STUDIES ON THE RESPIRATION OF SEA-URCHIN SPERMATOZOA

I. THE EFFECT OF 2,4-DINITROPHENOL AND SODIUM AZIDE

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Humphrey (1950) has established by microspectroscopic examination the existence of cytochrome spectra in oyster spermatozoa, and has further shown that cytochrome inhibitors such as cyanide and azide suppress the oxygen uptake of these spermatozoa. In sea-urchin spermatozoa, however, Barron, Nelson & Ardao (1948*b*) have reported a contradictory result that, though the respiration is markedly depressed by cyanide, it is completely resistant to azide, even at high concentrations. On the other hand, Utida & Nanao (1954) have reported that the normal respiration of sea-urchin sperm is not affected by sodium azide, whereas the augmented respiration caused by 2,4-dinitrophenol (DNP) is completely abolished by this inhibitor. Since the presence of the cytochrome system in sperm cells has repeatedly been confirmed in a number of animals including sea urchins (Ball & Meyerhof, 1940; Zittle & Zitin, 1942; Mann, 1945; Rothschild, 1948), it is curious that azide fails to inhibit the normal respiration of sea-urchin spermatozoa.

Since Gray (1928) discovered that dilute sperm suspensions of sea urchin consume oxygen at a higher rate than dense ones, much work has been done to clarify the nature of this Dilution Effect (cf. Rothschild, 1951). Upon dilution of a dense suspension with sea water, there can be observed an increase in motility, and at the same time a sudden burst of respiration, which gradually subsides and settles to a steady level of oxygen uptake.

The present work has revealed that sodium azide exerts an inhibitory effect only on this initial burst. Consequently, during the subsequent course of low respiratory activity, the inhibitory effect is not noticeable. The effect of DNP on the Dilution Effect has also been investigated.

MATERIAL AND METHODS

The sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*, were used. After sexing electrically (Iwata, 1950), semen was obtained in a clean syracuse dish by introducing isotonic KCl solution into the body cavity. The semen was then centrifuged for 5 min. at 3000 r.p.m. and the packed sperm thus obtained was diluted to desired densities with filtered sea water. Oxygen consumption was measured by the usual Warburg technique with flasks of about 20 and 10 ml. capacities. The gas phase contained air. The shaking rate was 100 c.p.m. with a 4 cm. stroke and the

temperature was 20° C. All reagents were prepared afresh before each experiment and were adjusted to pH 8·2 if necessary.

For sperm counts a haemocytometer with Thoma ruling was used. It was shown that 1 ml. of the packed sperm contained about 5×10^{10} spermatozoa.

RESULTS

Initial burst of respiration. The Dilution Effect was examined in the spermatozoa of *Pseudocentrotus*. 1 ml. of dense suspension was put in the main chamber of the flasks and 0·5 ml. of sea water in the side arm. The time of mixing was taken as zero time ($t=0$). The results are shown in Fig. 1, in which the respiratory activity is

