OXIDATIVE PHOSPHORYLATION BY A CELL-FREE PARTICULATE SYSTEM FROM UNFERTILIZED ARBACIA EGGS

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Previous studies on the mechanism of energy generation and transfer in the egg of *Arbacia punctulata* have shown that the following substances concerned with oxidative disposal of foodstuffs are present: Cytochrome oxidase (1), flavindinucleotide (2), diphosphopyridine nucleotide (3), and diphosphothiamine (4, 5). The eggs can also oxidize pyruvate, the rate being greater for fertilized than for unfertilized eggs (4, 5).

The present paper deals with the preparation from these eggs of a cell-free particulate system which can utilize energy derived from oxidation of oxalacetate, α-ketoglutarate, or succinate for phosphorylation.

Experimental Methods

Particulate systems capable of aerobic phosphorylation have been prepared from mammalian tissues by a number of investigators (6–8). The essential steps in all methods are: homogenization of tissue in an isotonic or slightly hypotonic medium, differential centrifugation and washing of the resultant tissue particles, and measurement of oxygen consumption and inorganic phosphate disappearance in a medium containing adenosine mono- or diphosphate, plus hexokinase to effect transfer of the high-energy phosphate bonds generated by oxidation to a stable form such as glucose phosphate or fructose phosphate.

Preliminary experiments during the 1948 season showed that the methods used to prepare cell-free oxidative phosphorylating systems from mammalian tissues could not be used for the same purpose in the case of *Arbacia* and other marine eggs, owing first to the breakdown of echinochrome granules and second to the tendency of the homogenized material to become more acid than pH 7.0 to 7.4, which is the optimum for the mammalian phosphorylating systems so far studied, and third to the fact that the egg cytoplasm is osmotically equivalent to sea water, which has a substantially higher ionic concentration than the media employed for homogenization of mammalian tissues.

Accordingly, a homogenization medium designed to minimize these difficulties was worked out: Citrate was included to stabilize the echinochrome granules and make it possible to remove them with minimal loss of echinochrome (9, 10), a non-toxic buffer was included to maintain the pH of the homogenate at the level desired, and the concentration of KCl was increased from the 0.1 M used in the mammalian system to 0.4 M. Up to the present time the optimum procedure has been as follows:—

Unfertilized *Arbacia* eggs were washed once with 100 volumes of sea water, then three times with the same volume of a solution which was 0.5 M in NaCl, 0.02 M in

$NaHCO_3$, and adjusted to pH 7.9. Buffering to pH 7.9 was important, as eggs washed with unbuffered solutions developed acid products which led to homogenates which were unable to induce phosphorylation.

After the last wash, when the eggs had settled, the supernatant fluid was siphoned off, and the eggs were transferred to a 15 ml. centrifuge tube which had previously been chilled to 5°C. The eggs were packed by centrifuging at 1000 *g* for 30 seconds at 5°C. From this point on, all operations except the final incubation step were performed at 5°C.

The packed eggs were then suspended in 5 volumes of homogenizing solution. This egg suspension was then alternately drawn into and forced out of a syringe three times through a No. 18 needle, a procedure which caused disintegration of 95 per cent or more of the eggs. The homogenizing solution was 0.4 M in potassium chloride, 0.01 M in sodium citrate, 0.05 M in glycylglycine, and was adjusted to pH 7.4.

The suspension of particles resulting from the homogenization was centrifuged in the angle head of a refrigerated centrifuge at 5°C. for 8 minutes at 1000 *g* (2400 R.P.M.; average radius, 14.5 cm.). The centrifuging produced three layers: a red bottom layer containing echinochrome granules and a few unfragmented eggs, a yellow-white middle yolk layer, and a hazy pinkish white supernatant layer which was used as the cell-free particulate system. The bottom two layers were discarded. An attempt to fractionate the supernatant layer further was postponed on account of the scarcity of eggs. The fraction stainable with Janus green (5 per cent of total egg volume), which is usually referred to by Harvey (11) as the mitochondrial fraction, was discarded with the yolk layer.

The measurements of oxygen uptake and inorganic phosphate disappearance were carried out as follows: All reagents[1] of the test system (Table I) were put into chilled Warburg vessels; the 0.6 ml. of the particulate system (equivalent to 0.1 ml. packed eggs) was added last. The flasks were quickly attached to the manometers, transferred to a bath at 20°C., and incubated with shaking at 53 cycles per minute and 7 cm. amplitude and oxygen consumption recorded for 60 minutes, following a preliminary 5 minute equilibration period. At the end of the incubation period the flasks were removed. 2.6 ml. chilled 11 per cent trichloracetic acid was added to a 1 ml. aliquot of the incubation mixture and used for determination of inorganic phosphorus according to Fiske and SubbaRow. The rest of the flask contents were used for determination of the final pH by glass electrode. The contents of parallel control flasks were analyzed at the beginning of the incubation period to determine the amount of inorganic phosphorus initially available. The initial apparent inorganic phosphorus content of these flasks averaged 175 micrograms, of which 96 micrograms were added as phosphate buffer; the source of the additional inorganic phosphorus is undefined and requires further investigation.

