STIMULATION OF RESPIRATION OF RABBIT, HUMAN AND COCK SPERMATOZOA BY LIGHT AND CERTAIN CHEMICALS

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Summary. Rabbit, human and cock spermatozoa collected and washed without exposure to light respired very slowly or not at all. Brief exposure to white light of less than a minute's duration stimulated normal rates of respiration. Ubiquinone (UQ, Co-enzyme Q), seminal plasma, in-utero incubation, and dinitrophenol each markedly stimulated the respiration of spermatozoa protected from light. Ubiquinone₁₀ was effective with cock spermatozoa while UQ₁ was superior to UQ₃ or UQ₄ with rabbit spermatozoa. Ubiquinone₁ appeared more effective than UQ₃ for human spermatozoa.

INTRODUCTION

In the course of experiments on the effect of the female tract on metabolism of spermatozoa (Hamner & Williams, 1962, 1963), it was observed consistently that respiration of washed spermatozoa virtually came to a stop after 3 hr in the Warburg respirometer. In view of the reports that exposure of spermatozoa to light for several hours inhibited motility and respiration (Norman & Goldberg, 1959; Norman, Goldberg & Porterfield, 1960), experiments were carried out in which spermatozoa were completely protected from light during collection, washing and respiration measurements. A prolongation of the period of active respiration was the expected result. As already reported briefly (Hamner & Williams, 1961; Williams & Hamner, 1962) the observed result was quite the opposite. Rabbit, cock and human spermatozoa protected from light failed to respire or respired at a greatly diminished rate. It appeared that under the usual conditions for studying sperm respiration, most, if not all, the oxygen uptake resulted from the effect of laboratory light on spermatozoa. The present report concerns the time of exposure necessary for maximum stimulation of oxygen uptake, the effect of in-utero incubation, seminal plasma, ubiquinones, and dinitrophenol on sperm respiration.

MATERIALS AND METHODS

Rabbit spermatozoa were collected with an artificial vagina, and washed, in a totally dark room and the semen was received into test tubes painted black. The temperature of the artificial vagina was 44° C and was critical, since lower

temperatures prolonged the collection time and higher temperatures affected the spermatozoa adversely. The semen was passed into centrifuge tubes painted black through a double layer of cheese-cloth to remove the plasma clot. The semen was diluted to twice its original volume with calcium-free Krebs'-Ringer phosphate (KRP), pH 7.0 (Umbreit, Burris & Stauffer, 1957), and centrifuged at 142 g for 8 min. The supernatant was poured off and the packed spermatozoa were resuspended to twice the original volume with KRP and centrifuged at 10 g for 2 min to remove the major portion of the contaminating epithelial cells, leucocytes and amorphous yellow pigments. The supernatant containing spermatozoa was centrifuged at 142 g for 8 min. The supernatant solution was removed and the washed spermatozoa were resuspended to the desired volume in KRP solution. Cock and human spermatozoa were washed in the same way as rabbit spermatozoa except that the slow centrifuging step was omitted. Tyrode's solution, pH 7.25, was used as the suspending medium for cock spermatozoa.

For each experiment all ejaculates were collected, washed and pooled in total darkness. Portions were then removed from the pool and exposed to light. For exposure to white light, 2 to 4×10^8 washed spermatozoa in 1 ml KRP in a 50 ml Pyrex beaker were irradiated for various lengths of time with a 100 Watt tungsten filament lamp suspended 20 cm above the beaker while the contents were stirred gently. Except for experiments on time of exposure to light, the oxygen uptake of spermatozoa exposed to light was measured in unpainted flasks. No increase in temperature was observed during light exposure.

Oxygen uptake studies were carried out in a Warburg constant volume respirometer at 37° C with a total liquid volume in each flask of 2.6 ml containing 2 to 4×10^8 spermatozoa per flask. Two-tenths ml of 20 % potassium hydroxide was placed in the centre well; 2.5 mg streptomycin and 2 mg glucose were added to each flask. Results are expressed as Z_{O_2} or $\mu l O_2$ taken up per 10^8 sperm cells/hr.

The spermatozoa used in the studies on light exposure for various times were exposed to the light source for the desired length of time and returned to painted Warburg flasks. The flasks were painted with a thick coat of flat black paint. The manometers were painted from the flask to 0.5 in. above and below the T-joint. Ubiquinones (3 mg) were dissolved in 0.2 ml hot 95 % ethanol, diluted to 10 ml with hot water obtaining an opalescent but uniform solution, 300 $\mu g/ml$. Usually 0.1 ml (30 μg) were added per Warburg flask. The ubiquinones were of high purity, each free of other members of the series.

