed such a system is in the egg. It must not be forgotten that the glycolytic and respiratory systems produce not only energy but also structural materials and intermediates for other metabolic systems.

It has been demonstrated in the experimental part of this thesis with some probability that glucose-1-phosphate is transformed to glucose-6-phosphate. Glucose-6-phosphate and fructose-6-phosphate are interconvertible. 1,6,fructosediphosphate is broken down to triosephosphate. Respiration is inhibited by iodoacetate. Phosphoglyceric acid is converted to pyruvate. Fluoride inhibits this reaction and also the respiration. Lactic acid accumulates under anaerobic conditions. Citrate, alpha ketoglutarate, succinate and malate are metabolized and respiration is inhibited by malonate. Cytochrome c is both reduced and oxidized. The presence of cytochromes b and a is established. Other work mentioned in the literature survey has shown the presence of cytochrome oxidase and inhibition of respiration by cyanide and carbon monoxide. It seems

probable that the respiration of sea urchin eggs is similar to that well known in adult organisms. This respiratory system then does not arise anew in each ontogeny but is transferred intact through the egg.

It must be admitted, of course, that the demonstration of any enzymatic system is not tantamount to showing that it is actually used. This is a much more difficult problem and the solution of it in an unequivocal fashion can probably be done only by the use of methods involving labeled compounds in the intact organism. Should the point seem of sufficient importance for the solution of some problem this could of course be done.

The idea that the respiratory system is incomplete in the embryo received at one time a great impetus from the work of Needham. For a full discussion of the problem his book (1942) should be consulted. His idea was that the glycolytic system involving phosphate arose anew in each ontogeny. During early life its place was taken by a simpler system for the breakdown of glucose not involving phosphate. Since it is now generally believed that energy transfer in the organism involves phosphate the existence of a glucolytic system of the type proposed by Needham would be of great interest. Needham supported his idea with many experiments in which he tried to differentiate between the pathway of glucose and that of glycogen, which he concluded did involve phosphate. A favorite reagent to inhibit this nonphophorylative glucolysis was l-glyceraldehyde. The recent work of

Lardy, Wibelhaus and Mann (1949) has to some extent clarified the mode of action of this compound. Under the influence of aldolase it condenses with phosphodihydroxyacetone to form l-sorbose-1-phosphate, a powerful inhibitor of hexokinase. If this is indeed the case then l-glyceraldehyde, instead of inhibiting nonphosphorylative glucolysis inhibits the first step involving phosphorylation. Meyerhof and Perdigon took up the problem in 1940 and succeeded in demonstrating the Meyerhof scheme in the chick embryo. More recently Novikoff, Potter and LePage (1948) have isolated the intermediates of this scheme from chick embryos in the expected quantities. It would thus appear that there is no reason to believe that embryonic respiration in the chicken utilizes another mechanism than the adult.

The ideas of Needham have no doubt had a considerable influence on embryologists working with the sea urchin in making them interpret any anomolous results in terms of mechanisms not present in adults. It would appear now that this is not necessary.

B. An Interpretation of the uniformity of Glycolytic Mechanisms

Pertinent to the problem discussed above is the observation that the mechanisms for breaking down starch and glycogen are extraordinarily uniform in the most distantly related organisms. This is true not only of animals but also of plants. The possible exceptions are organisms that have not been fully studied, such as the fungi, or certain bacteria which can be easily interpreted as having lost part of their enzymatic equipment. The demonstration of such a uniformity can be regarded as one of the main contributions of biochemistry to general biology.

The cause of such a uniformity, which has been found now to extend to many other basic biochemical mechanisms, could be due to the common origin of all living things. On the other hand, an explanation in terms of the limitations to which any given biochemical mechanism is subject should be examined.

It will be pointed out here that the reactions of glycolysis are so interdependent that not much variation is possible. It is not possible to show with any completeness that no other mechanism than the one that exists is possible, but only that if others can be constructed they must be of a radically different nature.

At the start it is neccesary to assume certain facts. The glycolytic mechanism, let it be assumed, has to be easily reversible, proceeds from glycogen (or starch) to pyruvate and generates ATP.

The first point to notice is that a hexose has to be split into a three carbon compound. The only chemical mechanism that breaks carbon to carbon bonds in a reversible manner would seem to be an aldol condensation. This is of course what is done by the enzyme aldolase. The fact that an aldol condensation occurs limits the product (or substrate) to a keto sugar. Several points of interest now present themselves.

