GLUCOSE-6-PHOSPHATE AND 6-PHOSPHOGLUCONATE DEHYDROGENASES FROM EGGS OF THE SEA URCHIN, ARBACIA PUNCTULATA

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Glucose-6-phosphate dehydrogenase (Zwischenferment) and 6-phosphogluconate dehydrogenase, first studied in yeast by Warburg and his coworkers (1, 2), have since been found in a number of cells and tissues. These have received extensive study because they provide a pathway for formation of pentose phosphate from hexose phosphate and because of the possibility that oxidation of glucose-6-phosphate by this means may provide energy for function. (See references 3–6.)

The present experiments deal with the occurrence, the properties, and the relation to over-all metabolism of these two enzymes in eggs of the sea urchin, *Arbacia punctulata*; they are part of a general survey of the enzymes of *Arbacia* eggs (7).

Experimental Methods

The eggs were obtained and handled as previously described (8, 9). Homogenates were prepared as before (9), except that citrate was omitted from, and 0.004 M ethylenediamine tetraacetic acid included in, the homogenizing medium to minimize breakdown of the echinochrome granules; the homogenates were a light orange-pink and the supernatant fractions obtained therefrom by centrifugation at 20,000 *g* for 30 minutes were clear and of a straw yellow color. The homogenates and other fractions to be tested were kept at ice water temperature; full activity was retained for 6 to 8 hours; 20 to 40 per cent of the initial activity of the homogenate or supernatant fractions was lost by a single freezing and thawing; this procedure appeared relatively more damaging to the phosphogluconate dehydrogenase than to glucose-6-phosphate dehydrogenase. The values recorded in the tables and figures were calculated by use of the conversion factors previously derived (10).

The sources of the special reagents were: Sigma Chemical Company, triphosphopyridine nucleotide (TPN), 83 per cent purity (assayed by yeast Zwischenferment), barium glucose-1-phosphate, and yeast Zwischenferment (lot No. 54-137, virtually free of phosphogluconate dehydrogenase); Pabst Laboratories, adenosine diphosphate; Nutritional Biochemicals Corporation, barium glucose-6-phosphate monohydrate, 86 per cent purity (assayed by yeast Zwischenferment free of phosphogluconate de-

hydrogenase) and barium fructose-6-phosphate; Schwarz Laboratories, diphosphopyridine nucleotide (DPN), 65 per cent purity (manufacturer's value) and magnesium fructose-1,6-diphosphate.

Barium 6-phosphogluconate was prepared by one of the authors (M. E. K.) from glucose-6-phosphate according to the bromine oxidation method (11, 12). Aliquots were converted to the sodium salt by suspension in water and addition of sodium sulfate. Solutions (0.01 M) of the sodium salt were titrated over the range pH 2–10; two acid groups were present, one with pK′ about 3.4 (carboxyl), one with pK′ about 6.7 (phosphate pK'_2); the two groups were present in equal amount corresponding to approximately 95 per cent of that expected for the monobarium salt of phosphogluconic acid. No residual glucose-6-phosphate was revealed by test with a sample of yeast Zwischenferment free from phosphogluconate dehydrogenase, and the reducing power toward the Nelson copper reagent was negligible.

Protein was determined according to Lowry *et al.* (13) and pentose according to Mejbaum (14) with arabinose as standard and a 40 minute heating period.

Enzymatic reduction of TPN or DPN was followed at 340 mμ in the Beckman spectrophotometer with silica cells of 1.0 cm. lightpath; for all calculations the extinction coefficient for the reduced triphospho- and diphosphopyridine nucleotides was taken to be 6.22×10^6 cm.^{2} per mole (15). The initial temperature of the reaction mixture was adjusted to 19°C., and the temperature again measured after completion of the measurement; the average temperature is recorded with the data.

Preliminary experiments with the egg fractions were made to establish the range in which reduction was proportional to the concentration of egg fraction, *i.e.* enzyme; all rate measurements were made under such conditions.

Each spectrophotometric run was initiated by addition of substrate at zero time; readings were taken successively at 10 seconds, 1 minute, 2 minutes, and appropriate further intervals. The values for extinction were extrapolated to zero time; the calculations of rate were based on the average per minute change in extinction for the 2 minutes following addition of substrate, during which the course of the reaction was in general rectilinear with time under the conditions chosen.

