

THE RÔLE OF OXYGEN IN THE METABOLISM AND MOTILITY OF HUMAN SPERMATOZOA

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The experiments reported here are a continuation of the work on human spermatozoa which was begun with the object of studying the metabolic behavior of these cells. In previous papers (7, 8), the metabolism was shown to be almost exclusively glycolytic, the oxygen consumption being of such small magnitude that it could not properly be interpreted as a true respiration. Furthermore, no spectroscopic evidence of cytochrome could be found and it was observed that, in certain cases, motility was profoundly depressed in the presence of pure oxygen. These observations have been confirmed in every essential respect by Ross et al. (12) except that their mean figures for glycolysis are higher than those given by this author (7). Since the latter results were published, higher and more consistent figures² have been obtained from the combined spermatozoa of a small new group of donors. These figures are in closer agreement with those of Ross et al. and show, furthermore, that the level of aerobic glycolysis is virtually the same as that of the anaerobic.

The present extension of these studies was undertaken to determine more precisely the behavior of the spermatozoa under aerobic conditions and particularly to examine the link between glycolysis and respiration in these cells. The experiments entailed 1, determination of the activity of certain dehydrogenase systems; 2, the measurement of oxygen consumption in the presence of different substrates, and 3, testing for the presence of the cytochrome complex by indirect methods. Lastly, the toxic effect of high oxygen tensions was investigated from the point of view of a possible formation of hydrogen peroxide by the spermatozoa.

METHODS. The spermatozoa suspensions were prepared by methods described previously (7, 8). For the measurement of oxygen consumption, the cells were suspended in glucose-free Ringer-phosphate solution (pH 7.35) and shaken in Warburg manometers at 38°C. Various substrates were added directly to the cell suspensions in the Warburg vessels prior to temperature equilibration. The final concentration of each substrate was M/100. Cell motility was determined before and after every experiment by methods already

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² The new figures are as follows:

No. of expts.	60
mm ³ CO ₂ /10 ⁸ cells/hr. in N ₂	25
mm ³ CO ₂ /10 ⁸ cells/hr. in O ₂	24

It should be stated, however, that figures as high as these were obtained previously from some individual specimens (7).

described (8). For the determination of cytochrome and cytochrome oxidase activity, the p-phenylene diamine was added to the cell suspensions according to the techniques outlined by Keilin and Hartree (5) and Stotz et al. (13). These experiments will be described in detail below.

A modified Thunberg technique, which allowed simultaneous measurement of glycolysis and methylene blue reduction, was used to measure the dehydrogenase activity of the spermatozoa in the presence of certain substrates. The cell suspensions were prepared for the measurement of glycolysis (0.03M bicarbonate being substituted for phosphate) and equilibrated for 20 minutes at 38°C. with 95 per cent N₂—5 per cent CO₂. Methylene blue was placed in the side-arms of the Warburg vessels and added to the suspensions in the main vessels when equilibration was complete. Thereafter, at intervals, the manometers were removed temporarily from the water-bath to determine when reduction of the dye was complete.

EXPERIMENTS. The dehydrogenases of the spermatozoa were investigated using a variety of substrates which included glucose, succinate, pyruvate, fumarate and lactate among others. Only in the presence of glucose and succinate was any rapid or marked reduction of the methylene blue seen.³ The fastest reduction times were seen invariably in the presence of glucose and to a lesser degree when succinate was present. In the absence of substrate or in the presence of the other substrates mentioned above no marked reduction of the dye took place over a period of many hours. These results are in marked contrast to those of Lardy and Phillips (6) who showed that the reduction of methylene blue by bull spermatozoa took place rapidly in the *absence* of substrate and in the presence of succinate and fumarate but was inhibited when glucose was added.

The importance of the above results for human spermatozoa lies not so much in the reduction time values but in the active reduction in the presence of succinate. This was the first positive evidence obtained of the presence in the cells of the succinic dehydrogenase and, therefore, of the probable presence of the cytochrome system (2). This evidence was amplified further when malonate (M/100), a known inhibitor of the succinic dehydrogenase (11), was added to the spermatozoa suspensions reducing the dye in the presence of succinate. Malonate almost completely inhibits this reduction but has no effect on the reduction time in the presence of glucose.