Assuming that we are dealing with hexoses, it might appear that any ketohexose of the d series could be an intermediate. Actually this does not seem to be the case. Fischer and Baer (1936) have studied the nonenzymatic condensation of dihydroxyacetone and d-glyceraldehyde and discovered the unexpected fact that instead of four ketohexoses only two were obtained in detectable amounts. The conventional structural formulae of the four possible ketohexoses of the d series are as follows:

HCHOH	HCHOH	HCHOH	HCHOH
C = O	C = O	C = O	C = O
HCOH	HOCH	HCOH	HOCH
HCOH	HCOH	HOCH	HOCH
HCOH	HCOH	HCOH	HCOH
HCHOH	HCHOH	HCHOH	HCHOH
Psicose	Fructose	Sorbose	Tagatose

The two products were fructose and sorbose. The structural requirement is that the hydroxyls on carbon atoms 3 and 4 be trans to each other. Now it is of interest that these two sugars are naturally occuring while psicose and tagatose

are known only as synthetic products. In the presence of aldolase fructose and sorbose can be obtained, but not psicose and tagatose. Thus the enzymatic and nonenzymatic mechanisms are alike in this respect and an explanation is at hand for the exclusion of two otherwise possible intermediates from the glycolytic mechanism. Fructose rather than sorbose is the actual intermediate. The reason for this is not quite clear but seems to be connected with the optical specificity of the enzymes of glycolysis. Aldolase in the presence of phosphodihydroxyacetone and d-glyceraldehyde forms fructose. If l-glyceraldehyde is present instead the product is sorbose (Imanga, 1937 and Lardy, Wibelhaus and Mann, 1949). Thus the choice of fructose instead of sorbose would appear to depend on the optical specificity of triosephosphate isomerase which forms d rather than l-glyceraldehyde from dihydroxyacetone.

A point of interest, although it may be a minor one, is that if a ketohexose is split by the reverse aldol condensation the two products are different. They are however the keto and enol forms of the same compound and readily interconvert even nonenzymatically under mild conditions. Were the sugar a heptose or a pentose this would not be possible. In the case of a hexose the two products are able to proceed rather directly along the same metabolic pathway.

The above discussions show that a ketohexose must be an intermediate in the metabolic scheme. Yet the sugar

residues of starch and glycogen are glucose, an aldohexose. A conversion must occur and this explains the necessity of the other steps before the formation of 1,6,fructosediphosphate.

We have assumed that we start with glycogen or starch. The degradation of the molecule proceeds, as shown by Cori and Cori (1936), not hydrolytically but phosphorolytically. The rationale of this has often been discussed in connection with the reversibility of the reaction and need not be further discussed here. From the structure of glycogen the phosphorolytic split must give us glucose-1-phosphate (on paper at least glucose-4-phosphate is also possible. The nonenzymatic phosphorolysis of glycogen does not appear to have been studied and therefore nothing can be said on the chemistry involved here). Glucose-1-phosphate must now be transformed into fructose. There are three enzymes involved in this operation. The reason for this is not difficult to see. Under mild alkaline conditions glucose, mannose and fructose are mutually interconvertible. This is believed to proceed through an en-diol form common to the three sugars. The mechanism of the enzymatic conversion is not known, but is probably the same.

If such is the case, then obviously glucose-1-phosphate cannot be directly transformed into fructose, since the phosphate on carbon atom 1 blocks enolization. The shift of the phosphate to position 6 liberates the one position

and enolization can occur, giving rise to fructose-6-phosphate. The further phosphorylation of fructose-6-phosphate is of course connected with the ultimate transfer of the phosphate to ADP.

This sort of discussion could be extended to greater length if desired. The reason for bringing it up at this point is to stress that the organism does not have at its disposal an infinite number of possible mechanisms for natural selection to operate on and therefore the uniformity of biochemical mechanisms is not unexpected. Not only are the individual mechanisms subject to a variety of restrictions, but probably even more stringent are the requirements of interlocking mechanisms. For example, the above discussion does not explain why the storage polysaccharide is usually not a fructosan. This would obviate the necessity of several steps that actually occur in the glycolytic mechanism. However, there may be reasons for this as well. Suppose for example that galactose is needed by some other metabolic system. Galactose is synthesized by conversion of glucose-1-phosphate to galactose-1-phosphate. A route from fructose to glucose-1-phosphate may then have to exist in any case due to a requirement for galactose. Our knowledge of biochemistry is as yet slight, but it is enough to make us suspect that reasonable explanations based on sound chemical principles may eventually be found for the existence of certain systems and for the absence of others.

C. The Embryological Significance of Respiration

With the exception of the viruses all living organisms respire. From this it could be concluded that respiration, while a necessary condition of embryonic development, has no direct effect on the course of growth and differentiation. This conclusion, however, is too sweeping. Two aspects of the study of respiration would seem to be of direct embryological importance.

The problems of growth and differentiation have proved to be very difficult, probably because we have no idea which phenomena are the ones to study for the solution of these problems. The study of respiration, on the other hand, is by comparison an easy matter. Many embryological phenomena are accompanied by changes in the respiration, and detailed studies of such changes may provide us with a clue to the nature of other changes linked with the respiratory ones. These studies, while laborious, if pursued in connection with the study of other phenomena that may appear pertinent to the problem may yield results of interest.

