THE RESPIRATORY AND GLYCOLYTIC ENZYMES OF SEA-URCHIN EGGS

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A number of workers have studied the carbohydrate metabolism of sea-urchin eggs and have expressed different opinions about the pathway of carbohydrate breakdown in these eggs. The choice has rested between the breakdown of hexose via the 'hexose monophosphate shunt' and a glycolytic mechanism like that in yeast and muscle, followed by oxidation via the tricarboxylic acid cycle. Rothschild (1951) has summarized the evidence on this question. In listing the evidence in favour of the latter view, Rothschild included work reported in the author's Ph.D. thesis (Yčas, 1950). Some of these results are presented in this paper. The evidence for a normal glycolytic and respiratory system has since been greatly extended by Cleland & Rothschild (1952*a*, *b*).

MATERIALS AND METHODS

Unfertilized eggs were obtained from *Strongylocentrotus purpuratus* and *Lytechinus pictus*. The animals were collected in the vicinity of the Kerckhoff Marine Biological Laboratory of the California Institute of Technology at Corona del Mar, California. Eggs were obtained either by putting the ovaries in sea water or by making the animals shed by injection of isotonic (0·55M) KCl into the coelomic cavity (Tyler, 1949). The eggs were strained through bolting silk, washed several times in sea water, and centrifuged. Fresh homogenates were prepared by suspending the eggs in cold 1/15M-phosphate buffer, pH 6·8, and homogenizing in a glass homogenizer. The concentration of homogenate is given as percentage by volume of eggs in the homogenate. The volume of eggs was determined by centrifuging egg suspensions in graduated centrifuge tubes. In two cases homogenates prepared from lyophilized eggs were used. O_2 uptake was measured with the conventional Warburg apparatus at 22° C.

RESULTS

Örström & Lindberg (1940) showed that immediately after fertilization of the eggs of *Paracentrotus lividus* there is a breakdown of polysaccharide material in amounts sufficient to provide substrate for the increased respiration. The breakdown of this polysaccharide, presumably glycogen, was found to be phosphorolytic. As no accumulation of phosphate esters was detected, they expressed the belief that

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glucose-1-phosphate was formed from glycogen and then dephosphorylated before being further metabolized by some unknown system.

Since such a dephosphorylation of glucose-1-phosphate appears rather improbable, an attempt was made to determine if the well-known conversion of glucose-1-phosphate to glucose-6-phosphate and fructose-6-phosphate could be demonstrated in homogenates.

The homogenate was prepared from lyophilized, unfertilized eggs of *Lytechinus pictus*. The lyophilized powder was added to 60 times its weight of distilled water, homogenized, and then dialysed against a solution of 0·5% KCl and 0·26% Na_2CO_3 for 5 hr. in the cold. Mg^{++}, cysteine (Najjar, 1948) and the potassium salt of glucose-1-phosphate were added. Aliquots were taken at 0 and 90 min. after addition of substrate, hydrolysed for 15 min. in 1N-HCl at 100° C. and inorganic phosphate determined by the method of Fiske & Subbarow (1925). Under these conditions glucose-1-phosphate is hydrolysed completely, while glucose-6-phosphate is scarcely affected (Umbreit, Burris & Stauffer, 1945). In addition, fructose was determined by the method of Roe (1943), and calculated as fructose-6-phosphate. The results are shown in Table 1.

Table 1

Time (min.)	No. 1		No. 2	
	a	*b*	*a*	*b*
0	21	1·7	24	1·5
90	12	6·0	26	1·7

No. 1, 6 ml. egg homogenate, 1 ml. 0·1 M-cysteine, 1 ml. 0·1 M-$MgCl_2$, 1 ml. solution of 8 mg. of the potassium salt of glucose-1-phosphate and 1 ml. water. No. 2 as no. 1, substrate and water additions replaced by 4·4 mg. glucose and 25 μmoles KH_2PO_4 in 2 ml. *a*, μmoles of inorganic phosphate found after 15 min. hydrolysis in 1N-HCl at 100°. *b*, μmoles fructose-6-phosphate. Temperature 23° C.

