STUDIES ON THE RESPIRATION OF SEA-URCHIN SPERMATOZOA

II. THE CYTOCHROME OXIDASE ACTIVITY IN RELATION TO THE DILUTION EFFECT

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In the previous paper (Mohri, 1956), it was shown that in sea-urchin spermatozoa, the outburst of respiration which occurs on dilution of a sperm suspension can be suppressed by sodium azide. However, the low respiration which follows the outburst is not affected by this poison, although both are inhibited by cyanide.

Similar observations have been made on other materials as regards azide action. As far as the eggs of sea-urchins are concerned, the respiration of unfertilized eggs was found to be cyanide-sensitive but azide-insensitive, while the increased respiration after fertilization was sensitive to both inhibitors (Fischer, Henry & Low, 1944; Robbie, 1946). Runnström (1932) and Örström (1932) found that the addition of sufficient amounts of dimethyl-phenylendiamine (dpphd) caused similar increases in the O₂ uptake of unfertilized and fertilized eggs. This was considered to show that, although the capacity of the echinoderm oxidase (similar to but not identical with cytochrome oxidase, cf. Borei, 1945) is the same in unfertilized and fertilized eggs, in the former, the enzyme is not saturated with substrate, owing to a block of some kind in the carrier chain. From the experiments with sodium azide and phenylendiamine, Stannard (1939) also assumed that the cytochrome oxidase of the frog muscle functions in the active but not in the resting state.

The experiments reported in this paper were therefore designed to investigate the possibility that the cytochrome-cytochrome oxidase system is involved in the respiratory Dilution Effect in sea-urchin spermatozoa.

MATERIALS AND METHODS

Spermatozoa of the sea-urchin, Hemicentrotus pulcherrimus, were used throughout. The semen was centrifuged for 5 min. at 3,000 r.p.m. The packed sperm was then diluted with filtered sea water. The manometric technique was similar to that described in the previous paper (Mohri, 1956), using vessels of about 20 ml. capacity and at 20°C.

Dpphd was usually employed as substrate, but p-phenylendiamine (pphd), hydroquinone, ascorbic acid and cysteine were also examined. All reagents were made up immediately before use in filtered sea water. The pH was adjusted to 8.2. Since no cytochrome e was added, the reaction depended on the intracellular cytochromes only.
RESULTS

Effect of dpdhd on dense and dilute sperm suspensions

In contradistinction to the situation with other substrates, the catalytic oxidation of dpdhd through cytochrome oxidase does not require addition of cytochrome c (Borei & Renvall, 1949). In the present experiments, dpdhd was introduced in the main chamber, and sea water was added up to 2-5 ml. Then 0.5 ml. of very dense sperm suspension was pipetted into the side arm and poured into the main chamber after 10 min. temperature equilibration. The converse procedure (0.5 ml. of dpdhd in the side arm, tipped after equilibration) was also tried, but little difference was observed between them. Experiments with other reagents were run mostly with the former technique, unless otherwise mentioned.

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Effect of dimethyl-p-phenylenediamine (dpdhd) on O₂ uptake of sea-urchin spermatozoa, *Hemicentrotus pulcherrimus*. Dilution (a) 1/20; (b) 1/200. Final concentrations of dpdhd: ○, 0; ●, 10⁻⁶ M; ×, 10⁻⁴ M; □, 10⁻² M.

The effect of dpdhd on the oxygen uptake of dense suspensions (1/20 or 2.5 × 10⁹ spermatozoa per ml.) is shown in Fig. 1a. A marked increase in oxygen uptake after the addition of dpdhd is readily recognizable over the range of 10⁻⁶ to 10⁻³ M. Since the autoxidation of dpdhd in sea water is negligible, the observed increase in oxygen uptake is not due to a catalytic action by copper ions in sea water. The respiration with dpdhd proceeds at a constant rate during the first hour. The maximum rate is reached in the range from 3·2 × 10⁻⁵ to 10⁻³ M, though the latter concentration causes early death of the spermatozoa, which is reflected in a reduction in O₂ uptake after the first hour. The maximum rate is higher than the initial...
rate of the control. With other respiratory stimulants such as 2,4-dinitrophenol (Mohri, 1956) or copper and zinc ions (Table 2; cf. Rothschild & Tuft, 1950), the effect does not become apparent until after about 1 hr.