Experimental Results

Glucose-6-Phosphate Dehydrogenase and Phosphogluconate Dehydrogenase Activity in Relation to Fertilization.—Approximately 1.8 to 3.0 micromoles TPN were reduced per minute by the homogenate from 1 gm. of unfertilized eggs, wet weight (10), with glucose-6-phosphate as substrate and about 40 to 60 per cent of this amount with 6-phosphogluconate as substrate. The amount of activity toward glucose-6-phosphate did not change significantly upon fertilization. About 95 per cent of the activity was found in the supernatant fraction, after centrifuging at 20,000 *g* (Table I).

Relation of Activity to Substrate Concentration.—The reduction of TPN was measured with varying concentrations of glucose-6-phosphate or 6-phosphogluconate (Figs. 1 and 2). Each of the crude enzymes in the supernatant fraction was half saturated when its substrate was 0.00004 M. The half-satu-

ration value for 6-phosphogluconate dehydrogenase of yeast is 0.00005 M (16); for glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of *E. coli* the values are 0.0003 M and 0.00003 M, respectively (4).

Pentose Formation.—Pentose was formed during oxidation of glucose-6-phosphate by TPN (Table II). Whether or not the intermediates in this oxidation

TABLE I

Glucose-6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase Activity from Arbacia Eggs in Relation to Fertilization

The initial concentrations of the components of the reaction system, in moles per liter, were: $MgCl_2$, 0.03; glycylglycine, 0.025; KCl, 0.08; glucose-6-phosphate, 0.0009; or 6-phosphogluconate, 0.001; TPN, 0.00004. The total volume was 2.17 ml.; egg fraction, 0.05 ml. from 0.01 ml. eggs; pH, 7.4. Temperature, 23°C. For preparation of homogenate (H), see Methods. The supernatant (S) and particulate (P) fractions were prepared by centrifugation at 20,000 *g* for 30 minutes at 5°C.

Date	Experiment No.	State of egg development	Egg fraction tested	Protein content of egg fraction per gm. original wet eggs	TPN reduction, micromoles per min. per gm. original wet eggs	
					With glucose-6-phosphate as substrate	With 6-phosphogluconate as substrate
1954				*mg.*		
6–30	2W	Unfertilized	H	82	2.24	
			S	32	2.40	
7–21	18W	Unfertilized	H	58	1.80	
			S	38	2.30	
7–23	20W	Unfertilized	H	94	2.15	
			S	50	2.40	1.40
7–28	24W	Unfertilized	H	123	2.22	
			S	66	2.82	1.53
8–25	40W	Unfertilized	S	58	2.63	1.35
7–1	3W	Fertilized	H	64	1.88	
			S	37	1.88	
			P	23	0.10	
7–29	25W	Fertilized	H	113	2.46	
			S	65	2.40	
8–4	30W	Fertilized	H	121	2.42	
			S	62	2.56	1.06

are the same as demonstrated for yeast or *E. coli* (4, 5) could not be ascertained because of limited experimental material.

Lindberg (17) found an increase in pentose upon incubation of cytolysates of *Echinocardium* eggs with hexose monophosphate or phosphogluconate; no TPN was added. Cohen (18) observed that extracts of *Chaetopterus* eggs could form in 10 hours an amount of pentose from glucose-6-phosphate equal to all the ribose initially present in the egg ribonucleic acid.

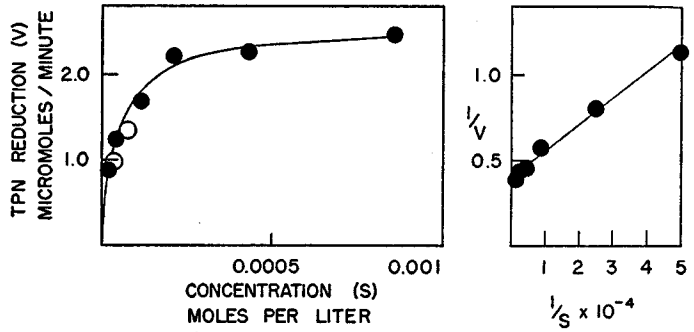

FIG. 1. TPN reduction by supernatant fraction from 1 gm. wet weight *Arbacia* eggs in relation to glucose-6-phosphate concentration. For experimental conditions, see Table I. Filled circles, fraction from unfertilized eggs; open circles, fraction from fertilized eggs. The half saturation values (K_m) are determined from the plot of $1/V$ against $1/S$ according to Lineweaver and Burk (32).

