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Activation of Respiration and Initiation of Motility in Rainbow Trout Spermatozoa

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ABSTRACT—It is well established that sperm motility of rainbow trout is initiated by the decrease in K^+ concentration surrounding sperm which triggers the intracellular cAMP-dependent initiation process. Present study showed that K^+ did not affect sperm respiration but inhibited flagellar movement and thus suggested that K^+ regulates sperm motility through its effect on flagellum. On the other hand, inhibitors of respiratory chain or uncoupler of oxidative phosphorylation affected sperm respiration and inhibited sperm motility, suggesting that energy producing system at mitochondria contributes to sperm motility. Motility was initiated even if O_2 was eliminated from dilution medium, although CO_2 suppressed both respiration and motility. This result suggested that sperm motility is not O_2-limited but CO_2 is responsible for the regulation of sperm motility through the activation of respiration. It is likely that regardless of K^+-dependent cAMP system at sperm flagella, there is another system at mitochondria: enhancement of respiration by the release from CO_2 suppression at spawning may relate to the initiation of sperm motility in rainbow trout.

INTRODUCTION

Spermatozoa are immotile in undiluted semen and initiate motility on dilution into appropriate medium. As factors to cause the phenomenon, many things in the seminal plasma have been proposed (see [1]). Rothschild [2] postulated that low O_2 tension in the seminal plasma is most likely responsible for the sperm immotility in the reproductive organ and that increase in O_2 tension surrounding spermatozoa at spawning causes initiation of motility. Carbon dioxide was also proposed as another possible factor from the results that CO_2 inhibits both respiration and motility in sea urchin sperm [3, 4]. Johnson *et al.* [4] also suggested that O_2 does not affect sperm motility, since motility initiation occurs when O_2 was eliminated by blowing N_2 gas over a thin layer of semen. These studies have focused on the contribution of energy supply system to the motility initiation; however it is still unclear which factor is the physiological initiator of sperm motility.

Morisawa and collegues recently proposed a motility initiation system from another point of view. They showed that motility of spermatozoa in salmonid fishes is suppressed by K^+ and spermatozoa become motile in the K^+ deficient medium [5]. However, K^+ can not inhibit motility of trout spermatozoa of which plasma membrane and mitochondria are removed with the detergent [6], implicating that the site of K^+ action is not mitochondria but flagella. By regulating flagellar motility with or without K^+, it is possible to separate the mitochondrial function from the flagellar function. Consequently, salmonid sperm seems to offer an especially convenient material for investigating which factor contributes to mitochondrial metabolism or flagellar mechanism in the initiation of sperm motility.

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For clarifying this point, we compared the respiration and motility in trout sperm in the presence or absence of K^+ and furthermore examined the effects of aerobic or anaerobic condition and CO_2 on the sperm respiration and motility. The results suggested that K^+ dependent initiation system is present in flagella, and that increase of energy supply at mitochondria by decrease of CO_2 may possibly contribute to the initiation of trout sperm motility.

MATERIALS AND METHODS

Mature male rainbow trout (*Salmo gairdneri*) was obtained from Oshino Branch of Yamanashi Prefectural Fisheries Experimental Station. They were kept in an aquarium with circulating and aerating water at 10°C. The semen was collected by inserting a pipette into the sperm duct. Collected sperm was preserved on ice without dilution for several hours during the experiments.

For investigating the effects of K^+, dilution, inhibitors of respiratory chain and uncoupler of oxidative phosphorylation on sperm respiration and motility (Figs. 1–3), 100 mM NaCl or KCl solution was kept without bubbling with any gases. With 3 ml of the above solutions 0.1 ml semen was diluted with various conditions in the chamber of oxymeter and oxygen consumption was measured. Each plot in Figures 2, 3 and 5 was calculated from the oxygen consumption in 5 sec after dilution. On a glass slide without cover 0.1 μl of semen was suspended in 50 μl of 100 mM NaCl solution and sperm motility was observed by light microscopy using dark illumination. NaN_3 and KCN were each dissolved in distilled water. CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) was dissolved in 4% ethanol which did not affect sperm motility and respiration.