For studies on spermatozoa incubated in the rabbit uterus the following technique was used: Pooled washed rabbit spermatozoa (7.9×10^8 spermatozoa/ml) protected from light were incubated *in vitro* for 6 hr in stoppered 13 $\times$ 75 mm painted test tubes in a 37° C water bath. A 1.5 ml sample taken from this pool was incubated *in utero* in the rabbit's uterus ligated at the cervix only. After the 6-hr incubation period 0.5 ml were transferred in the dark to painted and unpainted Warburg flasks from the in-vitro incubation tube. The ligated rabbit uterus containing the in-utero incubated spermatozoa was removed from the rabbit, and the spermatozoa were flushed from the uterus in the dark, washed and transferred to painted Warburg flasks.

RESULTS

The effect on respiration of exposing spermatozoa from three species to white light is shown in Table 1. The oxygen uptake in the dark was frequently nil. Spermatozoa protected from light showed rates of oxygen uptake considerably

TABLE 1
EFFECT OF LIGHT ON OXYGEN UPTAKE OF SPERMATOZOA

	Rabbit		Cock		Man	
No. experiments	24		11		6	
Light exposure	No	Yes	No	Yes	No	Yes
Mean ZO_2	1.7	5.4	4.8	9.0	2.6	9.2
Average difference between means, $\bar{d}$	3.7		4.3		6.6	
Sd	0.395		1.13		1.30	
Probability from <i>t</i> -test	<0.001		<0.005		<0.005	

t-test according to Snedecor (1961).

Light exposures were from 1 to 3 min followed by incubation in unpainted flasks. Unexposed spermatozoa were incubated in painted flasks.

less than half that of published values (Lardy & Phillips, 1943; MacLeod, 1943). Stimulation of oxygen uptake by light was highly significant statistically.

TEXT-FIG. 1. Oxygen uptake of rabbit spermatozoa exposed to white light, 100 watt bulb at 20 cm, at times indicated. Sperm count, 2.12×10^8 per flask. x, no light; o, 5 sec; □, 180 sec of light.

With cock spermatozoa which usually had an appreciable oxygen uptake in the dark, light exposure doubled the rate of oxygen uptake.

Text-fig. 1 shows the time of light exposure required to stimulate the subsequent oxygen uptake of rabbit spermatozoa incubated in painted flasks.

Five sec of light gave a readily detectable increase in oxygen uptake. Increasing light exposure to 5 min did not further increase the rate over that obtained with 3 min exposure.

Text-fig. 2 illustrates the time of light exposure required for maximum stimulation of the rate of oxygen uptake of cock spermatozoa. Exposure of 30 sec gave essentially maximum rate and extending the time to 5 min did not increase it further.

TEXT-FIG. 2. Oxygen uptake of cock spermatozoa exposed to white light, 100 watt bulb at 20 cm, at times indicated. Sperm count, 3.5×10^8 per flask. x, no light; O, 5 sec; Δ, 30 sec; □, 180 sec.

Since under natural conditions of mammalian reproduction spermatozoa are not exposed to light, the question came to mind—do spermatozoa normally respire after deposition in the female tract? Text-fig. 3 shows that incubation *in utero* stimulated a higher rate of oxygen uptake than continuous exposure to light in transparent Warburg flasks (middle curve).

Spermatozoa incubated in light *in vitro* for 6 hr lose their ability to take up oxygen. Since the spermatozoa showing the oxygen uptakes indicated by the two lower curves in Text-fig. 3 were incubated 6 hr in the dark, it is apparent that such incubation maintains the ability of spermatozoa to take up oxygen subsequently in response to light.

The initial studies on light stimulation utilized washed spermatozoa from which the seminal plasma had been removed as thoroughly and as rapidly as possible after ejaculation. As shown in Table 2 spermatozoa in whole semen respire at a good rate without exposure to light. Exposure of whole semen to

light in most instances did not further increase the rate of oxygen uptake. The rate of oxygen uptake of spermatozoa in whole semen in both light and dark was on the average greater than that of washed spermatozoa exposed to light.

