STUDIES ON CELL METABOLISM AND CELL DIVISION

V. Cytochrome Oxidase Activity in the Eggs of Arbacia punctulata

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In previous papers of this series (1–4) and allied publications, a study has been made of the effects of various agents on the respiration and cell division of the fertilized eggs of the sea urchin *Arbacia punctulata*. These experiments were part of a program directed toward gaining some insight into the respective rôles of individual enzyme systems in the utilization of oxidative energy for developmental processes in the sea urchin egg and other cells. For such investigations fertilized sea urchin eggs are especially suitable, owing to the fact that they depend almost exclusively on oxygen uptake for consumption of foodstuff, having little or no aerobic glycolysis or other metabolic activity of the anaerobic type under the conditions of experiment employed, even in the presence of cyanide and other respiratory inhibitors.

It was shown (3) that inhibition of division of fertilized *Arbacia* eggs took place at a level of respiratory inhibition which varied according to the type of inhibitor used. The significance of these experiments could not be assessed in terms of the individual oxidative enzymes of the eggs because, though effects of respiratory inhibitors on certain enzyme systems in other plant and animal cells have been worked out in detail in a few instances, no single known oxidative enzyme has been positively identified as being present in fertilized *Arbacia* eggs. For example, although it has been assumed, from the fact that the respiration of fertilized *Arbacia* eggs is partially poisoned by cyanide or carbon monoxide and stimulated by *p*-phenylenediamine, that such eggs contain a cytochrome oxidase-cytochrome system comparable to that in many other cells, no cytochrome bands have been observed in the eggs (4–6), and the presence of an enzyme system capable of oxidizing reduced cytochrome has not heretofore been demonstrated with certainty, though Ball and Meyerhof (5) recorded indications of the presence of such an enzyme in unfertilized *Arbacia* eggs.

The present paper is one of a series designed to correct this deficiency in

knowledge of respiratory enzymes in *Arbacia* eggs. It reports experiments to show that the eggs contain an enzyme which can oxidize reduced cytochrome c. This enzyme occurs in nearly equal amounts in unfertilized and fertilized *Arbacia* eggs, being present in both in a concentration sufficient to account for the respiration of the fertilized eggs even under the maximum degree of respiratory stimulation yet observed (1). The enzyme is inhibited—though in some instances to a degree differing greatly from that of the egg respiration—by cyanide, carbon monoxide, azide, and hydrogen sulfide, but not by such copper inhibitors as sodium diethyldithiocarbamate or 8-hydroxyquinoline.

In extension of previous experiments (4, 5, 7), a further effort to demonstrate the presence of cytochrome c and succinic dehydrogenase has shown that these oxidative catalysts, if present at all, occur in fertilized *Arbacia* eggs in concentrations disproportionately small in relation to the cytochrome oxidase activity, and too small to be of any apparent significance for the respiration of the egg.

From these experiments, certain tentative suggestions can be made regarding the probable nature of the oxidative systems operative in the fertilized *Arbacia* eggs.

EXPERIMENTAL METHODS

The cytochrome oxidase was prepared and used essentially according to Stotz (8) with the substitution of glycylglycine for part of the phosphate buffer in the test system and the use of a pH of 6.8 $\pm$ 0.1 to make the pH of the extraction and test system conform to what is believed from experiment to be the pH of the aqueous phase of the egg cytoplasm.

The details of the oxidase preparation were as follows: Ripe, mature eggs were obtained at Woods Hole during July and August, 1940 and, where necessary, fertilized according to methods reported in previous papers of this series. The volume of the eggs was, in every case, determined on the unfertilized eggs by the hematocrit method (9) (2700 times gravity for 5 minutes). Each cubic centimeter of eggs corresponded to approximately 5×10^6 eggs. The cytochrome oxidase values per cubic centimeter of eggs were converted to a wet weight and a dry weight basis using an egg density of 1.08 (10) and an egg solid content of 18 per cent (11). To carry out the preparation, the eggs, either unfertilized or at 30 minutes after fertilization at 20°C., were packed tightly by centrifuging at 2000 times gravity for 10 minutes. The eggs were then cytolyzed with 0.067 M Na_2HPO_4, using 5 cc. of phosphate solution for each gram of eggs; the resulting suspension was ground in a mortar with acid-washed sea sand at 5°C. for 20–25 minutes, using 0.4 gm. sand for each gram of eggs. The brei was then decanted from the sand, placed in cellophane tubing, and dialyzed overnight (22 hours) at 8°C. against 0.1 M phosphate buffer (pH 6.9) to reduce the concentration of any unidentified oxidizable substrates which might give large blank values in the manometric experiments. The resulting brei, after dilution with 0.1 M phosphate to a volume of 6.7 cc. per gm. of eggs,

was used as the enzyme preparation in the cytochrome oxidase experiments. It is of parenthetical interest that the liquid outside the dialysis tubes was pale yellow, and not red, at the end of the dialysis period, indicating that the echinochrome, though freely soluble in water, was tightly bound by the residual proteins of the brei.

Cytochrome c was prepared in this laboratory from beef hearts, according to the method of Keilin and Hartree (12), by Mr. T. V. Parke. After reduction with sodium hydrosulfite ($Na_2S_2O_4$) the cytochrome c was standardized spectrophotometrically (pH 6.0) at 550 mμ as described by Keilin and Hartree (12). It was preserved in solution (4.5×10^{-5} M, pH 5.0) in the ice box at 5°C. with a trace of toluene included to prevent bacterial contamination and destruction.

The manometric determinations of cytochrome oxidase activity were made with conical Warburg flasks and manometers. In all experiments, except those on effects of varying cytochrome c concentration, the main compartment of each flask received 0.5 cc. of 0.33 M glycylglycine buffer of pH 6.9 (final concentration 0.05 M), 0.5 cc. of the oxidase preparation, 1.0 cc. of 4.5×10^{-5} M cytochrome c (final concentration 1.36×10^{-5} M), and 1.0 cc. of water or a solution of the desired oxidase inhibitor in a concentration to give the final concentrations shown in the experimental section of the paper. The side arm contained 0.3 cc. of 0.22 M hydroquinone, metal-free cysteine, *p*-phenylenediamine, or sodium succinate according to the substrate desired; each substrate solution was brought to pH 6.9 before placing in the flasks. The flask center cups were left empty. For experiments concerned with effect of variation in cytochrome c concentration, the volumes were: 0.5 cc. oxidase, 0.5 cc. glycylglycine, 0 to 4.0 cc. cytochrome c solution (with water to make the total initial volume in the main compartment 5.0 cc.), and 0.5 cc. hydroquinone solution in the side arm. The flasks were equilibrated in the bath, at 20°C. unless otherwise specified, the substrate tipped in, and a preliminary period of 15 minutes allowed for minor pressure fluctuations to disappear. Readings of oxygen uptake were then made for the next 2 hours; all calculations here given are based on this 2 hour period, during which the total oxygen consumption was of the order of 50 to 200 c. mm. in the control flasks. The oxygen consumption followed a linear course under the conditions here defined, but use of higher final concentrations of the oxidase with hydroquinone as substrate led to the development of a cumulative inhibition of the enzyme by some unidentified oxidative product, possibly quinone.