The results obtained in dilute suspension (1/200 or 2.5 x 10⁸ spermatozoa per ml.) are illustrated in Fig. 1b. The addition of dpdph at concentrations of 10⁻⁶ and 10⁻⁴ M, which stimulate oxygen uptake of dense suspensions, has practically no effect on dilute suspensions, although for the same concentration of dpdph the spermatozoa die earlier in the dilute suspension. Furthermore, from a comparison of the curves in Fig. 1a and b, it can be noted that the oxidation rate with sufficient amount of dpdph in the dense suspension is almost comparable to that in the dilute one. These facts suggest that in dense suspension cytochrome oxidase functions only partially, owing probably to lack of saturation with substrate. Dpdph short-circuits this obstacle and brings the enzyme almost to a maximum activity, while in dilute suspension this situation is already attained leaving no room for dpdph to exert its effect.

Effect of other substrates. The effects of other substances which can be oxidized through the cytochrome-cytochrome oxidase system were examined in dense suspension (1/20). Autoxidation of these substances (except hydroquinone) was negligible in sea water in the range of concentration used. Among them, ppdph has a very similar stimulating effect to that of dpdph on the respiration of the sperm suspension (see also Fig. 4). The magnitude of oxygen uptake at the maximum stimulation is also the same as that found with dpdph, indicating a 100% mobilization of the enzyme. As shown in Fig. 2, a similar result is obtained with ascorbic

Fig. 2. Effect of ascorbic acid on O₂ uptake of sea-urchin spermatozoa, H. pulcherrimus. Dilution 1/20. Final concentrations of ascorbic acid: ○, 0; ●, 10⁻⁴ M; ×, 10⁻⁵ M; □, 10⁻⁶ M.
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acid at $10^{-3}$ M. At $10^{-2}$ M, however, ascorbic acid suppresses $O_2$ uptake from the beginning. In the case of hydroquinone, on the other hand, an extraordinary autoxidation occurs in sea water. According to Runnström (1932) and Örström (1932), if hydroquinone is added to sea water in which sea-urchin eggs are suspended, the hydroquinone acts as a true hydrogen carrier and no autoxidation occurs. Hydroquinone causes no increase in oxygen uptake of sea-urchin spermatozoa, but inhibits respiration at $10^{-3}$ and $10^{-2}$ M, unlike the previously mentioned substrates. The life-span of the spermatozoa is shortened by this substance. Zittle & Zitin (1942) also failed to detect cytochrome oxidase activity of homogenized bull spermatozoa with hydroquinone as a substrate. A marked spermicidal power of quinones was reported by Baker (1932).

As illustrated in Fig. 3, the results obtained with cysteine are not simple. When $10^{-5}$ M cysteine is used there is no change in oxygen uptake of the sperm suspension. From $10^{-4}$ to $10^{-3}$ M the initial oxidation rate is reduced to about the same level as that of the steady low respiration of the control after the initial burst. Above $10^{-2}$ M an increase in oxygen uptake can be observed. The stimulating action of cysteine and glutathione, both at $10^{-2}$ M, on oyster sperm respiration was reported by Humphrey (1950). Tyler (1953), on the other hand, found that amino acids including cysteine produce a prolongation of the life-span of sea-urchin spermatozoa. He suggested that this effect is due to the ability of amino acids to bind heavy metals, especially copper and zinc, present in the dilution medium. In the presence of amino acids or of other chelating agents, the initial rate of oxygen consumption is distinctly lower than that of the controls (Tyler & Rothschild, 1951; Tyler, 1953;
Rothschild & Tyler, 1954; see also Table 2). The increase in life-span of spermatozoa in the presence of cysteine is also confirmed in the present material (Table 1). This effect is obtained both at \(3.2 \times 10^{-3}\) M, which stimulates oxygen uptake, and at \(3.2 \times 10^{-4}\) M, which reduces the initial rate, although the latter concentration is more favourable than the former in keeping the spermatozoa alive.