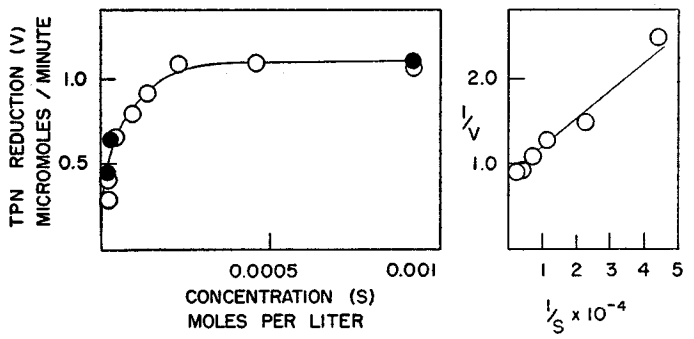

FIG. 2. TPN reduction by supernatant fraction from 1 gm. wet weight *Arbacia* eggs in relation to 6-phosphogluconate concentration. For experimental conditions and designation of results, see Table I and Fig. 1.

TABLE II

Formation of Pentose during Reduction of TPN by Supernatant Fraction from Arbacia Eggs

The initial concentrations of the components of the reaction system, in moles per liter, were: $MgCl_2$, 0.03; glycylglycine, 0.025; KCl, 0.08; glucose-6-phosphate, 0.000063; TPN, 0.00007. The total volume was 2.87 ml.; egg fraction, 0.1 ml. from 0.02 ml. eggs; pH 7.4; temperature, 25°C. The reaction was initiated by addition of substrate, allowed to proceed 10 minutes, and terminated by addition of 1 ml. 20 per cent trichloroacetic acid. Pentose formation was estimated (14) as the difference between that in this incubated sample and that in an identical non-incubated sample. For preparation of supernatant fraction, see Methods.

Date	Experiment No.	Stage of egg development	TPN reduced	Pentose formed
1954			*micromoles*	*micromoles*
7–23	20W	Unfertilized	0.12	0.07
7–28	24W	Unfertilized	0.16	0.06
7–29	25W	Fertilized	0.14	0.06
8–4	30W	Fertilized	0.15	0.06

Activity of Glycolytic Enzymes Relative to That of Glucose-6-Phosphate Dehydrogenase in Egg Extracts.—To estimate the ability of the egg extracts to degrade carbohydrate by the glycolytic pathway as compared to that *via* TPN, reduction of DPN was measured with fructose-1,6-diphosphate and ADP present.[1] This DPN reduction proceeded, in various trials, at 1 to 8 per cent the rate of TPN reduction by glucose-6-phosphate (Fig. 3). Thus, either aldolase or oxidizing enzyme, or both, are in very low concentration in unfertilized and fertilized *Arbacia* eggs.

The presence of certain other enzymes of the glycolytic cycle is indicated by the following experiments, in which the TPN reduction ensuing after

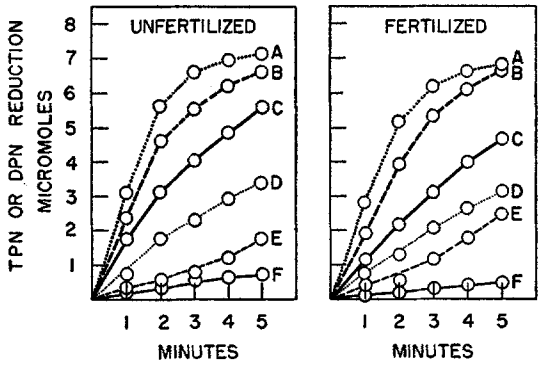

FIG. 3. TPN or DPN reduction by supernatant fractions from 1 gm. wet weight unfertilized or fertilized *Arbacia* eggs. Curves *A* to *E*, TPN reduction with substrates as follows: *A*, glucose-6-phosphate; *B*, fructose-6-phosphate; *C*, 6-phosphogluconate; *D*, fructose-1,6-diphosphate; *E*, glucose-1-phosphate. Curve *F*, DPN reduction with fructose-1,6-diphosphate as substrate and 0.001 M ADP present. The initial concentration of each substrate was 0.001 M. For experimental conditions see Table I.