With the same homogenate and fructose-6-phosphate there is a rapid disappearance of substrate and conversion of the phosphate into a less easily hydrolysable form, presumably glucose-6-phosphate (Table 2). One ml. of water containing

Table 2

Time (min.)	Fructose-6-phosphate (μmoles)	0 min. phosphate (μmoles)	180 min. phosphate (μmoles)
0	21	1·8	16
7	9·2	5·3	11

Disappearance of added fructose-6-phosphate in an egg homogenate. Temperature 23° C.

approximately 5 mg. of fructose-6-phosphate was added to 4 ml. of homogenate. The reaction was stopped by addition of 5 ml. of 10% trichloracetic acid to the samples at 0 and at 7 min. Phosphate was determined on aliquots after 0 and 180 min.

hydrolysis, and fructose as in the previous experiment. Under these conditions fructose-6-phosphate is hydrolysed completely and glucose-6-phosphate about 10% (Umbreit *et al.* 1945).

A decrease in fructose, estimated as fructose-6-phosphate, and in phosphate present after hydrolysis for 180 min. was observed, although the disappearance of fructose, when measured directly, is greater than that of the 180 min. phosphate.

These results are compatible with the assumption that the initial metabolism of hexose proceeds according to the classical Embden-Meyerhof sequence.

Lindberg & Ernster (1948) failed to find any accumulation of triosephosphate in egg homogenates incubated with iodoacetate; nor was the oxidation of glucose by these homogenates decreased by iodoacetate. They concluded that aldolase and triosephosphate dehydrogenase do not function in sea-urchin egg respiration.

Using the colorimetric method of Sibley & Lehninger (1949), aldolase activity is easily demonstrable in egg homogenates (Table 3).

Table 3. *Formation of triosephosphate*

Vessel	Homogenate (ml.)	Klett colorimeter reading
1	0·1	25
2	0·1	27
3	0·2	50
4	0·2	52
5	0·4	96
6	0·4	91
7	0·8	145
8	0·8	145

Composition of incubated mixture: 1 ml. glycylglycine buffer 0·1 M, pH 8·6, 0·3 ml. 1,6-fructose-diphosphate 20 mg./ml., 0·2 ml. 0·56 M-hydrazine, homogenate (100 mg. of lyophilized eggs of *Lytechinus pictus* in 6 ml. buffer) as above, and water to make to volume of 2·5 ml. Incubated 30 min. at 26° C., reaction stopped by addition of trichloracetic acid and triosephosphate determined by method of Sibley & Lehninger (1949). Blank had 0·8 ml. of homogenate, substrate added after trichloracetic acid.

The reaction of enzymes with iodoacetate is known to be rather slow. As the experiments of Lindberg & Ernster were conducted at 6° C., the reaction of iodoacetate with the dehydrogenase in their system may not have been sufficiently complete to show a detectable inhibition. To test this possibility the effect of pre-incubation with iodoacetate on the rate of reduction of methylene blue by egg homogenates was studied. Homogenates were pipetted into Thunberg tubes and iodoacetate added at various intervals of time before evacuation. The tubes were then evacuated simultaneously and, immediately after evacuation, methylene blue was tipped in from the side arms, the time to 90% decolorization being noted. Fig. 1 shows that iodoacetate inhibits the reduction of methylene blue by the endogenous substrate present in the homogenate and the inhibition increases with the period of pre-incubation with the inhibitor.

Using a more dilute homogenate, a reduction of methylene blue by 1,6-fructose-

diphosphate and an inhibition of reduction by iodoacetate is shown in Table 4. These results provide some evidence that an iodoacetate-sensitive triosephosphate dehydrogenase is present in the eggs and that it functions in respiration. The importance of an adequate length of time for development of iodoacetate inhibition is also demonstrated.