Table 1. Effect of cysteine on fertilizing capacity of sea-urchin spermatozoa, Hemicentrotus pulcherrimus

(Fertilizing capacity was tested after 4 hr. incubation at 20° C. of \(2.5 \times 10^6\) sperm per ml. suspension. To 5 ml. of variously diluted suspensions approximately 500 unfertilized eggs were added. Numbers represent percentage of fertilized eggs.)

<table>
<thead>
<tr>
<th>No. of sperm/ml.</th>
<th>Concentration of cysteine in sea water</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>9.8 \times 10^5</td>
<td>10</td>
</tr>
<tr>
<td>2.0 \times 10^5</td>
<td>1</td>
</tr>
<tr>
<td>3.9 \times 10^5</td>
<td>0</td>
</tr>
<tr>
<td>7.8 \times 10^4</td>
<td>0</td>
</tr>
</tbody>
</table>

Effect of azide on oxidation of dp-phd and of pp-hd

In the previous paper (Mohri, 1956) the respiration of sea-urchin spermatozoa was reported to be separable into two fractions, azide-sensitive and azide-insensitive, and that dilution initiates the former fraction. Since azide is one of the most powerful inhibitors of cytochrome oxidase, and furthermore since cytochrome oxidase activity is higher in dilute than in dense suspensions, it is quite justifiable to suppose that the azide-sensitive respiration is effected through cytochrome oxidase. To confirm this possibility the influence of azide on the enhanced respiration with substrates was examined. In this experiment, 0.5 ml. of a mixture of azide and dp-phd or pp-hd was put in the side arm and was tipped into the main chamber after equilibration. As shown in Fig. 4, azide at \(10^{-2}\) M is sufficient to eliminate completely the extra oxygen uptake (the effect of dilution as well as that of dp-phd or pp-hd), leaving only respiration which coincides in magnitude with the azide-inhibited control respiration.

The increased oxygen uptake with ascorbic acid can also be inhibited by azide, but not quite to the level of the azide-stable residue of the control. The oxidation by cysteine, on the other hand, is inhibited to a lesser extent, suggesting that the increase in oxygen uptake found at high concentrations of cysteine does not go through the channel of cytochrome oxidase.

Effect of \(ZnCl_2\) and of metal-chelating agents on oxidation of dp-phd

The addition of copper or zinc to dense sperm suspensions can enhance oxygen uptake, and this is counteracted by the addition of metal-chelating agents such as ethylenediaminetetra-acetate (versene) and diethyldithiocarbamate (dedtc) (Rothschild & Tuft, 1950; Rothschild & Tyler, 1954). Since dilution brings about an increase in cytochrome oxidase activity, there is a possibility that the heavy
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Fig. 4. Effect of sodium azide \((10^{-4} \text{ M})\) on oxidation of dpphd \((10^{-4} \text{ M})\) and pphd \((10^{-4} \text{ M})\) in sperm suspension of sea-urchin, *H. pulcherrimus*. Dilution 1/20. O, none; ⋄, dpphd; Δ, pphd; ●, azide; ×, dpphd + azide; □, pphd + azide.

Table 2. Effect of ZnCl₂ and of chelating agents on respiration of the sea-urchin spermatozoa, *Hemicentrotus pulcherrimus*, with and without dpphd

(Dilution was 1/20. The figures relate to the first hour at 20°C.)

<table>
<thead>
<tr>
<th>Conc. of dpphd</th>
<th>-µl. O₂/10⁶ spermatozoa</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0 10⁻⁴ M</td>
<td>44'95</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>56'2</td>
</tr>
</tbody>
</table>