EXPERIMENTAL RESULTS

Relation of Phosphorylation to pH.—The activity of the phosphorylating system was found to be sharply dependent on pH. Maximal phosphorylation

[1] Experiments have been started to determine whether certain of these components are necessary and to find the optimum concentrations, but lack of good material has necessitated the postponement of further work on this subject until next season.

with α-ketoglutarate as substrate was obtained when the pH of the cell-free particulate system at the end of the homogenization was 7.2–7.3, corresponding to a final pH of 7.0–7.1 after completion of incubation (Table II).

Dependence of Phosphorylation on Substrate for Oxidation.—In mammalian tissue the highest efficiency of phosphorylation, as measured by the ratio of phosphorus esterified to oxygen consumed, has been observed when α-ketoglutarate was used as substrate (6). It was of interest to determine whether

TABLE I

Composition of Incubation Medium for Measurement of Oxygen Consumption and Phosphate Removal by Cell-Free Particulate System from Unfertilized Eggs of Arbacia punctulata

Reagent	Concentration stock solution	Volume stock solution per flask	Final concentration in flask
	mM per l.	*ml.*	*mM per l.*
Magnesium chloride	200	0.2	14
Sodium fluoride	500	0.2	36
Potassium chloride	—	—	57*
Sodium citrate	—	—	1.4*
Sorensen phosphate, pH 7.2	20	0.2	1.4
Glycylglycine, pH 7.4	250	0.2	27*
Glucose	500	0.1	36
Nicotinamide, pH 7.1	112	0.1	3.6
Diphosphopyridine nucleotide‡	5.6	0.1	0.2
Cytochrome C‡	0.2	0.2	0.014
Adenosine triphosphate‡	10	0.2	0.7
α-Ketoglutarate,‡ pH 7.2	140	0.2	10

The above components plus 0.2 ml. yeast hexokinase solution were mixed in the main compartment of a chilled Warburg flask and the total volume was made to 2.2 ml. with water. Solutions of other reagents, such as substituted phenols, could be added in place of the water. Finally, 0.6 ml. of the cell-free phosphorylating system was added.

* This value includes the reagent introduced with the homogenizing solution.

‡ Source of reagents: diphosphopyridine nucleotide, Schwartz Laboratories; cytochrome C, Sigma Chemical Co. (no preservative); adenosine triphosphate, sodium salt, Rohm and Haas; α-ketoglutaric acid, National Biochemicals Corporation.

or not oxidative phosphorylation by the cell-free particulate system from *Arbacia* eggs could be demonstrated to be dependent on presence of α-ketoglutarate, or other substrates of the so called tricarboxylic acid cycle. This was found to be the case. In a typical experiment (Table III), the net amount of phosphorus esterified was (in micrograms per flask): no substrate, 13; with α-ketoglutarate, 101; with oxalacetate, 91; with succinate, 53; a second typical experiment is given to illustrate the reproducibility of the measurements. These data were obtained with a phosphorylating system made by carrying out the homogenization at pH 6.9. At higher pH values, the rate of phosphorylation without added substrate is somewhat higher. In ten experiments

with homogenates of pH 7.1 to 7.3, the fraction of available phosphorus esterified was 38 per cent without substrate and 57 per cent with α-ketoglutarate.

TABLE II

Relation of the pH of Homogenizing Solution to Phosphorylation by Cell-Free Particulate System of Unfertilized Arbacia Eggs

The initial inorganic phosphorus was 175 micrograms per flask. The other contents of each flask were as shown in Table I.

Homogenizing solution	No. of experiments	pH of particulate suspension before addition to flask	pH of flask contents after 60 min. incubation	Average per cent of initial inorganic phosphorus disappearing
Unbuffered	1	6.5	6.6	2
Buffered with glycylglycine	1	6.8	6.8	21
	3	7.0	6.8	30
	2	7.1	6.9	43
	4	7.2	7.0	61
	5	7.3	7.1	61
	2	7.4	7.2	51
	2	7.5	7.1	50

TABLE III

Effect of Substrate on Oxygen Consumption and Phosphorylation by the Crude Cell-Free Particulate System Obtained from 0.1 Ml. Unfertilized Arbacia Eggs

The substrates were added in an initial concentration of 10 mM per liter, and the initial available inorganic phosphorus was 175 micrograms.

Date	Substrate added	Oxygen consumption	Inorganic phosphorus disappearing
		c.mm. per hr.	*μg. per hr.*
7-18-49	None	24	13
	α-Ketoglutarate	34	101
	Oxalacetate	30	91
	Succinate	31	53
7-28-49	None	26	4
	α-Ketoglutarate	40	97

These data do not, for two reasons, permit calculation of significant values of the P/O ratio, atoms P esterified/atoms oxygen used. In the first place, there was a substantial oxygen consumption in absence of added substrate, which was only in part coupled with phosphorylation. Experimental procedures to eliminate this baseline oxygen consumption could not be worked out because of the shortage of eggs. In the second place the net amount of inorganic phosphate esterified represents the difference between phosphate uptake and phos-

phate liberation, as shown by data presented in the following paper. When phosphorylation is suppressed, there is a liberation of about 10 to 30 micrograms inorganic phosphorus per flask from some organic form.

