OXIDATIVE PATHWAYS FOR GLUCOSE IN EGGS OF THE SEA URCHIN

by

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Arbacia eggs have been much studied because they are animal cells which can be transformed, by fertilization, from resting cells to dividing cells, the cleavage proceeding synchronously among the members of a large population. The relation of metabolic events to fertilization and cleavage has received extensive attention[1,2,3]. The eggs contain a number of the glycolytic enzymes: hexokinase, isomerase, phosphofructokinase, aldolase, and oxidizing enzyme[4,5]; they contain the enzymes for oxidation of glucose-6-phosphate via the TPN shunt[5]; they also have particulate components capable of oxidative phosphorylation[6].

The importance of aerobic pathways has been indicated by previous experiments. Fertilized *Arbacia* eggs can cleave only under aerobic conditions[3,7,8], and form almost no lactate either aerobically or anaerobically[9,10]. The enzymes for oxidation of glucose-6-phosphate via the TPN shunt are in great excess relative to those for disposal of glucose-6-phosphate via the glycolytic pathway[5]. In view of this situation, it was of interest to study the oxidation of glucose-1-^{14}C, glucose-2-^{14}C, and glucose-6-^{14}C by the intact, living *Arbacia* eggs. The results are in general accord with the view that glucose is degraded more rapidly via the TPN shunt than via the aldolase step in unfertilized or just fertilized *Arbacia* eggs, with the glycolytic pathway becoming quantitatively more important as development proceeds.

EXPERIMENTAL METHODS AND MATERIALS

Preparation and handling of eggs

The experiments were carried out at Woods Hole during July and August, 1955. Eggs were obtained and handled as previously described[9]. Glucose oxidation was measured for a 2 hour period at three stages in development: (a) unfertilized; (b) beginning 25 minutes after fertilization, at which point the sperm aster stage had been reached and the mitotic processes of the egg begun; (c) beginning 24 hours after fertilization and development at 25°C, when the eggs had reached the pluteus stage and were actively swimming embryos[11]. Eggs or plutei were incubated in Warburg vessels at 25°C. The main compartment received 4 ml of sea water containing 2% eggs by volume; center well, 0.5 ml 0.3 *N* NaOH; side arm, 0.5 ml 0.1 *N* HCl. The vessels were shaken at 65 cycles per minute at an amplitude of 4 cm[9]. At the end of the incubation period, the HCl was tipped from the side arm into the main compartment and shaking was continued for 60 minutes to permit collection of all CO_2 in the center well. The various fractions were collected as described below.

Preparations of fractions

The contents of the center well were transferred quantitatively to a centrifuge tube, and the following additions were made: 1.6 ml of 0.1 *M* Na_2CO_3, to provide carrier carbonate; 0.5 ml of 0.5 *M* NH_4Cl, and 1.0 ml of 0.5 *M* $BaCl_2$. The $BaCO_3$ was collected on paper as a plate 2 cm in diameter, washed 3 times each with water, acetone, and ether, and dried in air at 50°C; the total sample weighed 75 mg, the paper alone 38 mg.

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The contents of the main compartment were frozen to be analysed when convenient. The acid-insoluble fraction, the barium salts insoluble in water, and the barium salts insoluble in 80% ethanol were later obtained by the conventional method[12]. After estimation of their radioactivity, the barium salts were dissolved in 0.2 *N* HCl and the barium was removed on Dowex-50. The solutions were concentrated and subjected to two-dimensional paper chromatography by the method of BANDURSKI AND AXELROD[13], employing first the acid solvent and then the alkaline solvent. Under these conditions, glucose-6-phosphate had R_F values of approximately 0.65 in the acid and 0.27 in the alkaline solvent. Ribose-5-phosphate accompanied the glucose-6-phosphate closely and was not clearly separable from it.