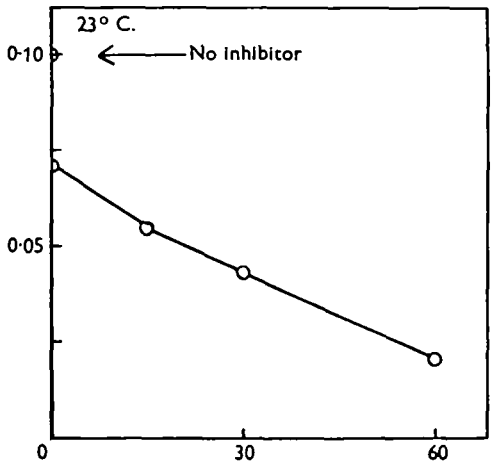

Fig. 1. Effect of pre-incubation with 0·03 M-iodoacetate on reduction of methylene blue by a 20 % homogenate of *Lytechinus pictus* eggs in M/15-phosphate buffer, pH 7·1. Ordinate: reciprocal of time in minutes till reduction. Abscissa: length of time in minutes homogenate exposed to inhibitor.

Table 4

Tube contents	Side arm	Time to decolorization (min.)
2 ml. homogenate 1 ml. water	1 ml. MB 1 ml. water	100
2 ml. homogenate 1 ml. iodoacetate	1 ml. MB 1 ml. water	>120
2 ml. homogenate 1 ml. water	1 ml. MB 1 ml. HDP	52
2 ml. homogenate 1 ml. iodoacetate	1 ml. MB 1 ml. HDP	>120

Fructose-1,6-diphosphate 0·05 M, iodoacetate 0·1 M, methylene blue 0·12 mg./ml., 5 % *Strongylocentrotus purpuratus* egg homogenate in 1/15 M-phosphate buffer, pH 7·1. Temperature 23° C. Iodoacetate added, tubes evacuated and allowed to stand 1 hr. before tipping sidearm contents.

The further metabolism of the phosphoglyceric acid formed by the oxidation of triosephosphate requires the participation of enolase. Since this enzyme is strongly inhibited by fluoride, an inhibition of the O_2 uptake of homogenates by fluoride should be demonstrable. Fig. 2 shows that such is the case. Enolase activity can be demonstrated more directly by the procedure described by Umbreit *et al.* (1945). A homogenate prepared from 0·5 g. of lyophilized eggs of *L. pictus* in 30 ml. of distilled water was dialysed in the cold against 0·5 % KCl and 0·26 %Na_2CO_3. One ml.

of this preparation was incubated with 1 ml. of 0·02 M-sodium phosphoglycerate for 10 min. at 23° C. Trichloracetic acid was then added, the incubation mixture filtered, and an aliquot treated with iodine and titrated with thiosulphate. The blank received substrate after addition of trichloracetic acid. Under these conditions, 1 ml. of the homogenate formed 0·30 mg. of phosphoenolpyruvate, which was reduced to 0·03 mg. in the presence of 0·01 M-NaF.

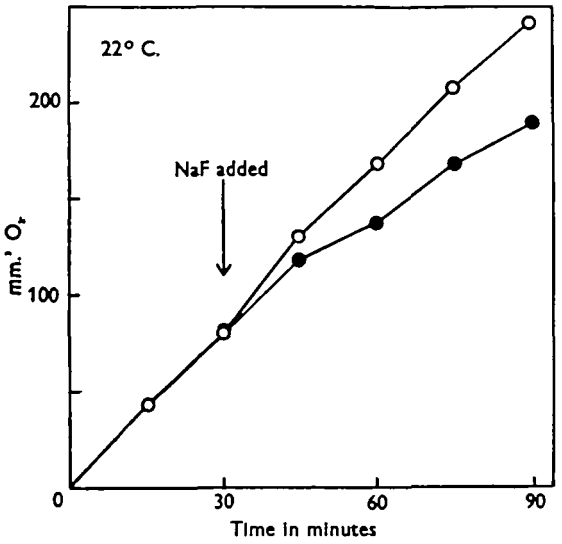

Fig. 2. Effect of NaF on the endogenous respiration of 2 ml. of a 25 % homogenate of *Strongylocentrotus purpuratus* eggs in M/15-phosphate buffer, pH 7·1. Light circles: control; dark circles: with 0·05 and 0·01 M-NaF.