Effect of succinate on the oxygen consumption. These results suggested a re-examination of the oxygen consumption of the spermatozoa particularly in relation to the presence of the cytochrome complex. It seems definitely established (2) that the succinic dehydrogenase is, structurally and chemically, intimately linked with the cytochrome system. When the dehydrogenase is reduced, it is oxidized by cytochrome C almost instantaneously (4) and not to any appreciable

³ There are certain objections to the use of intact cells for the measurement of enzyme activity, mainly that of the possible difference in penetration of the intact cell of different substrates. The figures given in table 1 serve merely to indicate that enzymatic activity towards a particular substrate is present and are not intended to demonstrate the *maximal* activity toward that substrate.

extent by molecular oxygen. As shown above, methylene blue can also act as the hydrogen acceptor. Therefore, it seemed probable that, if succinate was substituted as a substrate for glucose, the oxygen consumption of the spermatozoa would be increased. This proved to be the case. The figures in table 1 show the results obtained when oxygen consumption was measured in the absence of substrate and in the presence of glucose and succinate.

The figures obtained in the presence of glucose are similar to those reported previously (7). The increase in oxygen consumption in the absence of substrate, while small, is significant since it appeared in every experiment. The inhibiting effect of glucose has also been noted in ejaculated bull spermatozoa (6) but not in cells obtained from the epididymis (3). However, in the presence of succinate the human cells show a relatively high and stable oxygen consump-

TABLE 1
Oxygen consumption of human spermatozoa in the presence of different substrates

NO. OF EXPTS.	NO. SUBSTRATE	GLUCOSE	SUCCINATE	D-PHENYLENE DIAMINE (M/50)
20	1.43*	1.34	6.3	18

* The figures represent the oxygen consumption per $\text{mm}^3/10^8$ cells/hour.

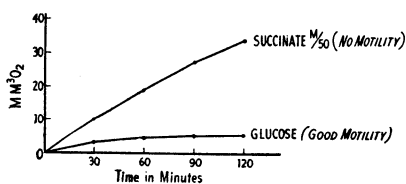


Fig. 1

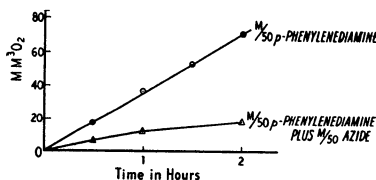


Fig. 2

Fig. 1. The oxygen consumption and motility of human spermatozoa in the presence of glucose and sodium succinate.

Fig. 2. The oxidation of p-phenylenediamine by human spermatozoa.

tion (fig. 1). In every case it was evident that the oxidation of succinate was vigorous and progressive compared to that of glucose. The addition of malonate or of azide inhibits the succinate oxidation to the autoxidation level. This evidence supplements that given above for the inhibition of methylene blue reduction by malonate in the presence of succinate. It also complements the observations of other authors (2, 4) which show the oxidation of succinate to be mediated by the azide-sensitive cytochrome complex.

Neither fumarate, lactate or pyruvate increases the oxygen consumption above the autoxidation level. The failure of the spermatozoa to oxidize lactate is to be expected since they produce as much lactic acid in oxygen as they do in nitrogen. Their failure to oxidize fumarate, on the other hand, suggests that the oxidation of succinate is a one-step process, namely, the removal of hydrogen to form fumaric acid and the subsequent accumulation of the latter compound.

In spite of the vigorous oxidation of succinate by the spermatozoa, any energy made available in this reaction is not coupled with motile activity. In *all* experiments in which succinate was substituted for glucose, the motility failed as rapidly as if no substrate was present.

The oxidation of p-phenylenediamine. As a further check on the presence of the cytochromes and cytochrome oxidase in the spermatozoa, the ability of the cells to oxidize p-phenylenediamine was determined. Preliminary experiments showed that the autoxidation of this substance was negligible but that it was rapidly oxidized by the spermatozoa. The concentration necessary to produce maximal oxidation was M/50. The results are shown in table 1.