The gas mixtures were prepared over water, passed through the flasks while the latter were shaken in the bath, and analyzed manometrically for oxygen by the method of Warburg and Kubowitz (13).

In extension of previous experiments with low oxygen tension, cyanide, and carbon monoxide on the respiration of the eggs, analogous experiments to determine the effect of azide on egg respiration were made by the Warburg direct method as previously described (3); the effect of hydrogen sulfide on egg respiration was determined by the method of Dixon and Keilin (14). In each case, eggs were obtained and fertilized in the usual way, concentrated by allowing to settle, then diluted to give a final egg concentration of 2 per cent by volume; in the course of this dilution a solution of 0.55 M glycylglycine in sea water at pH 8.0 (final concentration 0.05 M) was included to the extent of 10 per cent of the total volume. Each flask contained 0.5 cc. of a solution of the inhibitor solution. At 30 minutes after fertilization, 5.0 cc. of the egg suspension was added to the flasks, the temperature being maintained at 20°C. throughout.

In the course of this work on the eggs it was found, in running controls to ascertain

the division of the eggs at various hydrogen sulfide concentrations, that respiration experiments on the effect of hydrogen sulfide at pH 8 could be run without alkali in the center cup, the respiratory carbon dioxide being readily absorbed by the glycylglycine buffered sea water serving as medium for the eggs. Since the vapor pressure of carbon dioxide over sea water has in fact been found (15) to be negligible at pH values above 7.8, the method might be applied more generally for measurement of respiration under conditions where rather alkaline media can be used.

EXPERIMENTAL RESULTS

Under the conditions here employed the oxygen uptake by the cytochrome oxidase in presence of cytochrome c was linear over a 2 hour period and, with a given preparation, proceeded at nearly the same rate whether hydroquinone (0.02 M), cysteine (0.02 M), or phenylenediamine (0.02 M) was used as substrate (Fig. 1). The autooxidation of substrate in presence of heat-inactivated oxidase (100°C. for 10 minutes) and cytochrome c was, as indicated by the representative data of Fig. 1, between 5 and 10 per cent of that in the active preparation. This autooxidation was almost insensitive to the inhibitors to be mentioned below. For all subsequent discussion in the present paper, rates of oxygen uptake refer to the net values obtained by subtraction of the uptake by heat-inactivated enzyme plus cytochrome c plus substrate from the uptake by unheated enzyme plus cytochrome c plus substrate. In contrast to heart muscle (8) the eggs were readily freed, by simple washing and dialysis of the fragmented cells, of intermediate catalysts capable of causing a substantial oxygen uptake in absence of added cytochrome c (Fig. 1).

Cytochrome Oxidase Activity in Relation to Cytochrome c Concentration.—As previously shown by Stotz (8) for beef heart muscle, the activity of cytochrome oxidase from the eggs increased with the concentration of cytochrome and approached a maximum value at a concentration of cytochrome c somewhat below 10^{-4} M (Fig. 2). The cytochrome c concentration required for half activation of the oxidase was approximately 4×10^{-6} M. This value for unfertilized and fertilized *Arbacia* eggs at 20°C. may be compared with the value of approximately 6×10^{-6} M for half activation of cytochrome oxidase from beef heart muscle as determined by Stotz, Altschul, and Hogness (16) at 38°C.

Concentration of Cytochrome Oxidase in Unfertilized and Fertilized Arbacia Eggs.—When tested in the presence of excess cytochrome c the concentration of cytochrome oxidase was found to be nearly the same in unfertilized and in fertilized *Arbacia* eggs (Table I). Each unit of activity is defined, following Stotz, as 10 c.mm. oxygen uptake per hour at 20° C. Efforts to determine the activity of *Arbacia* egg cytochrome oxidase at 38°C. were invalidated by the fact that the oxidase, with hydroquinone as a substrate,

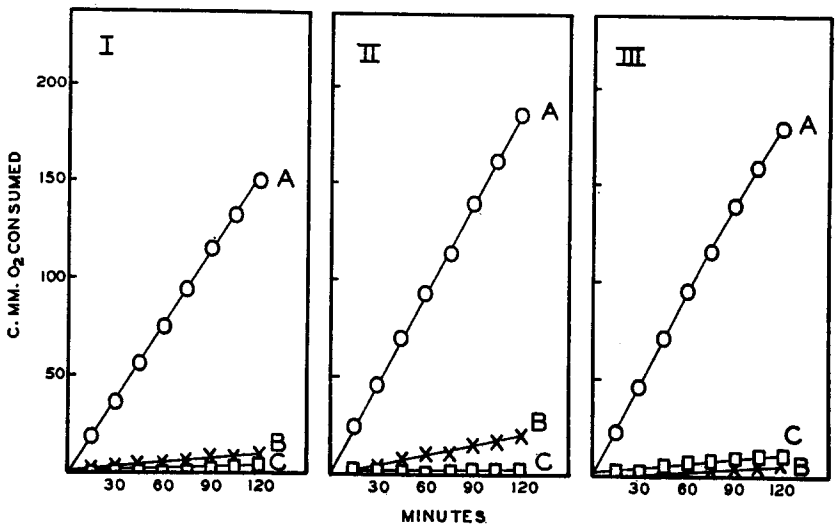

FIG. 1. Oxygen consumption at pH 6.8 by: *A*, cytochrome oxidase plus 1.36×10^{-5} M cytochrome c; *B*, heat inactivated cytochrome oxidase plus 1.36×10^{-5} M cytochrome c; *C*, cytochrome oxidase with no added cytochrome. The reductants for cytochrome c were: I, 0.02 M hydroquinone; II, 0.02 M *p*-phenylenediamine; III, 0.02 M cysteine. In this experiment all *A* and *C* samples were aliquots from the same oxidase preparation; the rates of oxidation with the various reductants may therefore be directly compared. Temperature, 20°C.