Perlzweig & Barron (1928) first reported the presence of lactic acid in sea-urchin eggs, and their results have been confirmed as follows. Eggs of *Stongylocentrotus purpuratus*, placed in evacuated Thunberg tubes at 23° C. for 3 hr., were found to accumulate an average of 0·86 mg. of lactate per ml. of eggs. Controls in oxygenated sea water only contained traces of lactate. The lactate was determined by the method of Barker & Summerson (1941). A rather weak lactic dehydrogenase activity with methylene blue as a hydrogen acceptor was found to be present in homogenates.

The results obtained with iodoacetate and fluoride agree well both qualitatively and quantitatively with those reported by Cleland & Rothschild (1952*a*, *b*), even though different species and experimental conditions were used. The anaerobic formation of lactate by eggs of *S. purpuratus* (860 μg./ml. eggs/3 hr. at 23° C.) was found to be larger than in the more extensive experiments of Cleland & Rothschild with *Echinus esculentus* (300 μg./ml. eggs/4 hr. at 20° C.). Since these authors have shown that slight cytolysis greatly enhances lactate formation, and because of differences in the experimental conditions, the difference is perhaps not significant.

Although Ball & Meyerhof (1940) showed that eggs were unable to metabolize succinate and malate, implying the absence of a tricarboxylic acid cycle, Crane &

Keltch (1949) demonstrated that oxalacetate, succinate, α-ketoglutarate, glutamate and citrate are rapidly oxidized by similar homogenates. A particulate cell-free system was prepared from unfertilized *Arbacia* eggs by Keltch, Strittmatter, Walters & Clowes (1949). This system was capable of esterifying orthophosphate during the oxidation of intermediates of the tricarboxylic acid cycle. Cleland & Rothschild (1952*b*) have found that egg homogenates oxidize added pyruvate, the effect on the O_2 uptake being more marked when the formation of pyruvate from endogenous substrate is inhibited by fluoride. It is therefore probable that a tricarboxylic acid cycle does function in sea-urchin eggs.

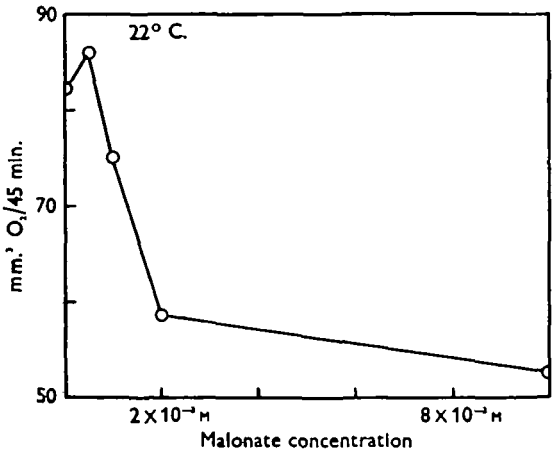

Fig. 3. Effect of malonate on the respiration of 2 ml. of a 25 % homogenate of *Strongylocentrotus purpuratus* eggs in M/15-phosphate buffer, pH 7·1.

In the present study it was found that homogenates of unfertilized eggs prepared in 1/15M-phosphate buffer, pH 7·1, were able to oxidize citrate, α-ketoglutarate, succinate and malate as measured by an increased O_2 uptake in the presence of these acids. Since these results are not essentially different from those obtained by previous workers, details of the experiments are not given. The endogenous O_2 uptake of homogenates was found to be inhibited by malonate (Fig. 3), which is further evidence for the existence of the tricarboxylic acid cycle.