Radioactive glucose: counting, and expression of results

Samples of glucose-^{14}C labeled in the 1,2, or 6-positions were purchased from the National Bureau of Standards through Dr. H. S. ISBELL. The total glucose concentration in the incubation medium was 0.0006 *M* in every experiment. Each radioactive sugar was adjusted, by addition of the appropriate amount of non-labeled glucose, so that the contents of one incubation flask, 4 ml of 2% egg suspension, contained 644,000 c.p.m. at the beginning of incubation. This is equivalent to a specific activity of 270,000 c.p.m. per micromole of glucose under the counting conditions used.

Counting was carried out with a Nuclear Instrument and Chemical Corporation gas tube (Model D46A) using Q-gas (98.6% helium – 1.4% methane). This tube had an efficiency of about 55% and a background of about 25 c.p.m. under the conditions used. The counts are corrected for self-absorption and coincidence loss.

All results are expressed in terms of the changes in a *single incubation flask* for a 2 hour period at 20°C. For comparison with measurements elsewhere, the following data may be useful[9]: Each flask contained the equivalent of 80 c.mm of unfertilized eggs, having a wet weight of 88 mg; the dry weight was 21 mg, of which 14 mg was protein. There were, on the average, 380,000 eggs per flask.

RESULTS

Oxidation of radioactive glucose to carbon dioxide

Values for the radioactivity collected as CO_2 when variously labeled glucose samples were used as substrates are given in Table I. The ratios of $^{14}CO_2$ from glucose-2-^{14}C to that from glucose-1-^{14}C, and from glucose-6-^{14}C to glucose-1-^{14}C were, respectively:

TABLE I

TOTAL RADIOACTIVITY RECOVERED AS $BaCO_3$ (FROM RESPIRATORY CO_2) AFTER 2 HOURS OF INCUBATION OF *Arbacia* EGGS OR EMBRYOS WITH GLUCOSE-1-^{14}C, GLUCOSE-2-^{14}C OR GLUCOSE-6-^{14}C

For details of technique and assay of radioactivity, see EXPERIMENTAL METHODS

Expt. No.	*Date 1955*	*State of egg development at start of incubation*	*O_2 consumed*	*Total c.p.m. in $BaCO_3$ from labeled glucose*				
				glucose-1-^{14}C	*glucose-2-^{14}C*	*$\frac{g\text{-}2\text{-}^{14}C}{g\text{-}1\text{-}^{14}C}$*	*glucose-6-^{14}C*	*$\frac{g\text{-}6\text{-}^{14}C}{g\text{-}1\text{-}^{14}C}$*
			c.mm					
15W	7–20	Unfertilized	10	3500	1100	0.31	320	0.09
16W	7–21	Unfertilized	18	4400	1300	0.30	260	0.06
20W	7–26	Unfertilized	12	5900	1900	0.32	340	0.06
		Means				0.31		0.07
11W	7–13	Fertilized 25 min	55	1200*	590	0.49	160	0.13
12W	7–14	Fertilized 25 min	51	1600	810	0.52	150	0.09
16W	7–21	Fertilized 25 min	56	3400	1700	0.50	350	0.10
19W	7–25	Fertilized 25 min	55	3400	2000	0.58	570	0.17
		Means				0.52		0.12
17W	7–22	24 h embryos	136	40900	13200	0.32	8300	0.20
21W	7–28	24 h embryos	140	52200	24100	0.46	17900	0.34
28W	8–12	24 h embryos	122	39600	19400	0.49	11900	0.30
		Means				0.42		0.28

* Values for unfertilized and fertilized fractions cannot be quantitatively compared: only values obtained on a single batch of eggs with the variously labeled glucose samples (same line of table) are directly comparable.

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for unfertilized eggs, 0.31 and 0.07; for eggs fertilized 25 minutes before the start of incubation, 0.52 and 0.13; for 24 hour embryos, 0.42 and 0.28. The significance of these ratios is discussed below.

Radioactivity in fractions other than carbon dioxide

Values for the radioactivity found in fractions other than $^{14}CO_2$ are given in Table II. The counts found in the various fractions are directly comparable for experiments having the same experimental number in Tables I and II.