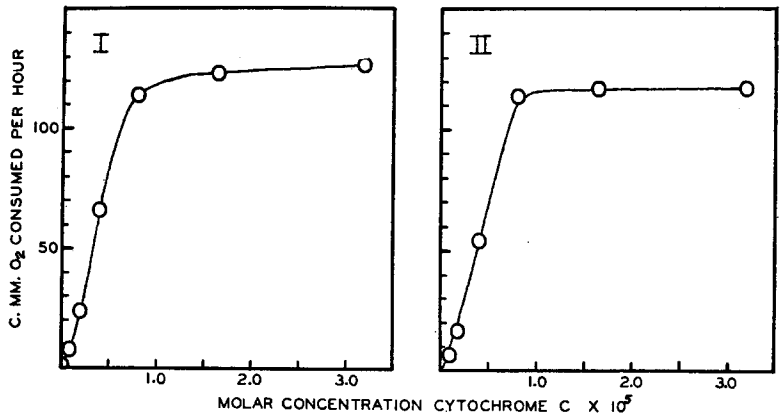

FIG. 2. Oxygen consumption at pH 6.8 by cytochrome oxidase from (I) unfertilized *Arbacia* eggs and (II) fertilized *Arbacia* eggs as a function of cytochrome c concentration with 0.02 M hydroquinone as reductant for cytochrome c. Temperature, 20°C.

was rapidly inactivated at this temperature, while the rate of autooxidation was relatively high. However, using the measurements at 20°C. with allowance for the temperature factor, the amount of cytochrome oxidase

activity present in the eggs compared favorably with that in various rat tissues, as determined by Stotz.

The Q_{O_2} values of Table I (cubic millimeters of oxygen taken up by cytochrome oxidase in 3.2×10^{-5} M cytochrome c and 0.02 M hydroquinone per hour per mg. original eggs, dry weight) may be compared with the following approximate Q_{O_2} values for the living whole eggs: unfertilized, 0.4–0.5; fertilized, 2; fertilized optimally stimulated by 4,6-dinitrocresol and other substituted phenols (1, 4) which act through the cyanide-sensitive egg respiratory system, 7–8.

Inhibition of Arbacia Cytochrome Oxidase by Carbon Monoxide.—The initial experiments with oxidase inhibitors were made to determine which

TABLE I

Analysis of Arbacia Eggs for Cytochrome Oxidase by Stotz (8) Method at 20°C. Cytochrome c, 3.2×10^{-5} M; Hydroquinone, 0.02 M

Exp. No.	Date	Cytochrome oxidase units* per mg. dry weight	
		Unfertilized eggs	Fertilized eggs
134 W	9–19–40	1.01	—
136 W	9–20–40	—	0.83
137 W	9–21–40	0.99	0.85
138 W	9–22–40	0.95	0.88

* The Q_{O_2} values corresponding to the cytochrome oxidase units may be obtained by multiplying the figures in the table by a factor of 10.

of the respiratory and division blocking agents previously (3) used for eggs could be considered to derive their physiological action from suppression of cytochrome oxidase activity. It was soon apparent, however, that the quantitative reaction of the oxidase to inhibitors was so different from that of the fertilized *Arbacia* eggs as to merit a considerable exploration of the properties of the oxidase with the hope that the resulting data might help to define the probable enzymic relationships in the living *Arbacia* eggs. The experimental results will first be presented; comparisons of the respective reactions of the eggs and the oxidase to inhibitors, together with theoretical considerations arising from the experiments with the various inhibitors, will be discussed in a separate section below, to which reference should be made for definition of terms employed in the captions accompanying the figures and tables.

The oxidase activity was strongly inhibited by carbon monoxide in the dark (Fig. 3). The inhibition was almost completely reversed (Fig. 4) by a carbon arc lamp. The relative inhibition by a given partial pressure

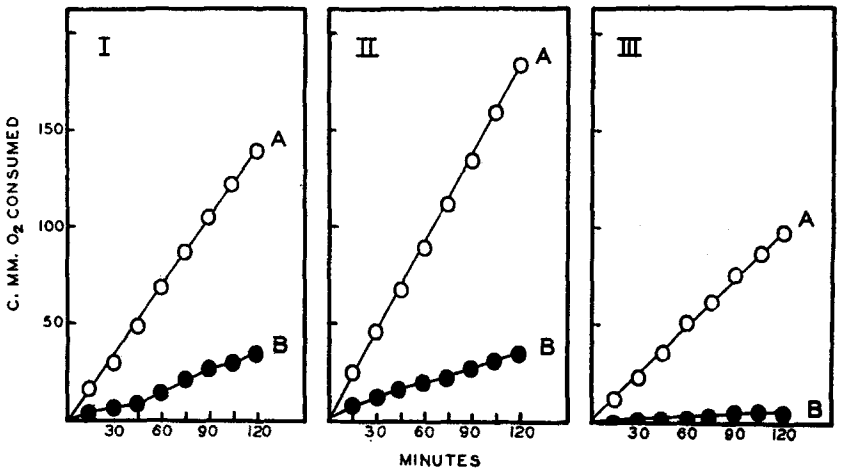

FIG. 3. Effect of carbon monoxide in the dark on oxygen consumption at pH 6.8 by cytochrome oxidase plus 1.36×10^{-5} M cytochrome c with various reductants for cytochrome c: I*A*, 0.02 M hydroquinone in air; I*B*, 0.02 M hydroquinone in 7.5 per cent O_2 — 92.5 per cent CO, K (See Table II) = 3.2. II*A*, 0.02 M *p*-phenylenediamine in air, II*B*, 0.02 M *p*-phenylenediamine in 6.8 per cent O_2 — 93.2 per cent CO, K = 1.8. III*A*, 0.02 M cysteine in air, III*B*, 0.02 M cysteine in 6.5 per cent O_2 — 93.5 per cent CO, K = 0.6. Temperature, 20°C.