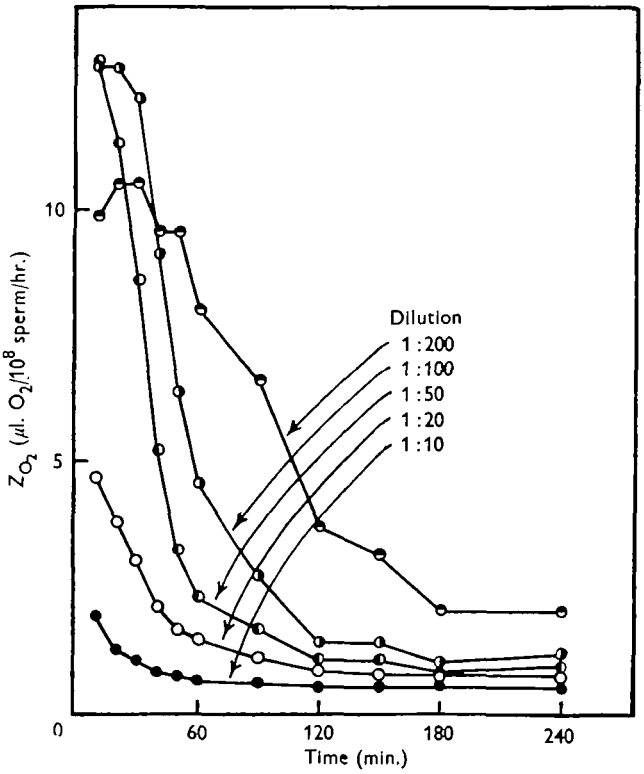

Fig. 1. Effect of dilution with sea water on the rate and the duration of initial O_2 uptake of sea-urchin spermatozoa (*Pseudocentrotus depressus*). 1 ml. of sperm suspension in the main chamber, 0·5 ml. of sea water in the side arm, tipped at $t=0$. Temp., 20° C.

expressed in terms of Z_{O_2} (μl. O_2 per 10^8 spermatozoa per hour). The presence of the Dilution Effect is readily noticeable. From these curves it can be said that the initial burst is more rapidly lost in dense suspensions than in dilute ones. Further dilution beyond 1:50 does not increase the height of the curve, though it results in a prolongation of the initial high respiratory activity. After the initial burst is over, the respiration is maintained at a fairly constant rate for several hours. It is

noteworthy that, in such a state, dense suspensions (especially in 1 : 10 and 1 : 20) still retain the capacity to exhibit another burst of respiration on further dilution, while in dilute suspensions (e.g. 1 : 200) this does not occur.

In addition, it was noted that in some cases, perhaps with poor material, the initial burst in dense suspensions failed to occur, the respiration proceeding at a steady low level from the beginning. The materials used by Utida & Nanao (1954) and by Barron *et al.* (1948*b*) must have been in such a state, as will be seen from the following sections.

