DINITROCRESOL AND PHOSPHATE STIMULATION OF THE OXYGEN CONSUMPTION OF A CELL-FREE OXIDATIVE SYSTEM OBTAINED FROM SEA URCHIN EGGS

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Various substituted phenols have been found to produce, in low concentrations, a two to three hundred per cent increase in the oxygen consumption of sea urchin eggs. As the concentration of a given substituted phenol is increased, the rate of oxygen consumption passes through an optimum and then declines to or below the normal rate. At concentrations larger than that for optimum oxygen consumption these agents produce a reversible block to the cell division of the fertilized eggs (1–3).

The mechanism of the two phases of the substituted phenol action, namely, the stimulation of oxygen consumption on the one hand and the inhibition of oxygen consumption and reversible block to cell division on the other hand, has received intensive study over the past several years. The analysis of the first phase of the effect has been hampered by the fact that the oxidative stimulation has been obtainable only with intact cells. Efforts to account for the second phase of the effect have revealed that the substituted phenols, when used at adequate concentrations, inhibit the following enzyme systems and cellular activities: *d*-amino acid oxidase (a flavoprotein) (4), *Zwischenferment* and cytochrome reductase (5), growth (6), and phosphate uptake by yeast cells (7), nitrogen assimilation (8), formation of adaptive enzymes (9), and certain other synthetic reactions (10).

Recently, Loomis and Lipmann (11), using a cell-free kidney granule preparation of the type previously employed by Green, Loomis, and Auerbach (12), have reported observations which bear on both phases of the substituted phenol effect. They found that 2,4-dinitrophenol at a concentration of 8×10^{-5} M substantially increased the oxygen consumption of this cell-free preparation. However, when the 2,4-dinitrophenol was used at a higher concentration, 1.8×10^{-4} M, the oxygen consumption was reduced to a normal level and inhibition of aerobic phosphorylation was observed. This observation was of especial interest in its bearing on the mechanism of action of substituted phenols for two reasons: first, it was the first case in which a stimulation of oxygen consumption in a cell-free system had been obtained by the use of substituted phenols; and, second, the inhibition of phosphorylation associated with a decrease in oxygen consumption may provide an explanation for the above mentioned inhibition of oxidative synthetic reactions which are dependent, directly or indirectly, on aerobic phosphorylation (13).

In view of the broad implications of the observations of Loomis and Lipmann, it seemed of interest to see whether either or both phases of the effects on the kidney granule preparation could be repeated with an analogous cell-free oxidative system obtained from the sea urchin egg, where there is the possibility of correlating observations on oxidative synthetic reactions with the mitotic activities of the egg.

The present paper deals with the study of effects of dinitrocresol and phosphate on oxygen consumption of a cell-free oxidative system obtained from the eggs of the sea urchin, *Arbacia punctulata*, which was carried out during the summer of 1948.

Experimental Methods

Mature *Arbacia* eggs were obtained at Woods Hole during July and August, 1948. They were shed, washed, and, where necessary, fertilized as described in previous papers from this laboratory (2, 14).

Preparation of Cell-Free Oxidative System from Arbacia Eggs.—While Lipmann and Loomis, in preparing a cell-free oxidative system from mammalian kidney, had at their disposal the information as to how such a cyclophorase system might be obtained (Green, Loomis, and Auerbach, 12), at the time that this work was undertaken no information was available as to how such a system could be obtained from sea urchin eggs. The cyclophorase system obtained from kidney was found to be extremely sensitive to the action of chemical and physical agents. It was destroyed by freezing, drying, or exposure to concentrations of salt greater than 0.1 N or solvents such as alcohol and acetone in concentrations greater than 10 per cent by volume or acidity below pH 4 or alkalinity above pH 10. The procedure employed by Green, Loomis, and Auerbach in obtaining a cyclophorase system from mammalian kidney was not found to be applicable in the case of the *Arbacia* eggs. After various procedures had been tried the following method was finally adopted:—

50 cc. of a 2 per cent suspension of unfertilized eggs in sea water was centrifuged at 1000 *g* for 2 minutes. The supernatant fluid was poured off and the packed eggs were chilled to about 5°C. in an ice water bath and all subsequent operations prior to the beginning of the manometric observations were carried out with ice cold solutions and containers. The packed eggs were admixed with 3 cc. of a solution containing 0.17 M KCl and 0.23 M sodium citrate (pH 7.7).[1] The resulting dense egg suspension was then alternately drawn into and forced out of a syringe through a No. 18 needle three times; this procedure broke up 95 per cent or more of the eggs.

