Identification of Sperm Stimulating Factor of Rabbit Oviduct Fluid.* (29546)

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The respiration of rabbit spermatozoa was markedly stimulated by incubation in the uterus of an estrous rabbit for 6 hours or in oviduct fluid(1,2). The present investigation was undertaken to determine the factor or factors responsible for this stimulation of sperm respiration and to find the optimum conditions for maximum stimulation.

Materials and methods. Semen samples from all species except bovine were pools of individual ejaculates. For each experiment semen pools were obtained from 6 New Zealand white rabbits 1 to 2 years old by artificial vagina, 4 human donors of proven fertility 30 to 45 years old, 2 Leghorn roosters by the method of Burrows and Quinn(3) and from a Jersey bull by electroejaculation. For repeat experiments semen pools from the same individuals were used. Immediately after ejaculation the semen was added to 2 volumes of calcium-free Krebs-Ringer phosphate buffer and the spermatozoa washed free of seminal plasma as previously described(4). Sperm respiration was studied in ordinary Warburg flasks using the method suggested by Pardee(5), and modified by Krebs(6) for studying the respiration of cells in the presence of CO_2. Each flask contained 1.2 to 2.8 $\times$ 10^8 twice-washed sperm, 2.5 mg streptomycin, 4 mg glucose, $NaHCO_3$ at 1.18, 2.36, 3.54 or 4.72 $\times$ 10^{-3} M amounts in equilibrium with 1, 2, 3 or 4% CO_2 respectively at pH 7.0. Because HCO_3^- is known to be in equilibrium with CO_2 and $CO_3^=$, the effects ascribed to HCO_3^- refer to all 3 molecular species. Calcium-free Krebs-Ringer phosphate solution with 0.1 M tris buffer, pH 7.0, was used with rabbit or human spermatozoa (the 0.1 tris buffer was omitted when rooster or bull spermatozoa were studied) to make a total volume of 2.4 ml per flask. To the side arm of each flask appropriate amounts of Krebs' "CO_2-buffer" (4 M 2,2'-Iminodiethanol with 0.1% thiourea) were added to give a 1, 2, 3 or 4% CO_2 atmosphere above the fluid after equilibrium. Flasks having no CO_2 in the atmosphere had 0.2 ml of 20% KOH in the center well. After all additions had been made to the flasks and the flasks connected to the manometers the flasks that were to have 2, 3 or 4% CO_2 in the atmosphere were gassed with 5% CO_2-95% O_2 for 1-2 minutes. The flasks were then allowed to equilibrate for 10 minutes with the stopcocks open, after which time the stopcocks were closed and 15 minutes allowed for the atmosphere above the fluid in the flask to reach the proper CO_2 concentration and equilibrium. Flasks containing no CO_2 and 1% CO_2 were closed the entire 25-minute period so that all CO_2 would be removed or increased to 1%. The pH in all flasks was determined at the end of each experiment insuring that the medium had remained at pH 7.0, and that the proper CO_2 equilibrium had been reached. Oxygen uptake was usually expressed as ZO_2 *i.e.*, μl O_2 taken up/10^8 sperm/hr. Motility of the sperm in each flask was checked to be sure that they were motile throughout the experiment. The oviduct fluid was collected by a published method(1). Dialysis of oviduct fluid was carried out in cellophane tubing against distilled water for 24 hours. The heating of oviduct fluid was done at 100°C for 10 minutes in a stoppered 13 by 100 mm tube. When whole or heated oviduct fluid was used in respiration studies determination of the HCO_3^- content was made(7), and a volume equivalent to 2.36 $\times$ 10^{-3} M HCO_3^- in the flask medium was used in conjunction with 2% CO_2 to maintain the pH at 7.0. Additions of oviduct fluid were made at the expense of the phosphate buffer.

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TABLE I. Stimulation of Rabbit Sperm Respiration by Whole, Heated, and Dialyzed Oviduct Fluid and $NaHCO_3$.

Exp No.	Buffer, no additions	Oviduct fluid			2% CO_2 2.36 × 10^{-3} M $NaHCO_3$
		Dialyzed	Whole	Heated	
340	4.1*	—	6.8	—	12.7
351	3.5	2.2	8.5	10.4	—
369	3.4	3.0	5.1	10.0	7.3
370	6.7	—	9.1	10.2	—
372	6.7	7.0	9.2	21.0	8.8
373	7.1	9.2	12.2	13.4	13.1
Avg	5.3	5.7	8.5†	13.0	10.5

* Values are ZO_2 (μl oxygen taken up/10^8 sperm/hr).
† Significantly different from mean of first column, $P < .005$.