There are a number of findings of interest. First, the radioactivity incorporated into the acid-insoluble fraction, containing nucleoproteins and a trace of lipid, does not differ significantly with the labeling of the glucose. This suggests that the incorporation of pentose into this fraction is relatively slow as compared to the randomizing of positions associated with recycling of intermediates. Also, there is no evidence that the radioactivity entering this fraction increases substantially as cleavage is initiated; if anything, the incorporation is somewhat less, the precise value being difficult to determine because of differences in permeability and in pool sizes between unfertilized and fertilized eggs. It seems clear, however, that the changes in nucleoprotein associated with initiation of mitotic processes after fertilization are associated primarily with that already available and not with synthesis *de novo* of the pentose components from hexose.

Second, the radioactivity incorporated into the individual or combined barium fractions does not differ significantly with the labeling of the glucose, although the pentose phosphate formed directly from glucose-6-^{14}C should be radioactive while that from glucose-1-^{14}C should not. The single possible exception is in the barium salts insoluble in ethanol from 24 hour embryos, where the radioactivity from glucose-1-^{14}C is less than from the other glucose samples. This difference apparently arises from the fact that oxidative tranformation of carbon 1 to $^{14}CO_2$ is quantitatively very large in the embryos, leaving a significant amount of unlabeled pentose phosphate in this fraction. The results are in general consistent with the view that hexose phosphates represent the major components of the pool, and that recycling of the products of

TABLE II

TOTAL RADIOACTIVITY RECOVERED IN VARIOUS FRACTIONS FROM *Arbacia* EGGS AND MEDIUM AFTER 2 HOURS OF INCUBATION WITH GLUCOSE-1-C^{14} GLUCOSE-2-^{14}C, OR GLUCOSE-6-^{14}C

Each value is the average of two experiments. The counts may be compared with those for $BaCO_3$ in Table I. For preparation of fractions and assay of radioactivity, see EXPERIMENTAL METHODS

Expt. No.	*State of egg development at start of incubation*	*Egg fraction*	*Total c.p.m. in fraction after incubation of eggs with:*		
			glucose-1-^{14}C	*glucose-2-^{14}C*	*glucose-6-^{14}C*
16W		Acid insoluble	1000	1300	1100
and	Unfertilized	Ba salts – H_2O insol.	3400	4900	4700
20W		Ba salts – EtOH insol.	5200	7200	6100
16W		Acid insoluble	300*	330	410
and	Fertilized 25 min	Ba salts – H_2O insol.	1300	2000	1600
19W		Ba salts – EtOH insol.	3000	5000	5000
17W		Acid insoluble	18000	26000	24000
and	24 h embryos	Ba salts – H_2O insol.	28000	30000	29000
21W		Ba salts – EtOH insol.	25000	40000	42000

* See footnote to Table I.

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glucose-6-phosphate oxidation is rapid relative to the initial decarboxylation by 6-phosphogluconate dehydrogenase. Another experimental observation is consistent with this suggestion: the only clear spots obtained when the barium salts insoluble in ethanol were chromatographed by the method of BANDURSKI AND AXELROD[13] had R_F values of 0.62–0.68 in the acid solvent and 0.27–0.31 in that alkaline solvent, corresponding to the mobilities of hexose phosphates under the present conditions (paper exposed first to the acid and then to the alkaline solvent). Spots with such R_F values were obtained by chromatography of the redissolved, de-cationized barium-ethanol insoluble fractions from unfertilized eggs, 25 minute fertilized eggs, and 24 hour embryos.