TEXT-FIG. 3. Comparison of rabbit sperm respiration stimulated by light and incubation *in utero*. Light exposure (middle curve) was obtained by ordinary room illumination of unpainted Warburg flasks. In-utero incubation was for 6 hr prior to respiration studies. Sperm count, 2.25×10^8 per flask. $\times$, *In vitro* (dark); $\circ$, *in vitro* (light); Δ , *in utero* (dark).

Seminal plasma appears to contain one or more substances that markedly stimulate the respiration of spermatozoa protected from light.

TABLE 2

EFFECT OF LIGHT AND SEMINAL PLASMA ON SPERM RESPIRATION

Experiment	Species	Washed spermatozoa		Whole semen	
		Dark	Light	Dark	Light
159	Man	2.4*	12.3	10.0	9.0
168	Man	1.2	6.4	11.0	9.0
201	Man	Not done	Not done	14.9	14.8
205	Man	2.6	6.4	12.4	19.2
169	Rabbit	3.8	7.2	10.0	13.6
170	Rabbit	4.0	7.6	5.1	6.4
204	Rabbit	0.5	3.2	2.6	3.8

* Values are Z_{O_2} and are corrected for oxygen uptake by seminal plasma which averaged 10 $\mu\text{l/hr/ml}$. Light exposure was from 3 to 5 min.

At this point it appeared desirable to attempt to duplicate with pure substances the stimulating effect on sperm respiration of light, uterine secretions and seminal plasma reported here and the effect of oviduct fluid reported earlier (Hamner & Williams, 1963). Because of the report by Page, Smith, Gale, Polin & Folkers (1961) that the 6-chromanol of hexahydro-UQ₄ prolonged

the motility of cock spermatozoa, various ubiquinones were investigated. Ubiquinone₁₀, as shown in Table 3, markedly stimulated the respiration of cock spermatozoa protected from light. Unfortunately the response to room light was poor in this experiment. Ubiquinone₁₀ also stimulated respiration of spermatozoa in the light.

TABLE 3
EFFECT OF UBIQUINONE₁₀ ON COCK SPERM RESPIRATION

Supplement to flask	$\dot{V}O_2$			
	Illumination	1st hr	2nd hr	3rd hr
None	None	0.0	0.5	0.0
30 μ g UQ ₁₀	None	11.0	9.3	7.9
None	Room light	3.3	3.3	0.0
30 μ g UQ ₁₀	Room light	10.4	11.0	7.6

Spermatozoa were exposed to room light only during manometric studies by incubation in unpainted flasks.

The effect of UQ₁ and UQ₃ on the respiration of rabbit spermatozoa was investigated. Using spermatozoa protected from light UQ₁ gave a greater rate of respiration than UQ₃ or controls. Table 4 shows the results of the experiment comparing the response to the co-enzymes with that of light. It appears that

TABLE 4
EFFECT OF UBIQUINONES ON RESPIRATION OF RABBIT
SPERMATOZOA PROTECTED FROM LIGHT

Treatment	$\dot{V}O_2$	
	30 min	60 min
Kept in dark	2.6	3.2
Exposed to light for 5 min	9.7	7.9
Dark + UQ ₁ , 30 μ g per flask	12.0	9.6
Dark + UQ ₃ , 30 μ g per flask	5.8	4.6

UQ₁ was more effective than light or UQ₃. In two additional experiments, UQ₁₀ was less effective than light in stimulating respiration of rabbit spermatozoa (Table 5).

The effectiveness of UQ₁ and UQ₃ in stimulating the respiration of human spermatozoa was compared in two experiments as shown in Table 6. As with rabbit spermatozoa, UQ₁ appears to be more effective than light or UQ₃ in stimulating respiration. Ubiquinone₃ stimulated respiration to about the same degree as light; however, the response to light was below average in these two experiments.

Compounds related to ubiquinones, i.e. tocopherol acetate, tocopherol phosphate and menadione had no consistent effect on the respiration of rabbit spermatozoa. In view of the possibility that light exposure may cause hydrogen

peroxide formation, catalase and hydrogen peroxide and glucose oxidase were added separately to Warburg flasks both in light and dark conditions without significant effect on the respiration of spermatozoa.

The possibility was considered that light may stimulate respiration by uncoupling phosphorylation from oxidation. The effect of the known uncoupling

TABLE 5
EFFECT OF UBIQUINONE₁₀ ON RESPIRATION OF RABBIT
SPERMATOZOA PROTECTED FROM LIGHT

<i>Treatment—additions per flask</i>	$\bar{Z}_{O_2}$	
	<i>Exp. 153</i>	<i>Exp. 162</i>
Kept in dark	2.0	1.0
Exposed to light for 3.5 min	4.6	8.1
Dark + UQ ₁₀ , 30 µg per flask	3.7	4.8

agent, 2,4 dinitrophenol (DNP), was compared with light in three experiments. In the dark DNP caused a greater stimulation of respiration than light in two of the three experiments (Table 7).