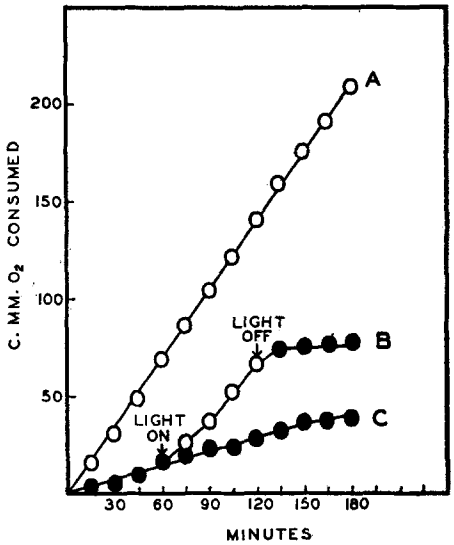

FIG. 4. Effect of carbon monoxide in the dark and in the light on oxygen consumption at pH 6.8 by cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone. *A*, control in air; *B*, in 7.5 per cent O_2 — 92.5 per cent CO, illuminated for period designated; *C*, in 7.5 per cent O_2 — 92.5 per cent CO, kept dark throughout experiment.

of carbon monoxide was apparently not greatly altered by variation in the concentration of cytochrome c or the concentration of hydroquinone used as reductant for the cytochrome c (Table II).

In experiments serving as controls for the carbon monoxide experiments, the oxidase was found to have full activity in 7 per cent oxygen–93 per

TABLE II

Inhibition of Arbacia Cytochrome Oxidase by Carbon Monoxide in the Dark at Two Concentrations of Cytochrome c and Two Concentrations of Hydroquinone. Temperature, 20°C.

Cytochrome c concentration	Hydroquinone concentration	$\frac{pCO}{pO_2}$	O_2 consumed in 2 hrs.		$K = \frac{n}{1-n} \cdot \frac{pCO}{pO_2}$
			CO absent	CO present	
moles per l. × 10⁶	*moles per l.*		*c.mm.*	*c.mm.*	
1.36	0.02	12.2	139	29	3.2
0.34	0.02	17.0	52	8	3.1
1.36	0.001	14.4	55	13	4.5

TABLE III

Inhibition of Arbacia Cytochrome Oxidase by Various Concentrations of Sodium Cyanide at Two Concentrations of Cytochrome c with Hydroquinone As Substrate and at One Concentration of Cytochrome c with p-Phenylenediamine as Substrate. Temperature, 20°C.; pH 6.9

Concentration total cyanide	O_2 consumed in 2 hrs. by oxidase with 1.36 × 10^{-6} M cytochrome c and 0.02 M hydroquinone	Inhibition	O_2 consumed in 2 hrs. by oxidase with 0.34 × 10^{-6} M cytochrome c and 0.02 M hydroquinone	Inhibition	O_2 consumed in 2 hrs. by oxidase with 1.36 × 10^{-6} M cytochrome c and 0.02 M *p*-phenylenediamine	Inhibition
moles per l. × 10⁶	*c.mm.*	*per cent*	*c.mm.*	*per cent*	*c.mm.*	*per cent*
0	98	0	61	0	171	0
1	57	41.9	39	36.0	73	57.2
4	29	70.4	29	52.5	50	70.7
16	14	85.6	23	62.3	41	76.0
64	10	89.8	20	67.2	31	81.9
256	0	100.0	0	100.0	4	97.5

cent nitrogen; it was about 5–10 per cent inhibited in 6 per cent oxygen–94 per cent nitrogen.

Inhibition of Arbacia Cytochrome Oxidase by Sodium Cyanide.—The oxidase activity was strongly inhibited at very low concentrations of sodium cyanide (Table III), the logarithm of the ratio of inhibited to uninhibited respiration varying approximately linearly with the logarithm of the cyanide concentration (Fig. 5). The relative inhibition by a given concentration of cyanide was apparently not greatly altered by variation in the concentration of cytochrome c (Table III) nor by use of *p*-phenylenediamine instead of hydroquinone as substrate.

Inhibition of Respiration and Cell Division of Fertilized Arbacia Eggs and Also Arbacia Cytochrome Oxidase by Sodium Azide.—To supplement earlier experiments (3) with low oxygen tension, carbon monoxide, and cyanide,

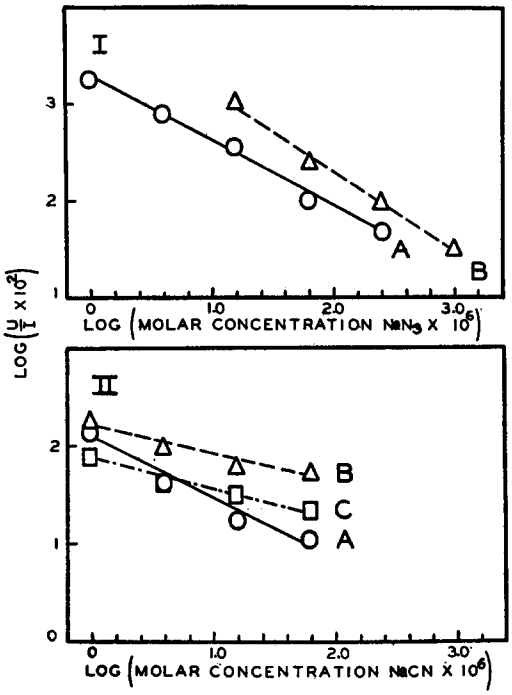

FIG. 5. Plot of log [inhibitor concentration] against log $\frac{U}{I}$ to test the equation $\frac{U}{I} = K$ [concentration inhibitor]$^{-a}$ for: I*A*, sodium azide at pH 6.8 on cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone; I*B*, sodium azide at pH 6.8 on cytochrome oxidase plus 0.34×10^{-5} M cytochrome c plus 0.02 M hydroquinone. II*A*, sodium cyanide at pH 6.8 on cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone. II*B*, sodium cyanide at pH 6.8 on cytochrome oxidase plus 0.34×10^{-5} M cytochrome c plus 0.02 M hydroquinone. II*C*, sodium cyanide at pH 6.8 on cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M *p*-phenylenediamine. Temperature, 20°C.

the effect of various concentrations of sodium azide on respiration and cell division of fertilized *Arbacia* eggs was determined and expressed (Fig. 6) by methods identical with those previously used for the other inhibitors. Attention is directed to two points of interest: First, approximately 50 per cent of the respiration of the fertilized eggs was insensitive to azide under the present conditions of experiment; secondly, 50 per cent inhibition

of cell division occurred at an azide concentration inhibiting respiration by only about 10 per cent; complete and reversible inhibition of cell division occurred at an azide concentration inhibiting respiration by about 50 per cent.