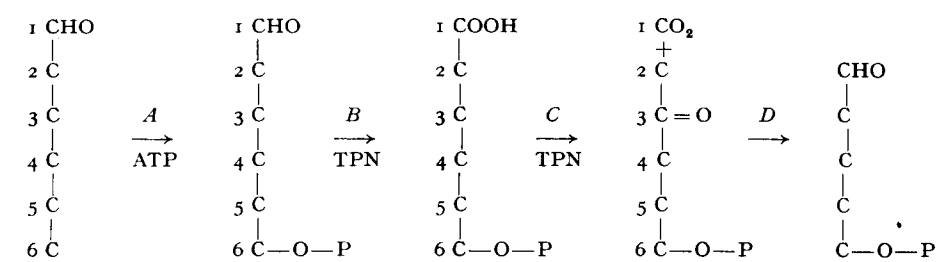
A third point of some interest is the rate of formation of CO_2, and of incorporation of radioactivity into the acid insoluble fraction, relative to the size of the pool in the barium salt fractions insoluble in water and alcohol. For unfertilized eggs, the ratios of counts in CO_2 and in the acid-insoluble fractions relative to those in the barium-salt fractions from glucose-1-^{14}C were, respectively (Expt. 16W): 4400/8600 = 0.51, and 1000/8600 = 0.11; for eggs fertilized 25 minutes, 3400/4300 = 0.79, and 300/4300 = 0.07; for 24 hour embryos (Expt. 21W), 52000/53000 = 0.98, and 18000/53000 = 0.34. There is thus some evidence that immediately upon fertilization the pathway toward CO_2 from a given pool becomes quantitatively more important, while that toward nucleic acids does not. Still later, at the 24 hour stage, both pathways are more efficiently used relative to the pool.

DISCUSSION

The results of Table I are consistent with the concurrent operation of two pathways for carbohydrate oxidation in *Arbacia* eggs. The one pathway includes the Embden-Meyerhof-Warburg-Cori glycolytic scheme and the tricarboxylic acid cycle: glucose → glucose-6-phosphate → fructose-6-phosphate → fructose-1,6-diphosphate → triose-phosphate → phosphoglyceric acid → via phosphopyruvate and tricarboxylic acid cycle to CO_2. Each molecule of glucose oxidized completely via this pathway would give the same amount of $^{14}CO_2$ whether labeled in the 1-, 2-, or 6- positions. The enzymes for this pathway are present in these eggs, the steps from fructose diphosphate to phosphoglyceric acid being limiting[5].

The second pathway is the Warburg-Dickens TPN shunt. The principal steps as summarized[14] from results on bacterial[15–18], higher plant[19], and animal[19–25] cells are:

Pathway for glucose oxidation via TPN Shunt



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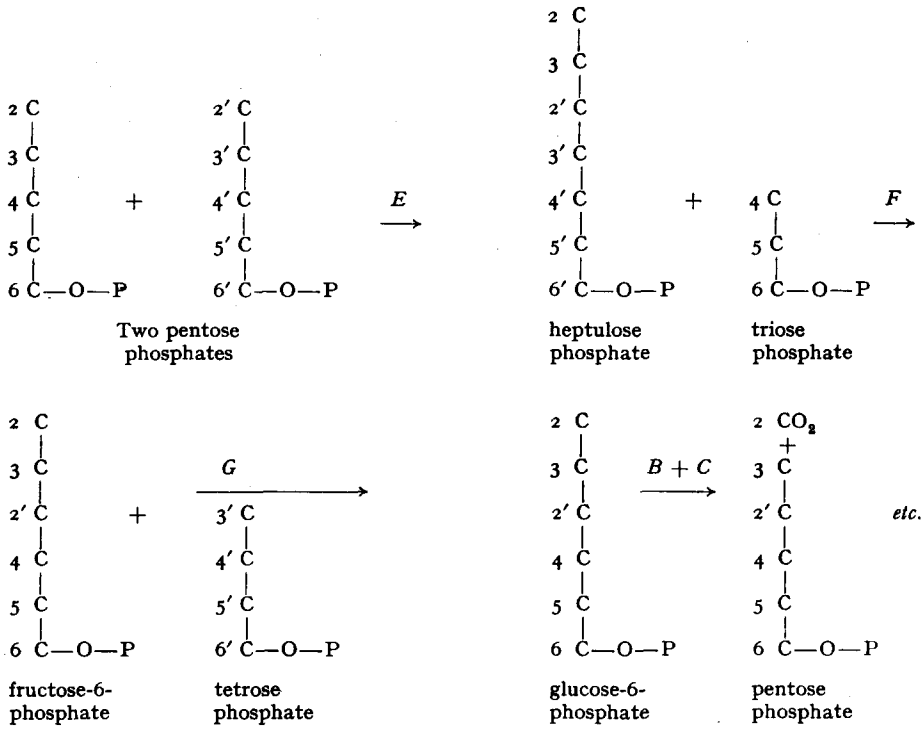