Though further investigation will be required to establish absolute values for the rate of P uptake and for the P/O ratio, it is of interest to compare the oxygen consumption, phosphate uptake, and P/O ratio of the cell-free particulate system from *Arbacia* eggs (Table III) with those published, while the present investigation was in progress, for *Strongylocentrotus drobachiensis* by Lindberg and Ernster (12).

With α-ketoglutarate as substrate the values for *Arbacia* (per gram wet eggs per hour) are: oxygen consumption, 340 c.mm.; P uptake, 1010 micrograms, P/O ratio, 1.07. The corresponding approximate values for the cell-free preparation from *Strongylocentrotus* (calculated from Lindberg and Ernster's Table III, Experiment 5, run at 6°C.) are: oxygen consumption, 114 c.mm.; P uptake, 210 micrograms; P/O ratio, 0.67. It is likely that the P/O ratios could in both instances be improved by elimination of the fraction of the oxygen consumption which is not coupled with phosphorylation.

The oxygen consumption of the homogenate is substantially larger than that of the unfertilized eggs from which it is derived. With α-ketoglutarate as substrate the oxygen uptake of the cell-free material derived from 1 gm. wet eggs averaged for ten experiments 340 c.mm. per hour at 20°C. This should be compared with oxygen consumption of intact eggs, 1 gm. wet weight of which consumed in the case of unfertilized eggs approximately 70 c.mm. and in the case of fertilized eggs approximately 310 c.mm. per hour at 20°C.

Earlier attempts to demonstrate succinate oxidation by cell-free homogenates of *Arbacia* eggs were unsuccessful (1, 13, 14). It was suspected that this might be due to the action of echinochrome, which in the oxidized form is a substituted naphthoquinone (15) capable of oxidizing sulfhydryl groups which are known to be essential for succinoxidase activity (16). Crane and Keltch (17) effected a partial elimination of echinochrome and in a preceding portion of this communication a method has been reported whereby the echinochrome content of the cell-free homogenate has been reduced to a minimum. As a result it has been possible to demonstrate that addition of succinate enhances oxygen consumption and phosphate esterification by a cell-free system derived from *Arbacia* eggs. Moreover, inhibition of succinate oxidation in heart muscle through a specific action of certain naphthoquinones on a respiratory step between cytochrome *c* and cytochrome *b* has been postulated (18). The bearing of these observations upon the mechanism by which echinochrome suppresses the succinoxidase activity of the eggs merits further investigation.

Miscellaneous Observations.—In an effort to achieve optimum conditions for phosphorylation, the make-up of the solution used to wash the eggs was varied, using a constant composition for the homogenization medium, and *vice versa*.

For washing, solutions of NaCl or of KCl, and mixtures of the two, were used at total concentrations of 0.4 and 0.5 M. Optimum results were obtained with 0.5 M NaCl. For homogenization, solutions of 0.4 M or 0.5 M KCl, of 0.4 M or 0.5 M NaCl, mixtures of NaCl and KCl of 0.5 M total concentration, or 0.9 M sucrose were tried. Each solution also contained 0.01 M citrate and 0.05 M glycylglycine. That containing 0.4 M KCl gave the optimum phosphorylation so far observed.

Certain homogenates were unable to esterify phosphorus; these were brown instead of the customary pinkish white at the end of the differential centrifugation. The brown color was not a function of the pH, as both the pinkish white active preparations and the brown inactive preparations were obtained in the range pH 6.9–7.2. It was observed that the brown homogenates came invariably from eggs of urchins which had been stored for some days inside the laboratory or of urchins which had nearly empty ovaries at the time of sacrifice.

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SUMMARY

1. A cell-free particulate system capable of effecting oxidative phosphorylation has been prepared from unfertilized eggs of *Arbacia punctulata*.

A substantial increase in phosphorylation can be produced by addition of α-ketoglutarate, oxalacetate, or succinate, the magnitude of the increase being greatest with α-ketoglutarate.

The activity of the phosphorylating system is sharply dependent on maintenance of a comparatively narrow pH range during both the preparation of the particulate system and its subsequent incubation with oxidizable substrate.

2. The maximum oxygen consumption of the cell-free particulate system derived from a given weight of unfertilized eggs is about three times that of the same weight of intact unfertilized eggs and approximately the same as that of an equal weight of fertilized eggs.

3. The data indicate that generation of high-energy phosphate bonds in the *Arbacia* egg is coupled, as it is in mammalian liver or kidney, with the functioning of the tricarboxylic acid cycle.

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