The cytochrome oxidase activity in the cell-free preparation was also inhibited by sodium azide (Table IV), the logarithm of the ratio of inhibited to uninhibited respiration varying linearly with the logarithm of the azide concentration (Fig. 5). The relative inhibition by a given concentration of azide was not greatly altered by variation in the concentration of cytochrome c (Table IV).

TABLE IV

Inhibition of Arbacia Cytochrome Oxidase by Various Concentrations of Sodium Azide at Two Concentrations of Cytochrome c with 0.02 M Hydroquinone as Substrate. Temperature, 20°C.; pH 6.8

Concentration total azide	O_2 consumed in 2 hrs. by oxidase with 1.36×10^{-6} M cytochrome c	Inhibition	O_2 consumed in 2 hrs. by oxidase with 0.34×10^{-6} M cytochrome c	Inhibition
moles per l. × 10^6	*c.mm.*	*per cent*	*c.mm.*	*per cent*
0	116	0	61	0
1	110	5.2	56	8.2
4	102	12.0	56	8.2
16	90	22.4	43	29.4
64	59	49.0	28	54.0
256	38	67.1	14	77.0

Inhibition of Respiration and Cell Division of Fertilized Arbacia Eggs and Also Arbacia Cytochrome Oxidase by Sodium Sulfide.—To supplement earlier experiments (3) with other inhibitors the effect of various concentrations of sodium sulfide on respiration and cell division of fertilized *Arbacia* eggs was determined (Fig. 6). Cell division was 50 per cent inhibited at a sulfide concentration inhibiting respiration by only about 10 per cent. Complete, but *not* reversible, inhibition of cell division occurred at a sulfide concentration inhibiting respiration by about 50 per cent. At the lethal concentration of sulfide the respiration was inhibited by about 80 per cent. Owing to the lethal action of the sulfide it was impossible to determine from the present experiments whether any fraction of the vital respiration was insensitive to sulfide.

The cytochrome oxidase activity was also inhibited by sodium sulfide, complete suppression of activity being produced at approximately 1×10^{-3} M sodium sulfide at pH 6.9 (Fig. 7). Experiments of this type, though repeatedly carried out, yielded rather unsatisfactory results because, at

concentrations above 1×10^{-3} M, the sulfide itself was rapidly oxidized by the heated and still more rapidly by the unheated oxidase preparation.

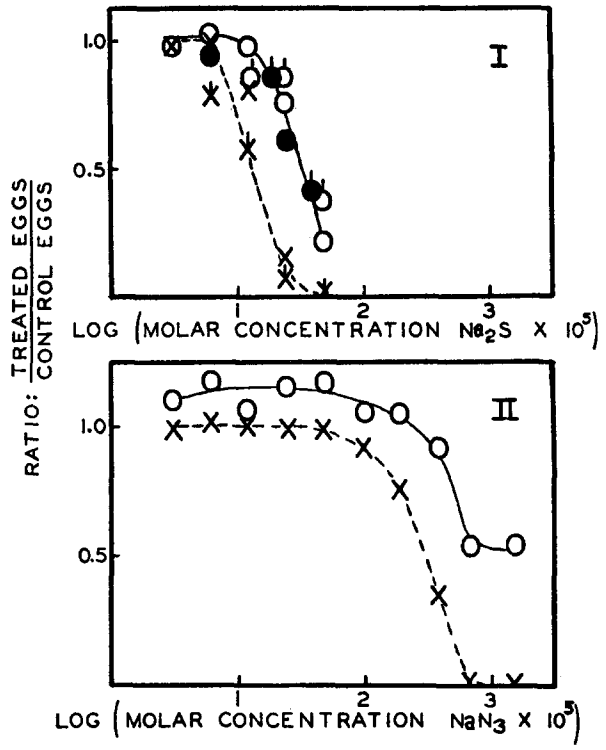

FIG. 6. Oxygen consumption and cell division of fertilized eggs of *Arbacia punctulata* in I, various concentrations of sodium sulfide at pH 7.9; II, various concentrations of sodium azide at 7.9. The reagents were added 30 minutes after fertilization. Temperature, 20°C. In Fig. 6

$$O\text{-}O = \frac{O_2 \text{ consumed in treated eggs}}{O_2 \text{ consumed in control eggs}}$$

$$X\text{-}X = \frac{\text{Cell division in treated eggs}}{\text{Cell division in control eggs}}$$

In I, the open circles represent measurements by the direct Warburg method; the solid circles represent simultaneous measurements by the Dixon-Keilin method on aliquots from the same egg sample. The plain circles refer to one experiment, the circles with bar to a second experiment on a different sample of eggs.

It is not at present clear whether this oxidation of sulfide is catalyzed by the cytochrome oxidase or by the echinochrome-protein complexes accompanying the cytochrome oxidase.

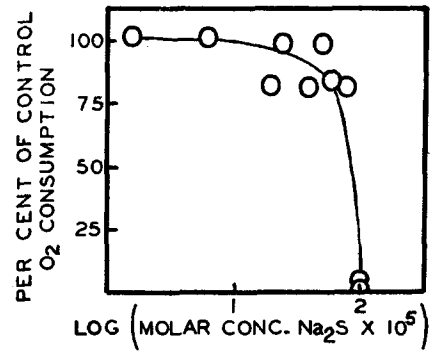

FIG. 7. Effect of various concentrations of sodium sulfide at pH 6.8 on cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone. Temperature, 20°C.

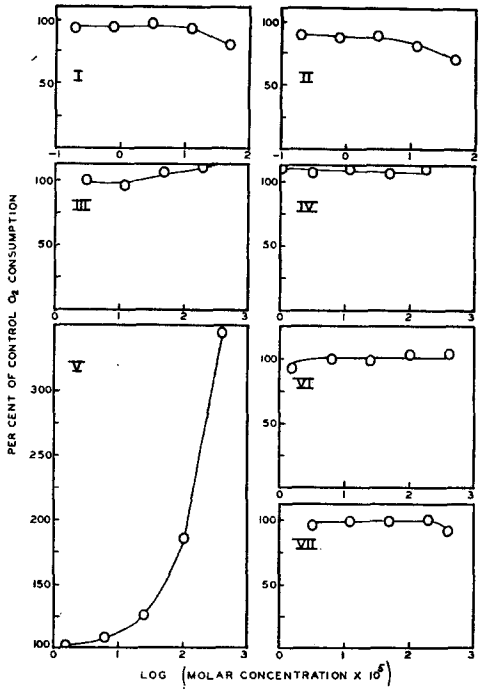

FIG. 8. Effect of various concentrations of each of seven agents at pH 6.8 on cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone. I, 2, 4-dinitro-*o*-cyclohexylphenol; II, 2, 4-dinitrothymol; III, 5-isoamyl-5-ethyl barbituric acid; IV, phenylurethane; V, sodium diethyldithiocarbamate; VI, iodoacetic acid; VII, 8-hydroxyquinoline.