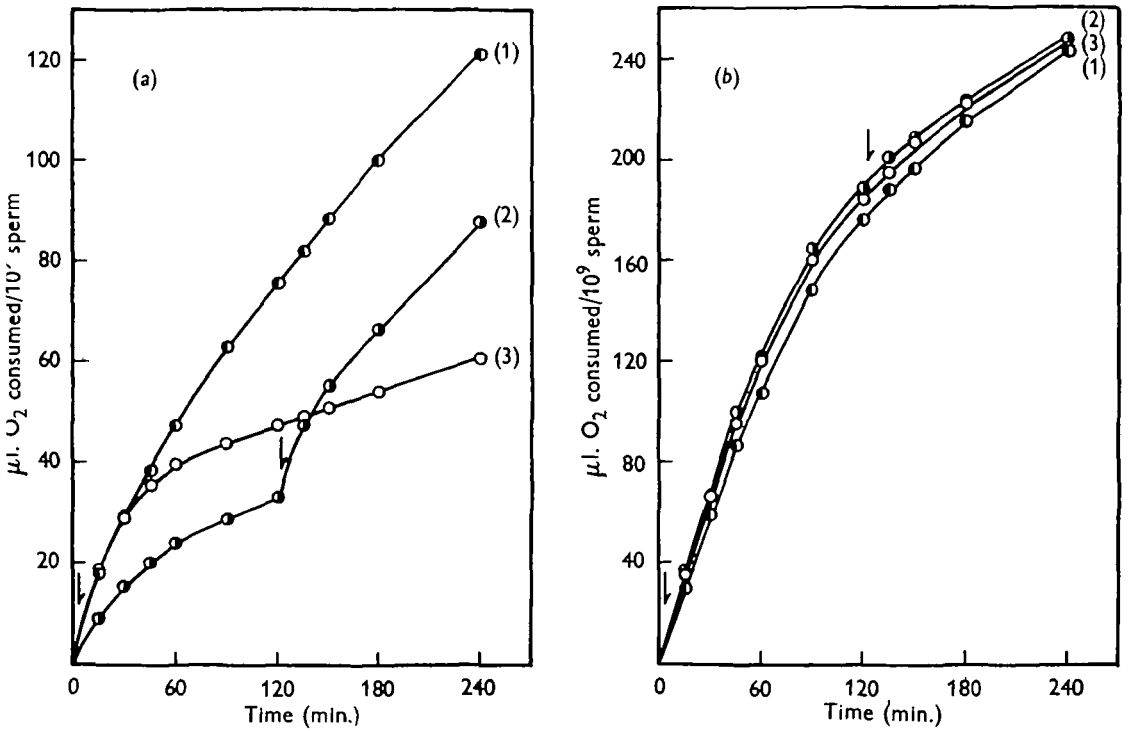

Fig. 2. Effect of DNP (1×10^{-4} M) on O_2 uptake of sea-urchin spermatozoa (*Pseudocentrotus depressus*). 2·7 ml. of sperm suspension in the main chamber, 0·3 ml. of sea water or DNP in the side arm, tipped at time indicated by arrows. (1), +DNP at $t=0$; (2), +DNP at $t=120$; (3), control. 20° C. *a*, dilution 1 : 20; *b*, dilution 1 : 200.

Effect of DNP on O_2 uptake of spermatozoa. The effect of DNP was examined in both dense (1 : 20) and dilute (1 : 200) sperm suspensions, using *Pseudocentrotus*. 1×10^{-4} M DNP, which was found to be optimal for increasing the oxygen uptake of the spermatozoa, was used throughout the experiments. Typical results with the dense suspension are shown in Fig. 2*a*, in which the curves represent the total oxygen consumed per unit quantity of spermatozoa. When DNP is added at $t=0$, during the first 30 min., no difference in the rate of oxygen uptake can be detected in comparison with the control. However, when the respiration of the control begins to come down to settle to the steady state, the difference becomes obvious

and the DNP respiration remains high for several hours. The addition of DNP during the steady state at 120 min. results in an instantaneous increase of oxygen uptake. The addition of the same volume of sea water as that of DNP (one-tenth of the total volume) at $t=120$ brings about no significant changes in the rate of oxygen uptake (cf. Utida & Nanao, 1954). Thus, the steady-state respiration of dense suspension can be enhanced considerably by DNP, while the initial burst is not altered in the presence of this reagent. In the dilute suspension, on the other hand, DNP does not increase the oxygen uptake, whether it is added at $t=0$ or $t=120$ (Fig. 2*b*), although lack of effect at $t=120$ is attributed to the fact that the sperm is, by this time, in a poor condition. This result was repeatedly confirmed. In Table 1, the degree of the DNP effect during the steady state of respiration is compared among different dilutions. The stimulating action of DNP decreases inversely with the increase of dilution and becomes almost unnoticeable at the dilution of 1:200.

Table 1. *Effect of 2,4-dinitrophenol (DNP) on oxygen uptake of sea-urchin spermatozoa* (Pseudocentrotus depressus)

(Oxygen uptake was measured 120 min. after preparation of suspensions. The figures given are μl. O_2 per 10^9 spermatozoa per hour. Temperature, 20° C. DNP, 1×10^{-4} M.)

Dilution	O_2 uptake		% (control = 100)
	Control	+DNP	
1:10	3·0	13·5	450
1:20	4·5	16·1	358
1:50	10·4	28·0	269
1:100	18·7	25·0	134
1:200	23·1	24·9	108

The above would suggest that there is some respiratory system in spermatozoa, which comes into full activity when diluted with sea water, and that DNP acts upon such a system in a manner resembling the Dilution Effect. However, the oxygen uptake per unit quantity of spermatozoa is lower in dense than in dilute suspensions, even after the addition of DNP.