The suspension of egg fragments was then further diluted with 2 cc. of 0.5 M KCl and centrifuged at 2000 *g* for 10 minutes in chilled cups. The centrifuging produced a hazy pinkish white supernatant layer, which constituted the cell-free oxidative system and was used as described below, and two layers of sediment which were discarded, a dark red layer at the bottom of the tube[2] and a yellow yolk layer.

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[1] In later experiments the pH of the KCl-citrate was adjusted to 7.0 with no difference in the results.

[2] This dark red layer contained some egg cells which were not fragmented by the

The method adopted in securing a cell-free oxidative system from fertilized eggs differed slightly from that employed with unfertilized eggs. Fifteen minutes after the eggs had been fertilized, 50 cc. of a 2 per cent suspension of the eggs was centrifuged at 1000 *g* for 2 minutes. The supernatant liquid was poured off and 3 cc. of a solution containing 0.17 M KCl and 0.23 M sodium citrate was added to the chilled, packed eggs. The egg suspension was allowed to stand for 3 minutes; the fertilized eggs were then broken up in the same manner as previously described for unfertilized eggs, except that the eggs were drawn into and forced from the syringe ten times. The suspension was diluted with 2 cc. 0.5 M KCl, centrifuged as described for unfertilized eggs, and the supernatant layer used as the cell-free oxidative system.

Measurement of Oxygen Consumption by the Cell-Free Oxidative System.—The main compartment of each Warburg flask contained the components shown in Table I, all of which had been held at 4°C. and were admixed in the Warburg vessel, the oxidative system being added last. The center well contained 0.2 cc. 10 per cent KOH.

After the flasks were filled they were attached to the manometers, placed in the bath at 20°C., and shaken at 60 cycles per minute with a 6 cm. amplitude. A 5 minute equilibration period was used prior to the beginning of the measurement.

EXPERIMENTAL RESULTS

Stimulation of Oxygen Consumption by 4,6-Dinitro-o-Cresol and/or Added Inorganic Phosphate.—The rates of oxygen consumption of cell-free oxidative systems obtained from both fertilized and unfertilized *Arbacia* eggs and set up as described in Table I, (A) alone, (B) with addition of dinitrocresol, (C) with addition of phosphate, and (D) with addition of both dinitrocresol and phosphate, are presented in Fig. 1.

With oxalacetate[3] as substrate a concentration of 6.4×10^{-5} M 4,6-dinitro-*o*-cresol (DNC) produced a marked stimulation of oxygen uptake by these cell-free oxidative systems, the stimulation being most marked during the period 30 to 60 minutes after addition of the reagent; with the preparation from unfertilized eggs, no stimulation was evident until 30 minutes after the measurements started. The maximum stimulation induced by the added DNC was approximately 50 per cent in both cases. It is of particular interest that the rate of oxygen uptake by these cell-free oxidative systems from either unfertilized[4] or

syringe treatment and the majority of the egg echinochrome in the form of unbroken pigment granules.

Preliminary experiments, in which the eggs were homogenized without removal of echinochrome, were unsuccessful. Accordingly, the above method of fragmentation of the eggs without destruction of pigment granules was employed. This procedure was first called to our attention by Dr. M. J. Kopac (15).

[3] Succinate, α-ketoglutarate, glutamate, and excess citrate were also found to be oxidized by the cell-free oxidative system, but oxalacetate gave the largest increase over the basal level without added substrate.

[4] In a single exploratory experiment, carried out in duplicate, the oxygen consumption of a cell-free oxidative system prepared from unfertilized eggs previously frozen

fertilized eggs is, in absence of added DNC, about twice that of the intact unfertilized eggs and half that of the intact fertilized eggs.