It must also not be forgotten that respiration, besides providing energy for the organism, can have other functions. As has been clearly shown by the use of isotopically labeled compounds, the intermediates of the respiratory process can provide a variety of structural materials for other biochemical systems. Changes in the respiration alone could disturb the steady state of the organism, with results sometimes pathological but perhaps also producing some of the normal

phenomena of development. Whether this is indeed the case will be decided by investigations that have yet to be carried out.

Bibliography

Ashbel, R. (1929) Boll. Soc. Ital. Biol. Sper. 4:1152

Ball, E. G. and Meyerhof, B. (1940) J.B.C. 134:483

Ballentine, R. (1938) Biol. Bull. 75:368

Barker, S. B. and Summerson, W. H. (1941) J.B.C. 138:535

Barron, E. S. G. and Goldinger, J. M. (1941) Biol. Bull. 81:289

Battelli, F. and Stern, L. (1912) Ergeb. Physiol. 12:96

Borei, H. (1945) Arkiv. Kemi min. Geol. 20A No. 8
(1948) Biol. Bull. 93:124

Brachet, J. (1933) Arch. Biol. 44:519
(1934) ibid 45:611
(1938) Biol. Bull. 74:93
(1947) Embryologie Chimique, Mason et Cie, Paris

Brachet, J. and Needham, J. (1935) Arch. Biol. 46:821

Broh-Kahn, R. H. and Mirsky, A. E. (1948) Proc. Soc. Exp. Biol. Med. 67:176

Brooks, S. C. and Chambers, E. L. (1948) Biol. Bull 95:262

Chaigne, M. (1934) Soc. Biol. 115:174

Clowes, G. H. A. and Krahl, M. E. (1934) Science 80:384

Connors, W. M. and Scheer, B. T. (1947) J. Cellular Comp. Phys. 30:271

Cori, C. F. and Cori, G. T. (1936) Proc. Soc. Exp. Biol. Med. 34:702

Cori, G. T., Cori, C. F. and Schmidt (1939) J.B.C. 129:629

Crane, R. K. and Keltch, A. K. (1949) J. Gen. Physiol. 32:503

Daleq, A. (1941) L'oeuf et son Dynamisme Organisateur, Albin Michel, Paris

Dickens, F. (1938) Bioch. J. 32:1645

Drabkin, D. L. (1950) J. B. C. 182:317

Heichel, E. , Wainio, W. W., Person, P. and Cooperstein, S.J., (1950) J. B. C. 183:89

Ellis, E. (1933) J. Cell. Comp. Physiol. 4:127

Epheussi, B. and Rapkine, L. (1928) Ann. Physiol. Physiochim. biol. 3:386

Fischer, H. O. L. and Baer, E. (1936) Helv. Chim Acta 19:519

Fiske, C. H. and Subbarrow, Y. (1925) J.B.C. 66:375

Geissman, T. A. (1949) Quart. Rev. Biol. 24:309

Henry, R. J. (1943) Bact. Rev. 7:175

Horecker, B. L. and Kornberg, A. (1946) J.B.C. 165:11

Hutchens, J. O., Keltch, A. K., Krahl, M. E. and Clowes, G.H.A., (1941) J. Gen. Physiol. 25:717

Imanga, H. (1937) Biochem. Z. 294:342

Jandorf, B. S. and Krahl, M. E. (1942) J. Gen. Phys. 25:749

Johnson, M. J. (1949) in Respiratory Enzymes, Lardy, H.A. Burgess Publishing Co., Minneapolis

Keilin, D. and Hartree, E. F. (1937) Proc. Roy. Soc. Biol. 122: 298 (1940) Proc. Roy. Soc. Biol. 129:277

Korr, J. (1939) J. Cellular Comp. Physiol. 10:461

Krahl, M. E., Jandorf, B.S. and Clowes, G.H.A. (1941) Jour. Gen. Phys. 25:733

Krahl, M. E., Keltch, A. K. and Clowes, G. H. A., (1941) Biol. Bull. 77:318

Krahl, M. E., Keltch, A. K., Neubeck, C. E. and Clowes,G.H.A. (1941) Jour. Gen. Phys. 24:597

Lardy, H. A. (1950) Feder. Proc. 9:187

Lardy, H. A., Wibelhaus, V. and Mann, K. (1949) in Lardy, H. Respiratory Enzymes, Burgess Publishing Co., Minneapolis

Leloir, L. F., Trucco, R. E., Cardini, C. E., Paladini, A. and Caputto, R. (1948) Arch. Biochem. 19:339

LePage, G. A. and Umbreit, W. W. (1945) in Manometric Techniques and Related Methods for the Study of Tissue Metabolism, W. W. Umbreit, R. H. Burris and J.F. Stauffer, Burgess Publishing Co., Minneapolis