The reactions are: *A*, hexokinase; *B*, glucose-6-phosphate dehydrogenase; *C*, 6-phosphogluconate dehydrogenase; *D*, pentose phosphate isomerase; *E*, transketolase; *F*, transaldolase; *G*, hexose phosphate isomerase.

According to this second pathway, carbon 6 appears as CO_2 only if triose phosphate re-enters the glycolytic scheme or if tetrose phosphate is further oxidized. Carbon 1 appears as CO_2 from step *C*, carbon 2 as CO_2 from the repetition of step *C* after a complete cycle; it should be noticed that appearance of $^{14}CO_2$ from carbon 2 represents oxidation of two glucose molecules, while CO_2 from carbon 1 represents oxidation of only one glucose. The ratio of $^{14}CO_2$ from glucose-2-^{14}C to that from glucose-1-^{14}C is actually found to be 0.31 in the unfertilized, and 0.52 in fertilized eggs.

In general, the observations of the present and previous papers[4,5] indicate that glucose oxidation during the early stages of cleavage of the *Arbacia* egg proceeds predominantly via the TPN shunt. The argument is as follows: First, glucose is utilized by egg extracts only when ATP is supplied[4]; glucose-6-phosphate is found as a product in both egg extracts and in intact eggs, indicating that glucose use proceeds via a phosphorylating pathway. Second, the amount of glucose-6-phosphate which can be oxidized in egg extracts via the TPN shunt is approximately twenty times that which can be oxidized via the DPN step in the glycolytic pathway. Third, the $^{14}CO_2$ formed by the intact eggs from glucose-1-^{14}C is 10–16 times that from glucose-6-^{14}C under the same conditions (Table I), agreeing well with the prediction from the enzyme content of the eggs. Fourth, the $^{14}CO_2$ production from glucose-2-^{14}C, being intermediate between that from glucose-1-^{14}C and glucose-6-^{14}C, is consistent with the operation of the two TPN oxidation steps followed by transketolase and transaldolase

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reactions as postulated for bacteria. The hexose phosphates are the only phosphorylated intermediates yet identified among the metabolic products of intact *Arbacia* eggs and embryos.

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SUMMARY

1. The $^{14}CO_2$ produced from glucose-1-^{14}C, glucose-2-^{14}C, or glucose-6-^{14}C by *Arbacia* eggs and embryos has been measured for a 2 hour period. The ratios of $^{14}CO_2$ from glucose-2-^{14}C to that from glucose-1-^{14}C and from glucose-6-^{14}C to glucose-1-^{14}C were, respectively: for unfertilized eggs, 0.31 and 0.07; for eggs fertilized 25 minutes at start of incubation, 0.52 and 0.13; for 24 hours embryos, 0.42 and 0.28. These observations, in conjunction with those of previous papers, indicate that glucose oxidation during the early stages of cleavage of the *Arbacia* egg is predominantly via the TPN shunt, with the glycolytic pathway becoming quantitatively more important as development proceeds.

2. Incorporation of radioactivity into the acid-insoluble and the barium fractions insoluble in water and insoluble in alcohol was also determined. It was found that rate of incorporation into the acid-insoluble fraction, containing nucleoprotein, was not increased just after fertilization, but did increase very markedly in later development.

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