TABLE I

Contents of Main Compartment of Warburg Flasks

Reagents	Final concentration of reagents* in experiments			
	A	B	C	D
	mM/liter	*mM/liter*	*mM/liter*	*mM/liter*
KCl	360	360	360	360
$MgCl_2$	14	14	14	14
Glycyl glycine (pH 7.0)	3.6	3.6	3.6	3.6
Oxalacetate	2.9	2.9	2.9	2.9
Glucose	36	36	36	36
NaF	18	18	18	18
Cytochrome C‡	0.014	0.014	0.014	0.014
Adenosine triphosphate§	0.7	0.7	0.7	0.7
4,6-dinitro-*o*-cresol (pH 7)	—	0.064	—	0.064
Sorensen phosphate (pH 7.4)	—	—	0.7	0.7

These components plus 0.2 cc. of hexokinase‖ were admixed and the volume made up to 2.2 cc. with water. Then 0.6 cc. of the cell-free oxidative system was added last.¶

The total Mg^{++} plus Ca^{++} of eggs used approximated the total citrate added.

The cell-free oxidative system in each flask was equivalent to 100 c.mm. eggs or 450,000 to 500,000 eggs. This volume of eggs would consume at 20°C. about 8 c.mm. oxygen per hour in the unfertilized state and about 31 c.mm. per hour in the fertilized state.

* The actual reagents added were: 0.3 cc. 3.34 M KCl, 0.2 cc. 0.2 M $MgCl_2$, 0.2 cc. 0.05 M glycyl glycine, 0.2 cc. 0.04 M oxalacetate, 0.1 cc. 0.5 M glucose, 0.2 cc. 0.25 M NaF, 0.2 cc. 0.0002 M cytochrome C, 0.2 cc. 0.01 M adenosine triphosphate, 0.2 cc. 0.009 M 4,6-dinitro-*o*-cresol, 0.2 cc. 0.01 M Sorensen phosphate, hexokinase, and oxidative system as shown.

‡ Cytochrome C was "Injection cytochrome C" purchased from Wyeth Incorporated. This contained 10 mg. per cc. cytochrome C and was diluted on the basis of a molecular weight of 12,000. The preparation also contained 0.01 per cent sodium ethyl mercurithiosalicylate, which gave a final concentration of 4.8×10^{-6} M in each Warburg flask. A control experiment showed that the presence of this mercurial does not account for the absence of phosphorylation by the enzyme system.

§ Adenosine triphosphate was the tetrasodium salt purchased from Rohm and Haas, which is stated by the manufacturer to be 99 per cent pure.

‖ The hexokinase used was prepared according to Berger, Slein, Colowick, and Cori (16). The purification was carried through the alumina adsorption stage. The resultant product was diluted approximately 3:1 with water before use.

¶ The cell-free oxidative system contained 0.3 M KCl and 0.14 M citrate. Consequently, the final KCl concentration was raised from the above stated 0.36 M to 0.424 M and citrate was present at a concentration of 0.03 M.

at −20°C. was higher than that of a similar cell-free system obtained from the chilled but unfrozen eggs. The oxygen consumption of the system from the frozen eggs was not increased by addition of dinitrocresol or phosphate, but the oxygen consumption of the system from the unfrozen eggs was raised to that of the former by addition of either dinitrocresol or phosphate.

Added inorganic phosphate also produced a stimulation of oxygen consumption by both systems and the addition of DNC with phosphate produced only a limited further stimulation in the fertilized system and little or no further stimulation in the unfertilized. These differences may possibly be in part accounted for by the fact that the phosphate originally present in the two systems differed, being higher in the unfertilized than in the fertilized system, with the result