Effect of Miscellaneous Agents on Arbacia Cytochrome Oxidase.—In view of the suggestion, made by Keilin and Hartree (17), that cytochrome oxidase may be a copper compound, the effects of two well known inhibitors of copper containing enzymes, 8-hydroxyquinoline and sodium diethyldithiocarbamate, were tested on the *Arbacia* cytochrome oxidase. Neither produced any inhibition of activity in the highest concentrations soluble in the medium used (Fig. 8). In fact, the diethyldithiocarbamate produced

TABLE V

Concentration of Sodium Cyanide, Sodium Azide, and Sodium Sulfide Required to Produce 50 Per Cent Inhibition of Respiration, Cell Division, and Cytochrome Oxidase Activity of Fertilized Arbacia Eggs. Temperature, 20°C. For Method of Calculation of Ion and Molecule Concentrations See Text and Reference 21

	Cyanide	Azide	Sulfide
	moles per l. × 10⁶	*moles per l. × 10⁶*	*moles per l. × 10⁶*
1. Total *extracellular* concentration of inhibitor for 50 per cent inhibition of respiration at pH 7.9	69	8200	350
2. Calculated *intracellular* concentration of inhibitor molecules at pH 6.8 corresponding to total concentration in item 1	66	5.2	39
3. Calculated *intracellular* concentration of inhibitor anions at pH 6.8 corresponding to total concentration in item 1	0.3	650	25
4. Total *extracellular* concentration of inhibitor for 50 per cent inhibition of cell division at pH 7.9	44	3200	130
5. Calculated *intracellular* concentration of inhibitor molecules at pH 6.8 corresponding to total concentration in item 4	42	2.0	15
6. Calculated *intracellular* concentration of inhibitor anions at pH 6.8 corresponding to total concentration in item 4	0.2	250	10
7. Total concentration of inhibitor for 50 per cent inhibition of cytochrome oxidase plus 1.36×10^{-5} M cytochrome c plus 0.02 M hydroquinone at pH 6.8	1.4	66	850
8. Calculated concentration of inhibitor molecules at pH 6.8 corresponding to total concentration in item 7	1.3	0.5	520
9. Calculated concentration of inhibitor anions at pH 6.8 corresponding to total concentration in item 7	0.1	66	330

a large increase in oxygen uptake in experiments with *Arbacia* egg cytochrome oxidase. This may be provisionally attributed to oxidation of the diethyldithiocarbamate by the oxidase or by the echinochrome-protein complexes accompanying the cytochrome oxidase. Keilin and Hartree (18), in a paper appearing after the completion of the present experiments, showed that diethyldithiocarbamate is oxidized to tetraethyldithiocarbamyldisulfide by a cytochrome oxidase preparation from beef heart muscle. They also showed the latter compound to be a powerful inhibitor of succinic dehydrogenase. Incidentally, while oxygen consumption of fertilized

Arbacia eggs was affected little or not at all by sodium diethyldithiocarbamate in concentrations up to 4×10^{-3} M, the cell division was about 10 per cent inhibited at 3×10^{-5} M and 50 per cent inhibited (partially irreversibly) at 4×10^{-3} M.

To clarify the results previously obtained on living *Arbacia* eggs, a number of other physiologically active agents were employed with the oxidase (Fig. 8). With the exception of sodium chloride (not shown), which produced a complete inhibition of oxidase activity in a concentration of 0.6 M at pH 6.8, none of the agents produced a substantial inhibition of *Arbacia* cytochrome oxidase activity until concentrations greatly exceeding the physiologically active concentrations were reached; this indicates that their physiological inhibition of egg respiration and cell division is attributable to their action on enzyme systems other than cytochrome oxidase.

Quantitative Comparison of Effect of Various Agents on Egg Respiration, Egg Cell Division, and Cytochrome Oxidase.—The concentrations of sodium cyanide, sodium azide, and sodium sulfide required to produce 50 per cent inhibition of fertilized *Arbacia* egg respiration, fertilized *Arbacia* egg cell division, and *Arbacia* cytochrome oxidase have been assembled (Table V) from the data of this and a previous paper (3).

In comparing the effects of these agents on the eggs and on the oxidase, it should be noted that cyanide and azide apparently penetrate fertilized *Arbacia* eggs only as undissociated molecules (19) and that the form of each of these agents which enters into complexes with metalloporphyrins may well be the anion (20). On the basis of theoretical considerations detailed elsewhere (21) the probable concentrations of anions (CN^-, N_3^-, and HS^-) and of undissociated molecules (HCN, HN_3, and H_2S) in the aqueous phase of the egg cytoplasm have been calculated (Table V), using pK′ values (22) of 9.2, 4.7, and 7.0 for hydrogen cyanide, hydrogen azide, and hydrogen sulfide (first hydrogen).

It has recently been shown by Fisher and Öhnell (23) that the effects of cyanide on a number of physiological processes conform to the equation $\frac{U}{I} = K[CN]^{-a}$, where U is the fraction of function uninhibited, I is the fraction of function inhibited, $[CN]$ is the molar total cyanide concentration in the medium, and K and a are constants. As an empirical approach, while postponing discussion of the probable significance of such numerical values until the mechanism of the inhibitor action is better understood, log [inhibitor concentration] has been plotted against log $\frac{U}{I}$ for cyanide and azide inhibition of cytochrome oxidase (Fig. 5). In these plots the

experimental points, though not accumulated specifically to test this possibility, are found to conform approximately to a straight line, as demanded by the above equation.

Attempts to Isolate Cytochrome c from Fertilized Arbacia Eggs.—Previous qualitative spectroscopic examination of brei from fertilized *Arbacia* eggs after reduction with sodium hydrosulfite failed (3) to reveal the presence of cytochrome c. These experiments have now been extended by repeated attempts to isolate cytochrome c by the method of Stotz (8). The resultant products displayed no specific light absorption at 550 mμ and were devoid of cytochrome c activity when examined manometrically by the Stotz method; control experiments in which known amounts of cytochrome c were carried through the testing process showed that cytochrome c, if present, could have been detected in concentrations down to approximately 2 micrograms per gram of wet fertilized *Arbacia* eggs. On the basis of these data and those of Fig. 2, it seems safe to conclude that cytochrome c cannot carry a significant fraction of the oxygen consumption of fertilized *Arbacia* eggs.