Keilin and Hartree (5) and Stotz et al. (13) have shown that the oxidation of p-phenylenediamine is mediated through cytochrome C and cytochrome oxidase and that its oxidation can be used as an indicator of the presence of such a system in cells. These authors have shown further that cytochrome B can be oxidized by molecular oxygen, but is insensitive to the inhibitory effect of cyanide and azide, and therefore can act independently of cytochrome oxidase. Stotz et al. (13) demonstrated a relatively cyanide-insensitive oxidation of p-phenylenediamine and showed conclusively that the remaining catalysis was due to cytochrome B. In the case of human spermatozoa approximately 20 per cent of the oxidation of p-phenylenediamine escapes azide inhibition (fig. 2). In line with the evidence of Stotz et al., this can be attributed to the presence of cytochrome B.

In view of the evidence given above, it is reasonable to conclude that in spite of the very small oxygen consumption of human spermatozoa, these cells contain a virtually complete respiratory system, namely, succinic dehydrogenase, cytochromes B and C and cytochrome oxidase,⁴ and are capable of carrying on oxidative processes if the appropriate substrate is present.

The effect of high oxygen tensions on motility. Any analysis of the rôle of oxygen in the metabolism and motility of human spermatozoa would not be complete without a consideration of the phenomenon first described by this author (7) and confirmed by Ross et al. (12), namely, that of the loss of motility which often occurs when these cells are exposed to oxygen at 38°C. for several hours (table 2). It has already been shown that the spermatozoa will retain maximal motility in nitrogen at 38°C. for many hours (7). This evidence, coupled with the low oxygen consumption of the cells and their ability to get all their motile energy from a process which involved the breakdown of glucose to lactic acid, suggested that the toxicity of oxygen may be related to a similar process which retards the growth of certain anaerobic bacteria in air. MacLeod and Gordon (9) have shown that certain of these bacteria under aerobic conditions produce

⁴ The absolute amount of these substances in the spermatozoa is exceedingly difficult to determine. Only a few milligrams of tissue (wet) are available for any given experiment and, since isolation of enzymes requires relatively large amounts of fresh material, certain extensions of this work on human spermatozoa are severely limited. As would be expected, the addition of cytochrome C to the intact cells in the presence of p-phenylenediamine causes no increment in the oxygen consumption.

enough hydrogen peroxide in the course of their metabolism either to destroy the growing colony or prevent its multiplication. Accordingly, experiments were designed to determine whether a similar phenomenon in human spermatozoa might be responsible for the loss of motility in high oxygen tensions. Two spermatozoa suspensions were set up in the usual way for measurement of aerobic glycolysis and equilibrated with 95 per cent O₂—5 per cent CO₂ for 15 minutes at 38°C. In similar fashion, another two suspensions of the same spermatozoa were set up, except that 0.1 cc. of dilute hemoglobin or catalase⁵ was added to each on the assumption that if any hydrogen peroxide was produced in the system, it would be destroyed instantaneously either by the peroxidase activity of the hemoglobin or the catalatic activity of the catalase. The manometers containing the spermatozoa exposed to oxygen were then run at 38°C. for periods up to 9 hours and the motility examined at the end of the experiment. Typical experiments are shown in table 2. In every case where loss of motility oc-

TABLE 2
Spontaneous loss of motility in 95 per cent oxygen and the protective effect of catalase

EXPT. TIME	ACTIVITY OF SPERMATOZOA AT END OF EXPERIMENT		
	95% nitrogen—5% CO ₂	95% O ₂ —5% CO ₂	95% O ₂ —5% CO ₂ and Catalase
<i>hours</i>			
8	4 (58%)	No motility	4 (55%)
8	3+ (50%)	No motility	3+ (52%)
7	4 (60%)	No motility	4 (50%)
9	3 (50%)	No motility	3 (49%)
5	3+ (32%)	1 (10%)	3+ (32%)
5	4 (68%)	2 (10%)	4 (70%)

The figures in parentheses denote the number of motile cells. The other figures denote the quality of motility, 4 being maximal motility.

curred in the presence of oxygen, the addition of hemoglobin or of catalase was sufficient to maintain a maximal motility comparable to that of the same spermatozoa under anaerobic conditions.

These results were indicative of the production of hydrogen peroxide by the spermatozoa. It followed, therefore, that its formation and the resulting toxicity must be within the limits of the small oxygen consumption of the spermatozoa. This assumption was tested by adding amounts of hydrogen peroxide with varying oxygen equivalents to spermatozoa at 38°C. and determining its effect on motility. It was found that a concentration of peroxide⁶ equivalent to between 10 and 20 mm.³ of oxygen was sufficient to destroy the motility of 200 million spermatozoa within 5 minutes. These oxygen equivalents are within

⁵ I am indebted to Dr. Kurt G. Stern of Yale University for a generous sample of catalase of known purity.