Results. The effect of heating and dialysis of oviduct fluid on the sperm stimulating factor was first investigated. Table I shows that dialysis completely removed the stimulating factor from oviduct fluid in 3 of 4 experiments, and the difference between the ZO_2 (μl O_2 taken up/10^8 spermatozoa/hour) in dialyzed oviduct fluid and buffer control compared to whole oviduct fluid was highly significant(8). Heating of oviduct fluid removed none of the activity. HCO_3^- gave ZO_2 values equivalent to those obtained with whole oviduct fluid. This information plus the fact that oviduct fluid had a pH of 7.8-8.0 which went to pH 9.0-9.3 in air at room temperature after 3 to 4 hours together with the observation that acidification plus aeration removed respiration stimulating factor suggested that HCO_3^- was involved.

Thirteen determinations of HCO_3^- content in oviduct fluid showed an average concentration of 28.2 × 10^{-3}M. Dialysis of oviduct fluid removed all of the HCO_3^-, but heating had no effect on the HCO_3^- content.

Since HCO_3^- appeared to be the stimulatory agent present in oviduct fluid, an attempt was made to establish if there was an optimum concentration of HCO_3^- which would give maximum sperm respiration in several species. Rabbit spermatozoa gave maximum respiration at 2.36 × 10^{-8} M HCO_3^- (Table II). However, human, rooster, and bull spermatozoa gave maximum respiration at 3.54 × 10^{-3} M HCO_3^-.

Oviduct fluid showed maximum stimulation of respiration of spermatozoa when diluted 1:6 in the Warburg flask (Fig. 1), and a sharp reduction in sperm respiration occurred above that concentration.

Two experiments designed to elucidate the

TABLE II. Effect of Increasing Bicarbonate Concentrations on Sperm Respiration.

Species	Control No CO_2 No $NaHCO_3$	1% CO_2 1.18 × 10^{-3} M $NaHCO_3$	2% CO_2 2.36 × 10^{-3} M $NaHCO_3$	3% CO_2 3.54 × 10^{-3} M $NaHCO_3$	4% CO_2 4.72 × 10^{-3} M $NaHCO_3$	No. of experiments
Rabbit						3
1st hr	4.2*	5.7	12.5	9.2	8.0	
Total 3 hr	13.2	18.6	25.9	24.6	17.3	
Human						3
1st hr	1.4	5.2	5.0	13.7	9.5	
Total 3 hr	4.3	9.4	9.7	19.7	13.7	
Rooster						3
1st hr	3.2	3.6	5.4	7.9	7.5	
Total 3 hr	8.5	14.0	13.2	16.1	11.6	
Bull						2
1st hr	6.9	10.1	12.0	15.4	12.7	
Total 3 hr	22.6	28.0	30.1	39.1	25.4	

* Values are μl oxygen taken up/10^8 sperm.

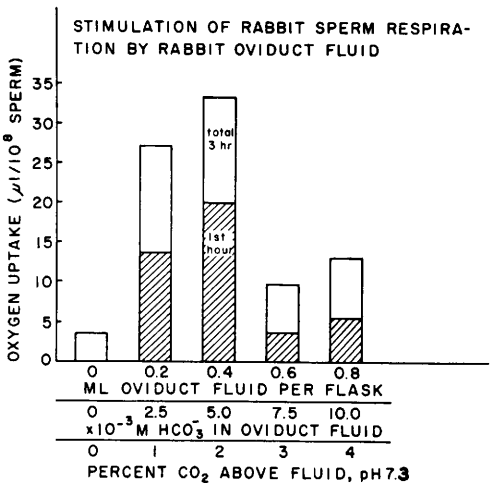

FIG. 1. Effect of increasing amounts of rabbit oviduct fluid on oxygen uptake by rabbit sperm. Bicarbonate was from an oviduct fluid pool 30 $\times$ 10^{-3} M in HCO_3^-.

mechanism by which HCO_3^- stimulates respiration of rabbit spermatozoa have shown that succinate stimulated respiration to the same extent as HCO_3^- in the presence of glucose with oxalacetate and malate were inactive (23.6 *vs* 20.7 μl taken up/10^8 sperm/3 hr for succinate and HCO_3^- respectively). In other experiments using pyruvate as substrate malonate at 2.5 $\times$ 10^{-3} M decreased the respiration rate of cells stimulated with HCO_3^- by 33% while at 5 $\times$ 10^{-3} M the rate was reduced by 75%.