Also in extension of previous experiments (5, 7) by the Thunberg method, it has been found manometrically that the cytochrome oxidase preparation has no succinic dehydrogenase activity; succinate, in a final concentration of 0.02 M at pH 6.8 and 20°C., caused no extra oxygen uptake when added to *Arbacia* cytochrome oxidase saturated with added cytochrome c.

DISCUSSION

The present experiments were undertaken with a view to establishing a basis from which the effects of carbon monoxide, cyanide, azide, and sulfide on fertilized *Arbacia* eggs could be used to clarify the mechanism by which energy from oxidative processes is utilized for the support of cell division in such eggs. A number of facts relevant to this objective have been established.

1. The eggs of *Arbacia punctulata* contain an enzyme capable of oxidizing reduced cytochrome c.

2. The amount of the enzyme, as measured by means of its activity toward cytochrome c as a representative substrate, is more than sufficient to account for the highest rate of oxygen utilization yet observed in the intact, living fertilized eggs.

3. In its rapid reaction with molecular oxygen, its light reversible inhibition by carbon monoxide, and its inhibition by cyanide, azide, and sulfide—but not by agents forming complexes with copper—the enzyme displays

properties which are those of an electromotively active iron-porphyrin compound.

4. The enzyme, when acting with cytochrome c as substrate, is completely inhibited by cyanide or azide, just as is the cell division of the living fertilized *Arbacia* eggs. In contrast, the respiration of the fertilized egg can be inhibited to a maximum of only about 70–80 per cent by cyanide and only about 50 per cent by azide.

5. In the equation $K = \frac{n}{1-n} \cdot \frac{p\text{co}}{p\text{o}_2}$, where n is fraction of respiration not inhibited, and $p$co and $p\text{o}_2$ are the carbon monoxide and oxygen partial pressures, the apparent values of the inhibition constant for the action of carbon monoxide upon the enzyme in the dark were found to be in the range of 0.5 to 5, depending on the concentration of cytochrome c and the nature and concentration of the reductant for cytochrome c. The corresponding value for the fertilized *Arbacia* eggs is very much larger than this, being of the order of 60 on the assumption that the respiration is completely sensitive to carbon monoxide.

6. Previous qualitative observations regarding the low concentration, or absence, of cytochrome c in the eggs are confirmed; further quantitative observations place the highest possible concentration of cytochrome c at a level too low to be of any probable significance for the respiration of fertilized *Arbacia* eggs.

When considered in conjunction with what is now known regarding the combination of cyanide and other nitrogenous materials with iron-porphyrin compounds, the present results appear to be of some potential significance, not only for respiration of *Arbacia* eggs, but in explaining the action of cyanide on cell respiration in general.

It was shown by Barron (24) that cyanide forms electromotively active hemochromogens when added to certain iron-porphyrin compounds. The oxidation-reduction potentials (E_0' values at pH 7.0) of such cyanide hemochromogens are, other factors being equal, much lower (100–300 mv. or more) than those of hemochromogens containing the same iron-porphyrin nucleus with other simple nitrogenous bases or proteins substituted for cyanide in the complex. These observations have recently been extended by Davies (20) and the theory has been developed in detail by Clark, Taylor, Davies, and Vestling (25). Continuing his previous work (26) on the parallelism between oxidation-reduction potential difference between catalyst and substrate system on the one hand and catalytic activity on the other, Barron (27) also showed that various hemochromogens could act as oxidative catalysts if supplied with a substrate system having a potential at an appropriate level below that of the hemochromogen.

In the light of these findings, it appears that the action of cyanide in inhibiting cell respiration may be interpreted on the hypothesis that the cyanide forms, with the iron-porphyrin of the cytochrome oxidase, a complex having a potential lower than that of the original oxidase. Whether or not the cyanide complex can then continue in part the function of the oxidase will depend, among other factors, on the potentials of the catalytic systems next lower in the oxidative chain. For example, if this system were exclusively cytochrome c (E_0' at pH 7, + 257 mv. (29)), the cyanide complex (E_0' from pH 5 to 8, −183 mv. for the cyanide complex with blood hemin) could not serve as catalyst and the respiration of the cell would be completely inhibited. On the other hand, if a catalyst with a potential somewhat below that of cytochrome c, and near or suitably below that of the cyanide hemochromogen were available, the cell would retain some capacity to consume oxygen, with cyanide hemochromogen partially substituting for the oxidase.[1] On this basis, the residual respiration displayed by certain cells in the presence of cyanide would remain, in certain instances at least, a metal-catalyzed oxidation and not, as hitherto implicitly supposed, a catalysis carried on by metal-free systems.

With this background in mind, a number of observations regarding the effects of cyanide and analogous inhibitors on the respiration and cell division of *Arbacia* eggs may possibly be given a provisional qualitative explanation on the basis of an assumption derived, by analogy, from the experimental data on cyanide referred to above. This assumption is that the inhibitors cyanide, azide, carbon monoxide, and possibly sulfide, change the state of intracellular binding of the iron-porphyrin which initially functioned as part of the enzyme acting as the terminal link at the oxygen end of the respiratory chain; as a result of the change the potential of the iron-porphyrin in its new linkage is lower than in the untreated cell. While the data necessary for the further elaboration of this assumption are not at present available it may be noted that both the ability of any given reagent to enter into complex formation with the iron-porphyrin and the magnitude of the potential shift obtainable with any given inhibitor

[1] The hypothesis here proposed regarding the mechanism of action of cyanide on cell respiration is considered by the authors to be an extension, to living systems, of the ideas developed by Barron for purely chemical systems. The possibility that cyanide (and other analogous) hemochromogens might act as physiological oxygen transfer catalysts was, so far as the authors are aware, first explicitly stated by Dr. E. G. Ball and one of the authors (M. E. Krahl) during a discussion of an evening paper presented at the Marine Biological Laboratory, Woods Hole, on July 18, 1939. The hypothesis has been further discussed by Ball (28) elsewhere.

depend, among other factors, on the particular structure of the porphyrin component.

It was observed that the vital respiration of fertilized *Arbacia* eggs was about 20 per cent insensitive to cyanide and about 50 per cent insensitive to azide. On the basis of the above hypothesis, this would be at least in part attributable to the difference in potential and in catalytic activity between the cyanide and azide iron-porphyrin complexes under the conditions operative in the *Arbacia* egg.