⁶ The oxygen equivalent of peroxide was determined by adding catalase to measured amounts of peroxide at 38°C. and measuring manometrically the oxygen produced.

the range of oxygen consumption which might be expected of the spermatozoa over a period of several hours. These experiments serve to demonstrate the sensitivity of the spermatozoa towards low concentrations of peroxide and indicate the nature of the chemical mechanism for the loss of motility which occurs when the cells are exposed to high oxygen tensions. Lardy and Phillips (6) have observed that bull spermatozoa show a similar sensitivity towards hydrogen peroxide.

Further investigation of the effect of oxygen on spermatozoa motility is in progress and preliminary results show that at low oxygen tensions (between 5 and 10 per cent) the toxicity is eliminated.

DISCUSSION. The results presented here and those published previously (7, 8) indicate that oxygen is not of primary importance in the metabolism of human spermatozoa and is not essential for the maintenance of motility. On the other hand, the presence in these cells of a more or less complete respiratory system would suggest 1, that they once possessed a high respiratory activity, or 2, that their ultimate function required oxidative activity. Recent work on the metabolism of bovine epididymal spermatozoa (3) shows that the sperm in the epididymis rapidly oxidize glucose but, when ejaculated, they not only fail to oxidize glucose but the respiration is actually inhibited by this substance (3, 6). It is possible that a similar metabolic change takes place in human spermatozoa in passage from the epididymis to the seminal fluid.

In view of the rate of p-phenylenediamine oxidation, the cytochrome system is potentially capable of carrying a large oxygen consumption. That it does not do so, except in the oxidation of succinate, cannot be explained at present although it is obvious that the link between glycolysis and respiration has been broken or perhaps never existed. The spermatozoa cannot oxidize lactate or pyruvate and there is no evidence of the Pasteur effect. In the latter respect, the metabolism of human spermatozoa is, like that of jejunal mucosa (11), unique among mammalian tissues.

The conclusion has already been made (8) that human sperm derive enough energy for motility from the breakdown of glucose to lactic acid. This conclusion receives additional support from the evidence given here that the oxidation of succinate, presumably an energy-yielding reaction, does not maintain motility in the absence of glucose.

The failure of motility which often appears at 38°C. in high oxygen tensions and which can be prevented by catalase (or peroxidase) points definitely to the production of hydrogen peroxide in the system. The evidence is only indirect since the peroxide cannot be detected chemically. But the small amount of peroxide necessary to destroy motility is not easily susceptible of chemical analysis if, indeed, it exists as such for any length of time. That peroxide should be produced at all in the course of the metabolism of the spermatozoa is a matter of considerable interest since no formation of this substance has as yet been demonstrated in the cells of higher organisms (10). But such cells invariably have an active respiration and contain enough catalase rapidly to destroy any peroxide which may be formed (10). The virtual absence of respiration in the

spermatozoa and their pronounced sensitivity towards peroxide poisoning suggests a deficiency of catalase in these cells.

Finding the source of the peroxide in the metabolism of the spermatozoa awaits further analysis of the enzyme systems. Autoxidation of a flavoprotein is a strong possibility since hydrogen peroxide is the end-product of such a reaction. Theorell (14, 15) has shown that the reoxidation of flavoprotein is normally accomplished by the terminal respiratory system (cytochrome C) but if it cannot do so, it will react directly with molecular oxygen, particularly at *high oxygen pressures*. Since the cytochrome system in the spermatozoa is so inactive and since the possible peroxide formation takes place only at high oxygen pressure, the mechanism suggested above is tenable.

SUMMARY

1. The rôle of oxygen is not of primary importance in the metabolism and motility of human spermatozoa.
2. These cells possess a complete terminal respiratory system but can not oxidize glucose or its anaerobic breakdown products, lactate and pyruvate.
3. Succinic acid is oxidized but this reaction is not coupled with motility in the sense that any energy made available can be used for motile activity.
4. In regard to the depressing effect of high oxygen pressure on the motility, evidence is presented suggesting the production of small amounts of hydrogen peroxide by the spermatozoa and a possible mechanism for its production is discussed.

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