metals act directly on the cytochrome-cytochrome oxidase system. By contrasting the effects of ZnCl₂ and chelating agents, versene, ddtc and glycine, on the oxidation with and without dpphd in dense suspension (1/20), the results turned out to be as described in Table 2; as already mentioned, ZnCl₂ at 10⁻⁸ M without dpphd, which would cause a marked increase in oxygen uptake if added after a decline, had no effect on the initial phase of respiration, while all chelating agents suppressed the initial rate of normal respiration to a low level. These facts fit in with the above supposition. However, the situation becomes more complicated after further experimentation. Addition of excess zinc ions to dpphd 10⁻⁶ M, under conditions of submaximal oxidation, fails to improve the rate. Furthermore, the chelating agents do not inhibit the extra oxygen uptake in the presence of dpphd. This might mean that the point where the heavy metals are concerned is not the terminal oxidase system, but may be some other link in the oxidation chain.
The results reported in this paper seem to support the view that the cytochrome-cytochrome oxidase system is involved in the respiratory Dilution Effect in sea-urchin spermatozoa. Rothschild (1948) also observed that the inhibition of sea-urchin sperm respiration by carbon monoxide is greater in dilute than in dense suspensions. Cytochrome oxidase, however, is located at the terminal point in the oxidation chain and the activation of the enzyme will, on this view, be an ultimate result. This may suggest the existence of some other mechanism responsible for saturating the terminal oxidase with substrate. In a sense, the latter can be considered as the real cause of the Dilution Effect.

As described above, copper and zinc have a stimulating effect on the respiration of sea-urchin spermatozoa (Rothschild & Tuft, 1950; Mohri, 1956). Since copper and zinc are normal constituents of sea water and the increase in oxygen uptake on dilution is reported to depend on the amount of these metals present in sea water (Rothschild & Tuft, 1950), it is quite likely that these metals play a primary role in the mechanism of the Dilution Effect. The possibility that they directly affect cytochrome oxidase was examined, but without success. Barron, Nelson & Ardao (1948) reported that very low concentrations of sulphhydryl-binding substances such as monooiodoacetate, p-chloromercuribenzoate and CdCl₂ accelerate the respiration of sea-urchin spermatozoa, whereas higher concentrations cause an inhibition. This is interpreted as showing that the addition of such SH-blocking reagents in small concentrations will cause the removal of the soluble SH groups regulating cellular respiration, resulting in an increase in oxygen uptake; with higher concentrations, however, SH groups in the protein moiety of enzymes will be destroyed, which results in inhibition of respiration. Copper and zinc will also serve as SH-binding reagents, and in fact the respiration of sea-urchin spermatozoa can be accelerated at \(10^{-9}\) M or \(10^{-8}\) M of both metals, though it is inhibited by \(10^{-4}\) M and stronger solutions. The following scheme, then, might be applicable to the mechanism of the Dilution Effect in sea-urchin spermatozoa. When shed and diluted with sea water, the spermatozoa come in contact with copper and zinc ions in sea water. These metals combine with the soluble SH groups which regulate the oxidation rate of cytochrome \(c\), eliciting the full activation of cytochrome oxidase. This scheme, however, is only tentative and further study is needed concerning the soluble SH groups.

**SUMMARY**

1. The activity of cytochrome oxidase in sea-urchin spermatozoa (Hemicentrotus pulcherrimus) was studied in relation to the Dilution Effect, using dimethyl-p-phenylene-diamine (dpdphd), p-phenylenediamine (pphd), hydroquinone, ascorbic acid and cysteine as substrates.

2. All substrates except hydroquinone cause a marked rise in oxygen uptake in dense sperm suspensions (1/20). It is shown that the maximum rate obtained with a sufficient amount of dpdphd in dense suspensions is almost comparable with the normal respiratory rate in dilute suspensions (1/200). The oxygen uptake of dilute suspensions is not affected by the addition of dpdphd.
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3. The increase in oxygen uptake caused by dpphd and pphd is completely eliminated by sodium azide (10⁻² M).

4. From the results of 2 and 3, it can be inferred that cytochrome oxidase exhibits its maximum activity only in dilute suspension, probably as a result of substrate saturation.

5. Heavy metals, copper and zinc, do not seem to affect the terminal oxidase system directly, but some other part of the oxidation chain, such as the soluble SH groups.

6. The effect of cysteine is rather complicated. In the range from 10⁻⁴ to 10⁻³ M, cysteine causes a reduction in the initial respiratory rate as already observed with other amino-acids, but above this range, it exerts a marked stimulatory effect on oxygen uptake.

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REFERENCES