For studying the effect of O_2 (Fig. 4a), N_2 gas was introduced into 200 ml of 100 mM NaCl solution in an Erlenmeyer flask from a N_2 gas cylinder. Amount of dissolved O_2 was checked at an appropriate time interval with an oxymeter. A closed chamber (Bellco: 0.75 ml) was filled with the solution containing various concentrations of O_2 using a syringe and 1 μl of semen was injected with a microsyringe, and then the motility of sperm in the chamber was observed under microscope.

The effect of completely-O_2-eliminated condition (Fig. 4b) was investigated in 100 mM NaCl solution containing various concentrations of $Na_2S_2O_4$, which was introduced into both a closed chamber and an oxymeter, and sperm motility and oxygen content were measured.

Effect of CO_2 on sperm motility was investigated (Fig. 5) as follows. CO_2 gas was bubbled into 200 ml of 100 mM NaCl solution in the flask for a few hours. pH value of the solution decreased during CO_2-bubbling and finally reached 6.0. Media containing various concentrations of CO_2 were prepared with mixing the CO_2 saturated medium with 100 mM NaCl solution and pH was adjusted to 6.0 with HCl. Each medium was introduced into the closed chamber and oxymeter, and sperm motility and oxygen consumption were measured. Amount of CO_2 and pH value in these media were checked with a carbon analyzer (Model 524 C, O. I. Corporation, U. S. A.) and pH meter respectively before experiment.

Oxygen consumption was measured with an oxymeter (Yanagimoto Co., Ltd.) for 30 to 60 sec at a chart speed of 30 or 60 cm/min. Solutions were buffered with 20 mM Hepes-NaOH at pH 8.0 (Figs. 1–4) and 6.0 (Fig. 5). Experiments were carried out at 10°C (Figs. 1–4) or 20°C (Fig. 5).

Tracks of sperm were recorded by VTR through a video camera connected with a microscope and percentage of motile sperm and swimming speed in Figures 4 and 5 were measured as described previously [7]. In Figures 1 and 3, the number of moving spermatozoa was evaluated in terms of grade (−, ±, +): grade +, at least over half of spermatozoa were motile in the field of view of microscope; grade ±, below half of spermatozoa were motile; grade −, all spermatozoa were immotile.

RESULTS

Effect of potassium

As shown in Figure 1, when spermatozoa were suspended into 100 mM NaCl solution at a dilution ratio of 1:30, in which spermatozoa initiated forward motility, they consumed oxygen at the rate of