With each of the inhibitors dealt with in this paper it was observed that, at critical concentrations of the inhibitor, cell division was inhibited relatively more than respiration, finally being completely suppressed at inhibitor concentrations which allowed a substantial portion of the respiration to proceed. On the basis of the general hypothesis advanced above, this is the result to be expected if the potential of the particular carrier by which that fraction of the electron transfer critical for cell division is keyed to the oxidase is relatively closer to that of the oxidase than the potentials of other carriers responsible for the bulk of the overall respiration. If confirmed by independent methods of investigation this suggestion provides a partial answer to the principal question posed at the start of the investigation; it may help to define the type, and specify the potentials of, certain of the oxidative catalysts which make energy available for the cell division cycle.

It was observed that the carbon monoxide inhibition constant was much larger for the fertilized eggs than for the cell-free cytochrome oxidase-cytochrome c system. This is what would be expected if the substrate for the oxidase in the eggs had a potential substantially below that of cytochrome c; since the eggs, as shown above, appear to contain no cytochrome c, this possibility is open.

It has previously been observed that the respiration of unfertilized *Arbacia* eggs is completely insensitive to carbon monoxide (30) and is relatively less sensitive to cyanide than that of fertilized *Arbacia* eggs (31). On the basis of the general hypothesis advanced above, this means that the increase of respiration on fertilization is concerned with the entrance of a carrier system having a potential higher than those operative before fertilization, establishing a better relationship between oxidase and carrier before poisoning but, at the same time, providing an unfavorable relationship of potential after addition of carbon monoxide or cyanide.

It is suggested that this may also be the explanation for the fact that the endogenous respiration of certain cells in absence of substrate is insensitive to carbon monoxide, the respiration of the same cells becoming carbon monoxide sensitive on addition of substrate.

It has been observed that the respiration of a number of tissues (32) is sensitive to cyanide but not to azide. This apparent paradox is immediately resolved, by the present hypothesis, on two grounds: first, the relative abilities of cyanide and azide to combine with the iron-porphyrin group in question and, secondly, the respective potentials of the resulting complexes with reference to the next available carrier in the respiratory chain.

It has been observed (4) that various substituted phenols alter the sensitivity of fertilized *Arbacia* egg respiration to cyanide. With the substituted phenol alone, without cyanide, the rate of oxygen consumption of the eggs rose to an optimum as the concentration of the substituted phenol was progressively increased; at still higher concentrations the rate of oxygen consumption fell below this optimum and, at sufficiently high concentrations, below the normal. Suboptimum respiratory-stimulating concentrations of the substituted phenol induced a respiration relatively more sensitive than the normal to cyanide. Greater than optimum concentrations of the phenol caused the sensitivity to cyanide to fall at first toward the normal and then to become less sensitive to cyanide than the normal respiration. This is the course of events to be expected if the substituted phenol brings into play, at low and suboptimum concentrations, a carrier system reacting directly with the oxidase, with high and greater than optimum concentrations blocking or reversing this process and finally bringing into play considerable amounts of a carrier having a potential somewhat lower than those operating in the egg untreated with the substituted phenol.

It is proposed to conduct further experiments to throw light on this, at present, somewhat speculative theory. The theory is advanced only because of the large number of hitherto puzzling facts which it appears to correlate and because of the numerous interesting experimental suggestions to which it gives rise.

SUMMARY

1. An enzyme capable of oxidizing reduced cytochrome c (*i.e.* a cytochrome oxidase) has been obtained from *Arbacia* eggs. In 0.02 M hydroquinone, the cytochrome oxidase was half activated at a cytochrome c concentration of approximately 4×10^{-6} M. The concentration of the cytochrome oxidase was found to be nearly the same in unfertilized and fertilized eggs, the amount of the enzyme—as measured by means of its activity toward cytochrome c as a representative substrate—being more than sufficient to account for the highest rate of oxygen utilization yet

observed in the intact, living, fertilized eggs, and of the same order as that in certain rat tissues.

2. The *Arbacia* cytochrome oxidase was strongly inhibited by carbon monoxide in the dark, the inhibition being almost completely reversed by light. The inhibition constant was not greatly altered by variation in the concentration of cytochrome c or the concentration of hydroquinone used as reductant for the cytochrome c, having a value of 3 to 5 under the conditions used. The inhibition constant was about 2 with *p*-phenylenediamine as reductant for the cytochrome c, but apparently had the surprisingly low value of about 0.5 with 0.02 M cysteine as reductant.

3. The cytochrome oxidase was completely inhibited by sufficiently high concentrations of sodium cyanide, sodium azide, and sodium sulfide. It was also completely inhibited in 0.6 M sodium chloride. It was not inhibited by two inhibitors of copper containing enzymes, 8-hydroxyquinoline and sodium diethyldithiocarbamate. It was also not significantly inhibited by 2,4-dinitrothymol, 2,4-dinitro-*o*-cyclohexylphenol, phenylurethane, 5-isoamyl-5-ethylbarbituric acid, or iodoacetic acid.

4. Quantitative examination of the fertilized eggs showed that cytochrome c, if present at all, occurred in a concentration of less than 2 micrograms per gram of wet fertilized *Arbacia* eggs. On the basis of these data and those of Fig. 2, above, it seems safe to conclude that cytochrome c cannot carry a significant fraction of the oxygen consumption of fertilized *Arbacia* eggs.

It was also found that, in contrast to similar preparations from certain other animal tissues, the *Arbacia* cytochrome oxidase preparation displayed no succinic dehydrogenase activity when tested manometrically in the presence of excess cytochrome c.

5. Extending previously reported (3) experiments with other inhibitors, the effects of sodium azide and sodium sulfide on the respiration and cell division of fertilized *Arbacia* eggs were determined, the eggs being initially exposed to the reagents 30 minutes after fertilization at 20°C. With either reagent cleavage was completely blocked by a concentration of reagent which reduced the respiration to approximately 50 per cent of the normal level.

6. On the basis of certain theoretical considerations regarding the possible mechanism of action of cyanide and other respiratory inhibitors it is suggested that a fraction of the respiration apparently concerned with supplying energy for division processes in the fertilized *Arbacia* egg may be keyed into the respiratory cycle through a carrier having a somewhat higher potential than those which carry the larger portion of the egg respiration.

The theory is also employed in an effort to resolve a number of hitherto apparently paradoxical observations regarding the effects of cyanide, azide, and carbon monoxide on cell respiration.

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