For a considerable time cytochrome was not observed in sea-urchin eggs, although cytochrome oxidase was known to be present as the terminal oxidase. In 1949 Rothschild reported that these eggs contained cytochromes *a* and b_1. This observation was easily confirmed using a hand spectroscope, although the absorption bands are rather faint. With the instrument available, the position of the cytochrome bands could not be determined with precision, but the approximate positions were at 560 and 600 mμ. No trace of the band of cytochrome *c* could be detected, even in preparations examined at the temperature of liquid air. Strong general light absorption begins at about the expected position of the cytochrome *c* band, so that failure to detect it spectroscopically cannot be considered as strong evidence for its

absence. Attempts to extract cytochrome *c* from several hundred ml. of eggs by the methods of Keilin & Hartree (1937), and Rosenthal & Drabkin (1943), failed to yield any cytochrome *c*. This evidence cannot, however, be considered conclusive, as the extractability of cytochrome *c* varies in different tissues and organisms. Keilin & Hartree's method, for example, fails in the case of yeast, although cytochrome *c* is present in this organism in high concentration and can be extracted by suitable methods (Keilin, 1933).

Recently Borei (1950) has made a new attempt to detect and measure cytochrome *c* in sea-urchin eggs, using a method proposed by Paul (1950). His results indicate that at most only very small traces of cytochrome *c* can be present. While this may well be so, it should be pointed out that Paul states (personal communication, 1951) that the method is subject to hitherto unsuspected errors, a fact which was presumably not known to Borei.

As mentioned above, the absorption bands of cytochrome are faint in the sea-urchin egg, making their spectroscopic study difficult. No change could be detected with certainty in the strength of the absorption bands after aeration, anaerobiosis, or activation with sperm.

A considerable concentration of the egg cytochromes can be achieved by two different procedures. The first is essentially a modification of that used by Keilin & Hartree (1938) to prepare cytochrome oxidase from heart muscle. The procedure, as applied to eggs, was as follows: unfertilized eggs of *Lytechinus pictus* were washed several times in sea water and their jelly coats removed by allowing them to stand in sea water at a pH of 3·5 (Tyler, 1941). The eggs were then centrifuged. Throughout the rest of the treatment the preparation was kept ice cold. Twenty ml. of M/15-phosphate buffer, pH 7·1, was added to 3·5 ml. of eggs, the latter being broken up in a Waring blender. The preparation was then centrifuged for 10 min. at 3000 r.p.m. and 1 ml. of sediment came down. This fraction did not show any cytochrome bands after reduction with hydrosulphite and was discarded. The cloudy yellow supernatant was brought to pH 6 with dilute acetic acid and immediately formed a precipitate, which yielded a further 1 ml. of sediment on centrifuging for 10 min. at 3000 r.p.m. On reduction this fraction clearly showed the absorption bands of cytochromes *a* and b_1. Further acidification of the supernatant to pH 5 gave another precipitate, which, however, showed only a very weak band of cytochrome b_1.

The cytochrome components can also be concentrated by a simple centrifugation procedure. Eggs of *L. pictus* were treated as in the first procedure to remove the jelly coats and 7 ml. of eggs suspended in 13 ml. of buffer and homogenized. The homogenate was then centrifuged for 1 hr. at 10,000 *g* in a refrigerated centrifuge, the resultant precipitate being resuspended in buffer and recentrifuged. The precipitate showed strong *a* and b_1 bands when examined with the hand spectroscope. Absence of a significant amount of cytochrome *c* was demonstrated by heating the preparation to 100° C. for 1 min. This procedure destroyed the *a* and b_1 bands, as is the case in other tissues. Cytochrome *c* is stable to such treatment, but could not be demonstrated in the heated preparation after reduction with hydrosulphite.

The above results indicate that the cytochromes of sea-urchin eggs are bound to sedimentable particles, as in other tissues.