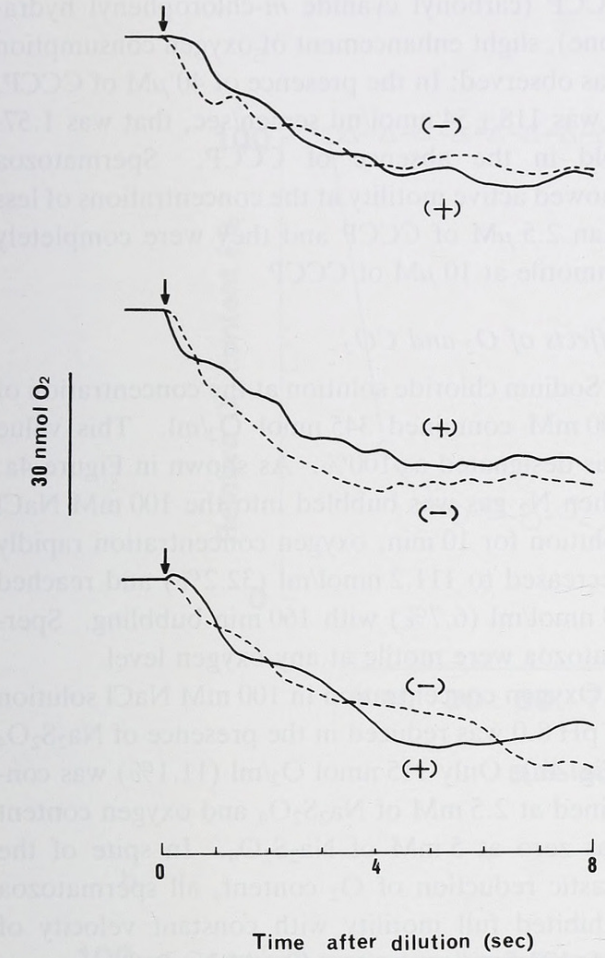


FIG. 1. Change in the oxygen consumption of rainbow trout spermatozoa in NaCl and KCl solutions. Semen at the volume of 0.1 ml was diluted with 100 mM NaCl solution (——) or 100 mM KCl solution (- - -) buffered with 20 mM Hepes at pH 8.0. Arrows indicate the time of adding the semen. Sperm motility was exhibited in parentheses.

66.9 ± 3.8 nmol/ml semen/sec from three experiments in Figure 1 in 5 sec after dilution and then the rate decreased. Spermatozoa diluted in 100 mM KCl (1:30 dilution) were completely immotile, however, they consumed oxygen at 67.1 ± 11.7 nmol/ml semen/sec in 5 sec and then the oxygen consumption became lower. Namely the rate of oxygen uptake of the sperm which were quiescent in the presence of K^+ was almost the same as that of the sperm which initiated motility in the absence of K^+.

Effect of dilution

Oxygen consumption of undiluted trout semen was almost zero (Fig. 2). When semen was diluted in 100mM NaCl solution (1:15 dilution), spermatozoa consumed oxygen at 45 ± 20 nmol/ml semen/sec. Oxygen consumption increased with the increase of a dilution rate and reached almost maximum at a dilution rate of 1:240 (365 ± 26 nmol/ml semen/sec). The level was maintained until a dilution rate reached 1:480.