Our colony of bucks yielded spermatozoa which when protected from light consistently showed a low Z_{O_2} of less than 2.0 for a series of over thirty experiments. During a subsequent period in a recent series of twenty-three experiments the Z_{O_2} values of spermatozoa in the dark increased to 5.0. Exposure to

TABLE 6
EFFECT OF LIGHT AND UBIQUINONES ON RESPIRATION
OF HUMAN SPERMATOZOA

<i>Treatment</i>	<i>Exp. 249</i>	<i>Exp. 252</i>
Kept in dark	2.1	3.1
Exposed to light for 3 min	6.2	4.8
Dark + UQ ₁ , 30 µg per flask	9.2	8.5
Dark + UQ ₃ , 30 µg per flask	5.7	6.0

light increased the average Z_{O_2} to 7.7. It was noted that several of the bucks in use gave semen heavily contaminated with epithelial cells and yellow amorphous material. A significant proportion of the spermatozoa was attached to the pigment particles. After elimination of these bucks and using clean semen, the average Z_{O_2} returned to 1.5 for the spermatozoa protected from light. Light exposure increased this to 5.5, hence the respiratory response to light was restored.

DISCUSSION

In view of the marked effect of light on the respiration of spermatozoa, it would appear that experimental re-examination of many of the previous results obtained on respiration of spermatozoa is perhaps necessary. White (1956) has

pointed out that certain widely held beliefs concerning sperm metabolism may be a function of the diluent used. For example, use of a low phosphate medium with ram spermatozoa results in high respiration and low glycolysis. Increasing the amount of phosphate gave low respiration and high glycolysis. Uncontrolled light exposure could lead to similar observations resulting from environmental conditions rather than the inherent metabolism of the sperm cell.

TABLE 7
EFFECT OF LIGHT AND 2,4 DINITROPHENOL ON RESPIRATION OF
RABBIT SPERMATOZOA

Treatment of washed spermatozoa	ΣO_2		
	Exp. 72	Exp. 261	Exp. 267
Dark	2.5	0.0	4.8
Light exposure (3 min)	11.5	2.9	6.3
Dark + DNP*	6.6	9.1	13.4
Light + DNP	12.8	Not done	15.1

* 2,4 Dinitrophenol = 4 μ g per flask.

The significance of the stimulation of respiration of spermatozoa by light, by substances in seminal plasma, and by in-utero incubation is not apparent at the present time. Some evidence for a relationship between stimulation of respiration by in-utero incubation and by capacitation has been obtained (Hamner & Williams, 1963). If exposure to ordinary room light and the consequent stimulation of respiration would capacitate spermatozoa, the phenomenon of capacitation would not have been discovered. It is possible, however, that irradiation with a specific wavelength of light of specific intensity would achieve capacitation and the high rate of respiration exhibited by spermatozoa after incubation *in utero*.

The effectiveness of ubiquinones in stimulating respiration of spermatozoa protected from light suggests that these co-enzymes and in-utero incubation affect the electron transport system rather than the citric acid cycle or glycolysis. The fact that UQ₁₀ stimulated respiration in an experiment in which the light response was poor (Table 3) suggests that light and the ubiquinones operate by different mechanisms. It is possible at present that seminal plasma and in-utero incubation stimulated respiration by virtue of their ubiquinone content.

A known uncoupler of oxidative phosphorylation, dinitrophenol, stimulated respiration of spermatozoa protected from light. This suggests that some of the agents reported herein that stimulate respiration may be uncouplers.

It should be emphasized that the present report on stimulation of respiration of spermatozoa by light is not necessarily in conflict with earlier reports on the detrimental effects of light on spermatozoa. Norman & Goldberg (1959) and Norman *et al.* (1960), using bull spermatozoa collected in light in the usual manner, found that several hours exposure to light markedly reduced the motility and fertilizing ability of spermatozoa. The wavelength for maximum damage

was 4400 Å. Collection methods and active wavelength were markedly different from those used herein, and the exposure times were hours in contrast to the 30 to 60 sec needed for stimulation of respiration.

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