No attempt was made to estimate sperm motility quantitatively, although a rough observation showed that DNP slightly reduces motility despite its stimulating effect on respiration.

Effect of azide on O_2 *uptake of spermatozoa.* The results obtained with sodium azide in *Pseudocentrotus* (Table 2) and *Hemicentrotus* (Fig. 3) will be given. Sodium azide, if added at $t=0$, produces a marked inhibitory effect on respiration at concentrations of 1×10^{-2} M and 1×10^{-3} M. Even at lower concentrations such as 1×10^{-4} M and 1×10^{-5} M, a slight inhibition can be noted. This is consistent with the results of Humphrey (1950) on oyster spermatozoa. Sperm motility in the two sea urchins is completely abolished at 1×10^{-2} M of azide and affected slightly by 1×10^{-3} M, azide thus differing from cyanide which has little effect on the motility of sperm cells.

Table 2. *Effect of sodium azide on the respiration of sea-urchin spermatozoa* (Pseudocentrotus depressus)

(Flask volume, 10 ml. 1 ml. of suspension in the main chamber, 0·5 ml. of azide in sea water in the side arm, tipped at $t=0$. Temperature, 20° C.)

Dilution	Conc. of azide	$-\mu$l. O_2			
		1st hr.	2nd hr.	3rd hr.	4th hr.
1:20	0	154·3	68·5	44·8	43·3
	1×10^{-2} M	35·4	38·4	41·5	39·2
	1×10^{-3} M	78·3	42·4	37·4	39·5
1:200	0	21·2	11·4	5·3	6·1
	1×10^{-2} M	2·2	6·7	6·0	6·9
	1×10^{-3} M	8·0	6·4	5·6	6·8

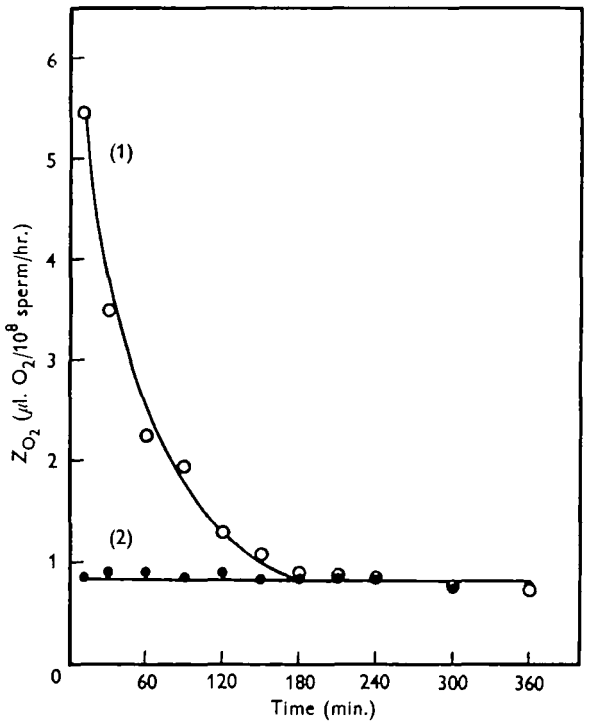

Fig. 3. Effect of sodium azide (1×10^{-2} M) on O_2 uptake of sea-urchin spermatozoa (*Hemicentrotus pulcherrimus*). 2·7 ml. of sperm suspension in the main chamber, 0·3 ml. of sea water or sodium azide in the side arm, tipped at $t=0$. Dilution 1:20. 20° C. (1), control; (2), +sodium azide.