conversion of the various substrates to glucose-6-phosphate[2] was measured (Fig. 3); the substrates tested, with the enzyme specifically indicated to be present by each test, were as follows: glucose-1-phosphate, phosphoglucomutase; fructose-6-phosphate, hexose isomerase;[3] fructose-1,6-diphosphate, a fructose-1,6-diphosphatase.

[1] In yeast and most animal cells fructose diphosphate is split into two triose phosphate moieties by aldolase; one of these, phosphoglyceraldehyde, is oxidized by its dehydrogenase (Warburg's oxidizing enzyme) with concomitant reduction of DPN (see reference 19). The test as here employed measures the rate of the slower of these two enzymatic reactions in the egg extracts.

[2] Enzymes for direct oxidation of each of these substrates are not excluded. Until these are demonstrated, the present results are interpreted in terms of the enzymes of the currently accepted Embden-Meyerhof-Warburg-Cori scheme (3).

[3] Hexose isomerase has also been demonstrated directly in *Arbacia* extracts, by measurement of fructose-6-phosphate formation from glucose-6-phosphate (20).

Inhibition of Glucose-6-Phosphate Dehydrogenase by Substituted Phenols.— Dinitro-, dihalo-, or trihalophenols reversibly suppress cleavage of *Arbacia* eggs at very low concentrations (21, 22); there is a close parallel between the ability of a series of these agents to suppress cleavage and to inhibit oxidative phosphorylation by a particulate enzyme system from the eggs (23). Since the glucose-6-phosphate of yeast (Zwischenferment) has been found to be one of a limited number of soluble enzymes sensitive to these agents (24), it was of interest to test the effect of 2,4,5-trichlorophenol[4] on oxidation of glucose-6-phosphate by *Arbacia* extracts. The glucose-6-phosphate dehydrogenase in the supernatant fraction from unfertilized or fertilized eggs was 50 per cent inhibited at about 6×10^{-5} M 2,4,5-trichlorophenol (Table III); this concentration is somewhat larger than the 1×10^{-5} M required to inhibit cleavage or oxidative phosphorylation at the same pH (23).

TABLE III

Inhibition of Glucose-6-Phosphate Dehydrogenase Activity of Homogenate (H) and Supernatant (S) Fractions from Unfertilized or Fertilized Arbacia Eggs by 2,4,5-Trichlorophenol

The experimental conditions were the same as those described in Table I (experiments 2W and 3W). The results are expressed as micromoles TPN reduced per minute per gram of original wet eggs.

Concentration trichlorophenol	Fractions from unfertilized eggs		Fractions from fertilized eggs	
	H	S	H	S
moles per liter				
0	2.24	2.40	1.88	1.88
0.00003	1.52	1.65	0.91	1.33
0.00012	0.50	0.55	0.43	0.54
0.0005	0.1	0.0	0.1	0.1
0.001	0.0	0.0	0.1	0.1

DISCUSSION

The present experiments demonstrate that the eggs of *Arbacia* contain glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase in substantial concentrations; the presence of these enzymes in eggs of European echinoderms had previously been postulated on the basis of less direct evidence (17, 25, 26). It is of interest to attempt to appraise the significance of these enzymes for the over-all oxidative metabolism of the eggs and for formation of the pentose required for ribonucleic acid synthesis.

[4] Trichlorophenol was used instead of one of the dinitrophenols because the latter absorb strongly in the range of 340 mμ, which interferes with the sensitivity of the test system.

The rate of glucose-6-phosphate oxidation *via* TPN is sufficient to account for an oxygen consumption of 1700 c.mm. per hour per gm. eggs, wet weight; the actual oxygen consumption of fertilized eggs is 310 and of unfertilized eggs 70 c.mm. per hour per gm. eggs, wet weight (see (7)). Thus, there is enough glucose-6-phosphate dehydrogenase to support a rate of oxygen consumption almost 6 times that observed for the fertilized, and 24 times that observed for the unfertilized, eggs. The amount of this enzyme in *Arbacia* eggs is as high as that in any mammalian tissue except lactating mammary gland (6).