Homogenates are able both to reduce and oxidize cytochrome *c*, and both the endogenous O_2 uptake and oxidation of succinate are increased in the presence of added mammalian cytochrome *c* (Fig. 4). The apparent absence of cytochrome *c* thus poses a problem as to the mode of action of the cytochrome oxidase long known to be present in sea-urchin eggs. It would be possible to explain these results by

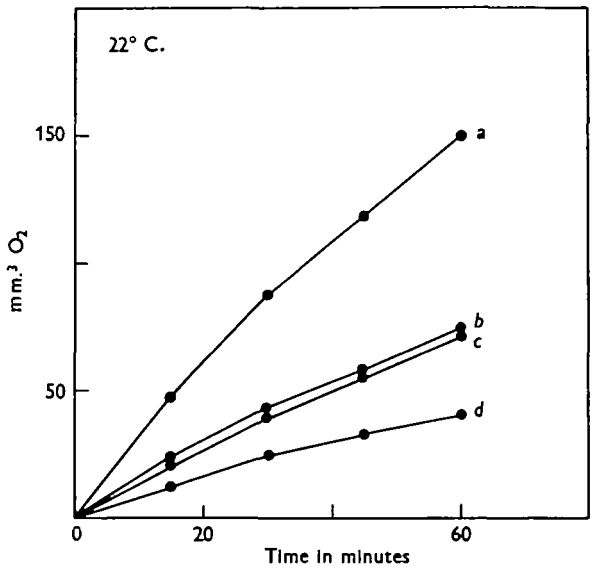

Fig. 4. Effect of cytochrome *c* and succinate on the respiration of a 25 % homogenate of *Strongylocentrotus purpuratus* eggs in M/15-phosphate buffer, pH 7·1. *a*, with 0·02 M-succinate and 5×10^{-5} M-cytochrome *c*. *b*, with 0·02 M-succinate. *c*, with 5×10^{-5} M-cytochrome *c*. *d*, no additions.

assuming the existence of cytochrome *c* in the egg and ascribing failure to detect it to technical difficulties. It would seem best, however, to suspend judgement on this point. The nature of the component b_1 has not been elucidated. Sanborn & Williams (1950) have described a cytochrome in the tissues of the larval *Cecropia* moth, which is believed by them to show some of the properties of both cytochromes *b* and *c*, including ability to react with cytochrome oxidase. Spectroscopically this component appears identical with the cytochrome *e* recently described by Keilin & Hartree (1949) and shown by them to be as universally distributed as the normal cytochrome system. Until more work is done on the cytochromes of sea-urchin eggs, especially on the properties of the b_1 component,* the nature of the final steps of oxidation cannot be considered clarified.

* Further discussion of cytochrome b_1 is to be found in Keilin & Harpley (1941) and in Rothschild (1949).

DISCUSSION

The above results show that at least a large part of the enzymatic equipment needed to metabolize carbohydrate according to the Embden-Meyerhof scheme and the tricarboxylic acid cycle is present in the sea-urchin egg, and that this is probably the major pathway of carbohydrate breakdown. The evidence for this conclusion has been greatly strengthened by Cleland & Rothschild (1952*a*, *b*). Nevertheless, it would be desirable to obtain more definitive evidence of the actual course of events *in vivo* than has yet been done. Using radioactive carbon Cohen (1951) has shown that both the usual glycolytic pathway and direct oxidation of glucose are important pathways in *Escherichia coli*. It is not impossible that the well-known changes in respiration occurring on fertilization and during development are associated with qualitative changes in metabolic pathways. The glycolytic pathway does appear, however, to be an important part of the respiratory mechanisms of the egg. An outstanding problem remains the apparent absence of cytochrome *c* and the nature and function of the b_1 component.

SUMMARY

1. Activity corresponding to phosphoglucomutase, phosphohexoisomerase, aldolase, triosephosphate dehydrogenase, enolase and lactic dehydrogenase has been demonstrated in homogenates prepared from unfertilized sea-urchin eggs (*Strongylocentrotus purpuratus* and *Lytechinus pictus*).

2. The presence of cytochromes *a* and b_1 has been confirmed. These cytochromes sediment in a relatively low centrifugal field.

3. No cytochrome *c* could be demonstrated, although cytochrome *c* is both reduced and oxidized by homogenates, and addition of cytochrome *c* increases the endogenous respiration and oxidation of succinate.

4. These results support the view that the usual glycolytic pathway operates in the sea-urchin egg and is the principal route of oxidation of carbohydrate.

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