Sodium azide, however, becomes non-effective in regard to oxygen uptake after 120 or 180 min. in both dense (1:20) and dilute (1:200) suspensions. This can be seen from Table 2 (compare the figures for 1st and 2nd hour against those for 3rd and 4th hour) and Fig. 3. In other words, the intense respiration associated with dilution is sensitive to sodium azide, but the subsequent lowered respiration is insensitive to it, as has been reported previously (Barron *et al.* 1948*b*; Utida &

Nanao, 1954). Furthermore, it can be noted that, in the presence of 1×10^{-2} M of azide, the inhibited level of respiration of the initial phase coincides with that of the later steady respiration. Consequently, from the standpoint of azide-action, it appears that two different components are at work in the respiration of spermatozoa, i.e. azide-sensitive and azide-insensitive ones, the initial burst consisting of both of them and the subsequent low respiration consisting of the latter alone.

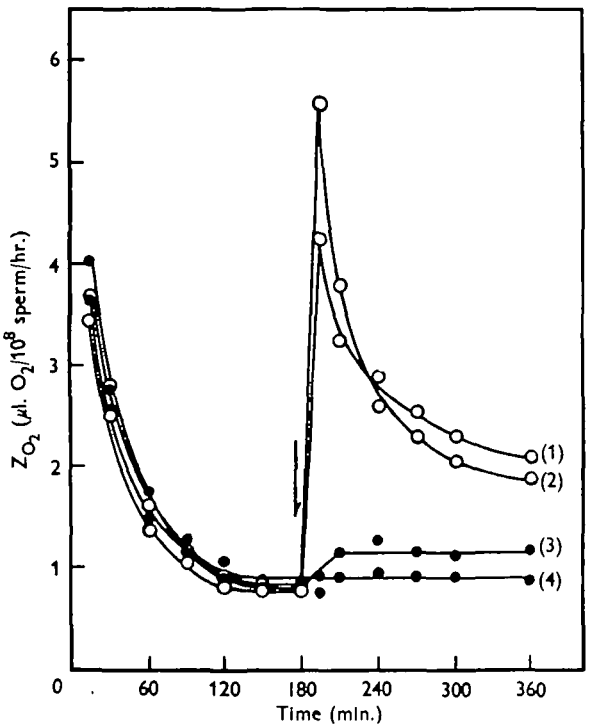

Fig. 4. Reversion from the azide-insensitive to the azide-sensitive respiration by the addition of DNP (1×10^{-4} M) and $ZnCl_2$ (1×10^{-4} M). *Hemicentrotus pulcherrimus*: 2·7 ml. of sperm suspension in the main chamber, 0·3 ml. of reagents in the side arm, tipped at $t = 180$. Dilution 1:20. 20° C. (1), +DNP; (2), +DNP+sodium azide (1×10^{-2} M); (3), +$ZnCl_2$; (4), +$ZnCl_2$ +sodium azide.

It has been mentioned that DNP can bring about an outburst of respiration if added to the low steady phase of dense suspensions. A similar effect was obtained by Rothschild & Tuft (1950) by the addition of trace metals such as copper and zinc, and the significance of the presence of these metals in natural sea water was suggested in relation to the Dilution Effect. Fig. 4 shows that the restored oxygen uptake after adding DNP and $ZnCl_2$ is also azide-sensitive. With DNP, however, the extra oxygen uptake is not completely eliminated by azide at 1×10^{-2} M, resulting in some rise in the level of the azide-insensitive residue. This is also seen from the experiment in which DNP is added at $t = 0$, as the following figures exemplify. For instance, figures of oxygen consumption in 1:20 suspension with

and without DNP are 166·2 and 154·3μl. respectively for the first hour, while those in the presence of azide (1×10^{-2} M) are 57·7 and 35·4μl. This difference is maintained for several hours. In other words, the azide inhibition of DNP-augmented respiration is not so thorough as respiration augmented by dilution, indicating some modification in metabolic systems by DNP. With $ZnCl_2$, on the other hand, this has not been observed.

With potassium cyanide, another cytochrome oxidase inhibitor, the respiration of sea-urchin spermatozoa is almost completely inhibited at the concentrations of 1×10^{-4} M and 1×10^{-5} M (after Robbie's method, 1946). The azide-stable respiration in dense suspensions is also inhibited by this inhibitor.