Further, the glucose-6-phosphate–TPN pathway is in great excess relative to the pathway by which glucose-6-phosphate may be converted directly to lactic acid: DPN reduction, with fructose-1,6-diphosphate added to the system,[1] proceeds at a rate approximately 5 per cent of that for TPN reduction by glucose-6-phosphate; this rate of DPN reduction could account for only about one-third of the oxygen consumption of the fertilized eggs. That the glycolytic system is also of low activity in the intact eggs is demonstrated by the fact that *Arbacia* eggs form only very limited amounts of lactate under either aerobic or anaerobic conditions (see references 10 and 27), although they contain preformed lactate as they are shed from the ovaries (28). Thus, the evidence presently available suggests that the glucose-6-phosphate dehydrogenase system may be the major pathway for utilization of carbohydrate in *Arbacia* eggs.

The degree to which carbohydrate oxidation actually supplies energy for development is not yet clear. Hutchens *et al.* (10) found that storage carbohydrate of *Arbacia* eggs was consumed during development, especially 8 hours or more after fertilization, but the coupling of this carbohydrate consumption with energy trapping has not been demonstrated. Hutchens *et al.* also offered evidence that protein was oxidized during development. Later, Keltch *et al.* (8) showed that oxidation of α-ketoglutarate or of oxalacetate by a particulate system from the eggs was coupled with phosphorylation. At the moment, therefore, there is evidence that developing *Arbacia* eggs may depend in part upon carbohydrate oxidation, for which the glucose-6-phosphate oxidation by TPN is quantitatively the major pathway, and in part upon amino acid oxidation *via* oxalacetate, α-ketoglutarate, and the tricarboxylic acid cycle. The contribution of fat oxidation has yet to be explored.

It is possible that other echinoderm eggs utilize the glycolytic pathway to a greater degree than *Arbacia* eggs (29, 30) but, as quantitative estimates of the relative activities of the glucose-6-phosphate–TPN and the glycolytic pathways have not been made in those eggs, this question is open.

The observed rate of pentose formation from glucose-6-phosphate was approximately 6×10^{-9} moles per minute per 0.02 ml. (95,000) eggs, or 0.6×10^{-18} moles per minute per egg. If each egg contains 6×10^{-12} moles ribose of

ribosenucleic acid (31), the enzyme present can form in about 2 hours an amount of ribose equal to that in the total ribosenucleic acid of the egg.

SUMMARY

1. Glucose-6-phosphate and 6-phosphogluconate dehydrogenases have been found in homogenates of *Arbacia* eggs; 95 per cent of the activity toward each substrate is recovered in the supernatant fraction after centrifuging at 20,000 *g* for 30 minutes.

2. With glucose-6-phosphate as substrate, the rate of TPN reduction by the supernatant fraction from 1 gm. wet weight unfertilized or fertilized eggs was 1.8 to 3.0 micromoles per minute; this rate is sufficient to support a rate of oxygen consumption 24 times that observed for unfertilized, and 6 times that for fertilized, eggs. Pentose was formed from glucose-6-phosphate at a rate 0.3 to 0.5 that of TPN reduction, when both rates were expressed as micromoles per minute.

3. The concentrations of glucose-6-phosphate and 6-phosphogluconate for half maximal activity were each approximately 0.00004 M for the respective enzymes in the supernatant fraction. Maximal activity toward 6-phosphogluconate was 50 to 60 per cent of that toward glucose-6-phosphate. Glucose-6-phosphate dehydrogenase activity was 50 per cent inhibited in presence of 0.00006 M 2,4,5-trichlorophenol.

4. Reduction of DPN by the supernatant fraction in presence of fructose-1,6-diphosphate and ADP was 0.1 to 0.2 micromoles per minute per gm. wet eggs, indicating that the glycolytic pathway can metabolize glucose-6-phosphate at about 5 per cent the rate at which it can be oxidized by the TPN system from unfertilized or fertilized *Arbacia* eggs.

5. Phosphoglucomutase, hexose isomerase, and a phosphatase for fructose-1,6-diphosphate also appear to be present in *Arbacia* eggs.

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