Discussion. Olds and VanDemark(9) first demonstrated that bull sperm respiration was stimulated by secretions from the bovine reproductive tract. Henle and Zittle(10) have reported that Ringer bicarbonate solution gave a 2-fold stimulation of respiration of bull epididymal sperm compared to Ringer solution. A lesser stimulation of ram sperm respiration has been reported(11) for Ringer bicarbonate solutions. Bull spermatozoa have been shown to be stimulated by CO_2 levels up to 2% while higher levels inhibited respiration(12). Our results showing that 3.54 $\times$ 10^{-3} M HCO_3^- gave maximum stimulation could be explained by the more thorough removal of HCO_3^- from the spermatozoa in our washing procedure. Bull spermatozoa in diluted semen have been reported to respire at a more rapid rate in presence of respired CO_2 than in its absence(13,14). However, bull spermatozoa washed twice failed to show a similar response to respired CO_2(14). This is not surprising since washing would remove the seminal plasma which contains HCO_3^-, which in turn has been shown to stimulate glycolysis of bull spermatozoa(15). Therefore the spermatozoa washed twice would not be expected to produce as much respired CO_2 as unwashed sperm remaining in seminal plasma.

A ZO_2 of 13.7 for washed human spermatozoa in presence of 3.54 $\times$ 10^{-3} M HCO_3^- is much higher than the values reported by Ross(16) or MacLeod(17) who reported values very similar to the ZO_2 obtained in the absence of CO_2 and bicarbonate. More recently Rothschild(18) showed the ZO_2 of human spermatozoa to range from 2.10 to 9.45. It is known that human spermatozoa possess the components of a respiratory system(19) and that they actively oxidize glucose(20). Thus it appears that an appropriate concentration of HCO_3^- is required for the maximum respiratory rate of human sperm.

Biological significance of the stimulation of sperm respiration by HCO_3^- is suggested by the following: 1. HCO_3^- markedly stimulates sperm of several species. 2. Oviduct and uterine fluids contain significant amounts of HCO_3^-(21). 3. Adequate oxygen is present in the female tract to support respiration(22). 4. Sperm must incubate *in utero* for several hours before achieving the capacity to fertilize *i.e.,* capacitation(23,24). Thus it is possible that an increased rate of respiration of sperm is important in capacitation or fertilization. Earlier results suggest that maximum rate of respiration in the female tract and capacitation are achieved simultaneously (1).

The fact that succinate stimulates respiration to the same extent as HCO_3^- suggests that HCO_3^- may be stimulating respiration by increasing the intracellular levels of tricarboxylic acid (TCA) cycle intermediates. The lack of effect by oxalacetate and malate may be due to failure to enter the cell. More direct experiments on incorporation of

HCO_3^- in TCA cycle intermediates are under way.

Respiration of many other mammalian cells has been shown to be stimulated by HCO_3^-(25,26). Thus the stimulating effect of HCO_3^- seems to be widespread in mammalian cells.

Summary. 1. Rabbit spermatozoa are stimulated to respire at a greater rate in oviduct fluid than in calcium-free Krebs-Ringer phosphate solution. From experiments involving dialysis, heating, and acid neutralizing of oviduct fluid, it appears that bicarbonate may be the sole stimulating factor present. 2. Bicarbonate stimulates maximally the respiration of rabbit sperm at 2.36×10^{-3} M and rooster, bull, and human sperm at 3.54×10^{-3} at pH 7.0. The respiration rate for human sperm of $ZO_2 = 13.7$ is the highest rate so far reported suggesting that reports that human sperm do not respire or respire at a very slow rate were due to improper bicarbonate concentrations. 3. Bicarbonate appears to stimulate respiration by increasing the concentration of tricarboxylic acid cycle intermediates. 4. Stimulation of respiration of sperm may be important in essential processes in the female tract.

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