DISCUSSION

The present experiments point out the necessity of paying more attention to the Dilution Effect in the analysis of the action of metabolic activators and inhibitors on the respiration of sea-urchin spermatozoa. DNP, for example, causes a four- to fivefold increase in oxygen uptake of the steady state at a dilution of 1:10, but not at a dilution of 1:200. In the experiments using nitrogen mustards, Barron, Seegmiller, Mendes & Narahara (1948*a*) have reported some difference in the mode of action of these substances according to the sperm density.

Furthermore, the present results indicate that azide differs in its effect on the initial burst and on the subsequent steady respiration. Much evidence is available to show that 'activity' respiration is more sensitive to azide than 'resting' respiration (frog muscle, Stannard (1939); sea-urchin egg, Fischer, Henry & Low (1944); nerve tissue, Gerard & Dotty (1950)). It is, therefore, of interest that the initial burst seems to act like 'activity' respiration, and the steady-state respiration seems to correspond to 'resting' respiration. This is true in both dense and dilute suspensions, but of special importance is the fact that the azide-stable respiration of a dense suspension, which continues for a long time if left undiluted, can be changed reversibly to the azide-sensitive one on further dilution.

No difference was obtained in the respiratory quotient (R.Q.) of the initial and the subsequent respiration measured by the Warburg direct method, both having the values characteristic of carbohydrate metabolism (0·91–1·03).

Recently it has been reported that DNP also causes an increase in oxygen uptake, which can be abolished by azide in such a low concentration as to be ineffective on normal respiration (grasshopper eggs, Bodine (1950); sea-urchin eggs and sperm, Utida & Nanao (1954); toad embryos, Ishida, Uramoto & Maruyama, unpublished; etc.), and it is known that one of the actions of DNP is to convert the mitochondrial apyrase from a latent to an apparent state (cf. Lardy & Wellman, 1953). Maruyama (1954) has demonstrated in the toad embryo that azide almost eliminates the activity of the apparent apyrase (with DNP), leaving the latent apyrase activity (without DNP) unaffected. There is a possibility, therefore, that some enzyme system essential for the respiration and motility of sea-urchin spermatozoa is in a latent state before dilution and is converted into an apparent state on dilution.

Aside from such a latent-apparent mechanism, azide is a powerful inhibitor of cytochrome oxidase *in vitro*. Therefore it is quite probable that the cytochrome-cytochrome oxidase system begins to work on dilution. This proposition has been investigated, and the results will be presented in the next paper.

SUMMARY

1. The effects of 2,4-dinitrophenol (DNP) and sodium azide on the respiration of sea-urchin spermatozoa (*Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*) have been investigated in relation to dilution.

2. The initial outburst of respiration following dilution is higher in its rate and longer in its duration the more dilute the sperm suspensions are. After the initial high respiration has diminished, oxygen uptake in dense suspensions continues at a very constant rate for a long period of time, during which spermatozoa remain motile. A high level of respiration can be restored by further diluting a suspension in the steady state. In dilute suspensions, however, once the respiration has dropped, it can never be reactivated.

3. DNP (1×10^{-4} M) causes a marked rise in oxygen uptake when given during the steady state, but has little effect during initial burst.

4. Conversely, only the initial high respiration is inhibited by sodium azide, while the subsequent steady respiration is not affected by this poison. This is especially the case in dense suspensions.

5. When the initial high respiration is inhibited by azide at 1×10^{-2} M, the reduced level always coincides with that of the steady-state respiration. The respiration of sea-urchin spermatozoa thus seems to be separable into two components, one azide-sensitive and the other azide-insensitive.

6. The extra increase in oxygen uptake over the steady-state level on adding DNP or Zn in dense suspensions is also ascribed to an increment in the azide sensitive component.

7. Potassium cyanide inhibits not only the initial increase but also the steady-state respiration.

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