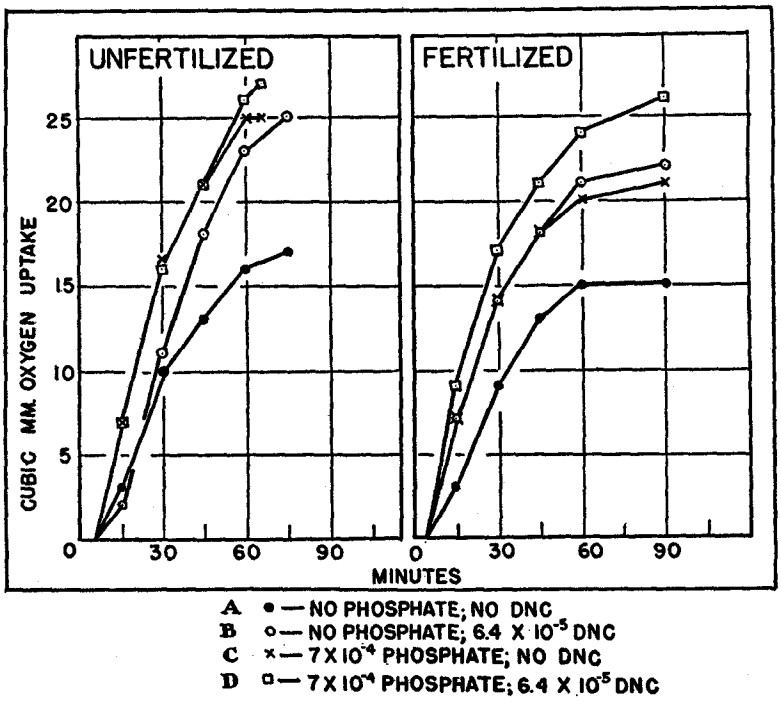

FIG. 1. Effect of 6.4×10^{-5} M 4,6-dinitro-*o*-cresol and/or 7×10^{-4} M inorganic phosphate upon oxygen consumption by cell-free oxidative systems from unfertilized and fertilized *Arbacia* eggs.

that in the fertilized system the phosphate added brought the total content of phosphate to a point between the initial and final levels of phosphate in the unfertilized system.

The stimulation of oxygen consumption by dinitrophenol or phosphate observed by Loomis and Lipman (11) with a cell-free particulate system from rabbit kidney has therefore been confirmed by the present observations with comparable cell-free systems prepared from both unfertilized and fertilized *Arbacia* eggs.

The major portion of the summer season at Woods Hole was expended in determining the conditions under which a cell-free oxidative system capable of

being stimulated by nitrophenols or phosphate could be obtained from *Arbacia* eggs. By the time that a method of obtaining such a system had been worked out the supply of available sea urchin eggs was exhausted and consequently it was necessary to postpone until next season experiments using different concentrations of dinitrophenol to determine whether there was an optimum point of oxygen consumption corresponding with the optimum points for the stimulation of the respiration of sea urchin eggs and of the Lipmann-Loomis oxidative phosphorylating system.

Preliminary Attempts to Obtain a Cell-Free, Aerobic, Phosphorylating System from Arbacia Eggs.—Attempts to determine conditions under which a cell-free, oxidative, phosphorylating system may be obtained from sea urchin eggs have thus far been unsuccessful, but will be resumed when experimental material is once more available. As stated above, no information was available in the literature as to how such a cell-free, phosphorylating system could be obtained from sea urchin or other marine eggs. The establishment of working conditions to produce such a system must necessarily precede the testing of the effect of substituted phenols on the phosphorylating mechanism.

In the experiments illustrated in Fig. 1 the ability of the system to transform inorganic phosphorus was also measured. No net disappearance of inorganic phosphorus was observed and the data are not being reported at present. As shown in Table I, the reaction mixtures used in the experiments presented in Fig. 1 contained glucose as an ultimate phosphate acceptor and yeast hexokinase to transfer phosphate from ATP to glucose. Other experiments were conducted in which succinate, α-ketoglutarate, glutamate, or excess citrate was substituted for oxalacetate as substrate. Also, arginine was introduced as a possible phosphate acceptor, but none of these procedures led to a recognizable phosphorylation.

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SUMMARY

1. A cell-free system capable of using oxygen with oxalacetate as substrate has been prepared from both unfertilized and fertilized sea urchin eggs. The oxygen uptake by this system is about twice that of an equivalent quantity of intact unfertilized eggs and half that of an equivalent quantity of intact fertilized eggs.

2. The oxygen consumption of this cell-free oxidative system can be stimulated by addition of suitable concentrations of 4,6-dinitro-*o*-cresol or by inorganic phosphate. This confirms, with a cell-free system obtained from sea urchin eggs, the observations of Loomis and Lipmann regarding stimulation of oxygen consumption by a system obtained from rabbit kidney.

3. A preliminary but unsuccessful attempt has been made to determine the conditions under which cell-free, aerobic, phosphorylating systems may be obtained from either unfertilized or fertilized sea urchin eggs.

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