Lillie, F. R. (1902) Arch. Entw. Mech. 14:477

Lillie, R. S. (1910) Amer. Journ. Physiol. 26:106
(1931) Biol. Bull. 60:288

Lindahl, P. E. (1936) Acta Zoologica 17:179

Lindhal, P. E. and Holter, H. (1941) C. R. Trav. Lab. Carlsberg (Ser. chim.) 24:49

Lindberg, A. (1943) Arkiv. kemi. mineral geol. 16A No. 15 23 A No. 2 (1946 ibid)

Lindberg, A. and Ernster, L. (1948) Bioch. Biophys. Acta 2:471

Loeb, J. (1906) Bioch. Z. 1:183

Loeb, J. and Wasteneys, H. (1912) Arch. Entw. Mech. 35:555

Lohman, K. (1933) Bioch. Zeit. 262:137

Lohman, K. and Meyerhof, O. (1934) Bioch. Zeit. 273:60

Loomis, W. F. and Lipmann, F. (1948) J.B.C. 173:807

Lugg, J.W.H. and Overell, B.I. (1947) Nature 160:87

Macleod, M. and Robinson, R. (1929) Bioch. J. 23:517

Meyerhof, O. (1911) Biochem. Zeit. 35:246
(1937) Naturwiss 25:443

Meyerhof, O. and Kiessling, W. (1933) Bioch. Zeit. 264:40

Meyerhof, O. and Perdigon, E. (1940) Enzymologia 8:367

Meyerhof, O. and Schultz, W. (1938) Bioch. Zeit. 297:60

Meyerhof, O. and Wilson, J. R. (1947) Arch. Bioch. 14:71

Najjar, V. A. (1948) J.B.C. 175:281

Needham, J. (1931) Chemical Embryology, Cambridge University Press, (1942) Biochemistry and Morphogenesis, Cambridge University Press

Needham, J. and Needham, D. M. (1930) Journ. Exp. Biol. 7:317

Needham, D. M. and Pillai R. (1937) Nature 140:65

Novikoff, A. B., Potter, V. R. and LePage, G. A. (1948) J.B.C. 173:239

Ochoa, S. (1944) J.B.C. 155.87

Orstrom, A. (1932) Protoplasma 15:566
(1935) ibid 24:177
(1941) Zeit. Physiol. Chem. 271:1

Orstrom, A. and Lindberg, O. (1940) Enzymologia 8:367

Pasteels, J. (1935) Arch. Biol. 46:229

Perlzweig, W. and Barron, E. S. G. (1928) J.B.C. 79:19

Rappoport, S. (1937) Bioch. Zeit. 289:406

Robbie, W. A. (1946) J. Cellular Com. Physiol. 28:305

Roe, J. H. (1943) J.B.C. 107:15

Rosenthal, O. and Drabkin, D. L. (1943) J.B.C. 149:437

Rothschild, Lord (1939) J. Exp. Biol. 16:49
(1949) ibid 26:100

Runnstrom, J. (1930) Protoplasma 10:106
(1932) ibid 15:548
(1933) Biochem. Z. 258:257
(1935) Biol. Bull. 68:327
(1949) Advances in Enzymology 9:241

Shapiro, H. (1941) Biol. Bull. 81:278

Sibley, J. A. and Lehninger, A. L. (1949) J.B.C. 177:859

Spallanzani, L. S. J. (1803) Memoires sur la Respiration, Geneve

Stott, F. C. (1931) J. Exp. Biol. 8:133

Stumpf, P. K., Zarudnaya, K. and Gree, D. E. (1947) J.B.C. 167:817

Szent-Gyorgi, A. (1948) Nature of Life Academic Press

Theorell, H. (1941) J. Am. Chem. Soc. 63:1820

Tyler, A. (1942) Quart. Rev. Biol. 17:197,339
(1949) The Collecting Net 19:19

Tyler, A. and Horowitz, N. H. (1937) Proc. Nat. Acad. Science 23:369 (1937)a Science 84:85

Tyler, A. and Humanson, W. D. (1937) Biol. Bull. 73:261

Tyler, A. and Schultz, J. (1932) J. Exp. Zool. 63:509

Warburg, O. (1908) Zeitschr. f. Physiol. Chem. 57:1
(1909) ibid 60:443
(1910) a ibid 66:305
(1910) b Bioch. Zeitschr. 29:414
(1910) c Zeitschr. f. physiol. Chem. 69:496

Warburg, O. and Christian, W. (1932) Bioch. Zeit. 242:206

Whitaker, D. M. (1931) J. Gen. Phys. 15:183

Whiteley, A. H. (1949) Amer. Nat. 83:249

Zielinski, M. A. (1935) Acta Biol. Exptl. Warsaw 13:35