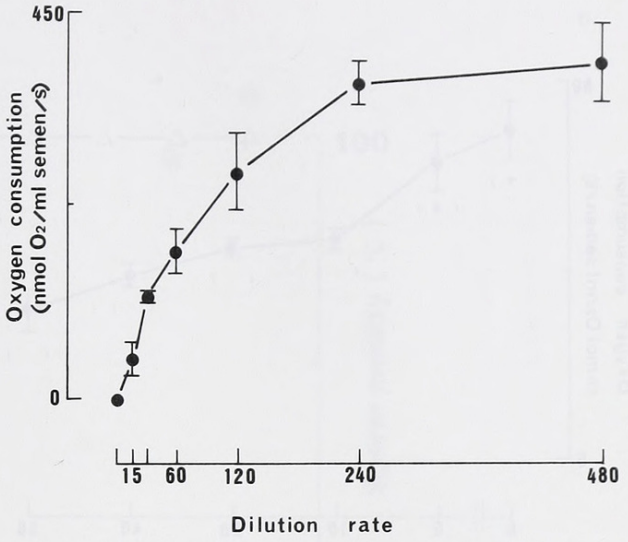


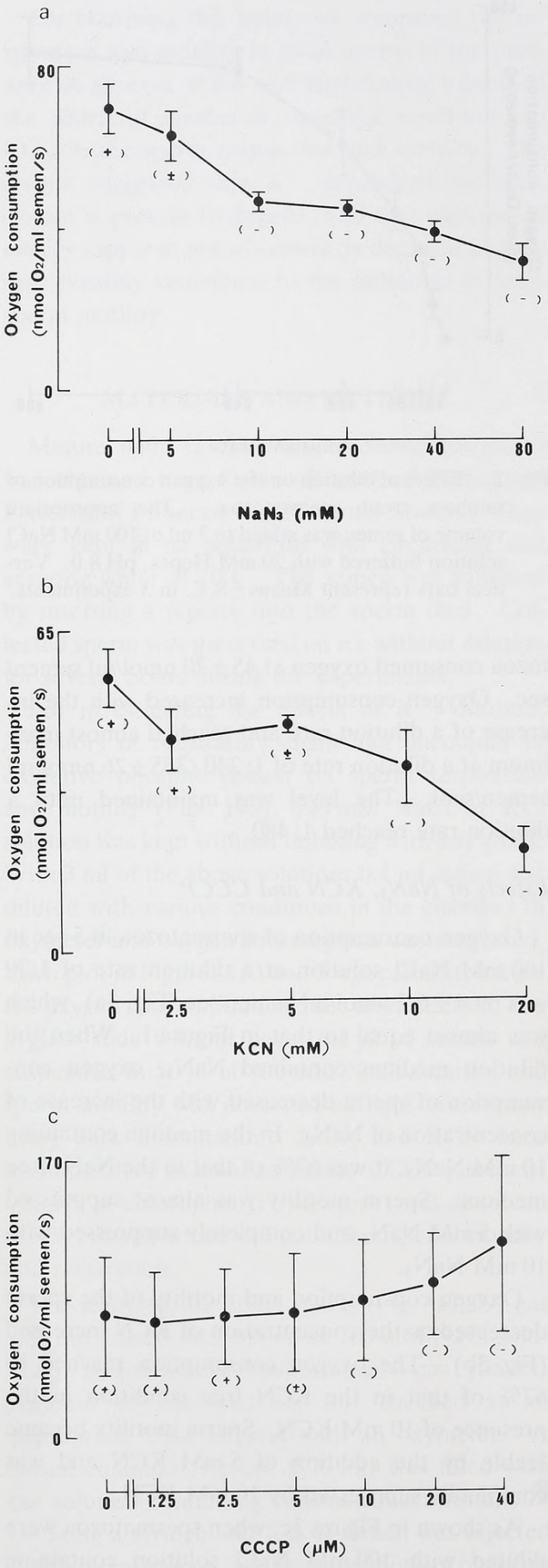
FIG. 2. Effect of dilution on the oxygen consumption of rainbow trout spermatozoa. The appropriate volume of semen was added to 3 ml of 100 mM NaCl solution buffered with 20 mM Hepes, pH 8.0. Vertical bars represent Means ± S.E. in 3 experiments.

Effects of NaN_3, KCN and CCCP

Oxygen consumption of spermatozoa in 5 sec in 100 mM NaCl solution at a dilution rate of 1:30 was 69.4 ± 6.2 nmol/ml semen/sec (Fig. 3a), which was almost equal to that in Figure 1. When the dilution medium contained NaN_3, oxygen consumption of sperm decreased with the increase of concentration of NaN_3: In the medium containing 10 mM NaN_3, it was 67% of that in the NaN_3 free medium. Sperm motility was almost suppressed with 5 mM NaN_3 and completely suppressed with 10 mM NaN_3.

Oxygen consumption and motility of the sperm decreased as the concentration of KCN increased (Fig. 3b). The oxygen consumption reached to 67% of that in the KCN free condition in the presence of 10 mM KCN. Sperm motility became feeble by the addition of 5 mM KCN and was completely suppressed by 10 mM KCN.

As shown in Figure 3c, when spermatozoa were diluted with 100 mM NaCl solution containing



CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone), slight enhancement of oxygen consumption was observed: In the presence of 40 μM of CCCP, it was 118 ± 54 nmol/ml semen/sec, that was 1.57-fold in the absence of CCCP. Spermatozoa showed active motility at the concentrations of less than 2.5 μM of CCCP and they were completely immotile at 10 μM of CCCP.

Effects of O_2 and CO_2

Sodium chloride solution at the concentration of 100 mM contained 345 nmol O_2/ml. This value was designated as 100%. As shown in Figure 4a, when N_2 gas was bubbled into the 100 mM NaCl solution for 10 min, oxygen concentration rapidly decreased to 111.2 nmol/ml (32.2%) and reached 23 nmol/ml (6.7%) with 160 min bubbling. Spermatozoa were motile at any oxygen level.

Oxygen concentration in 100 mM NaCl solution at pH 8.0 was reduced in the presence of $Na_2S_2O_4$ (Fig. 4b): Only 115 nmol O_2/ml (11.1%) was contained at 2.5 mM of $Na_2S_2O_4$ and oxygen content was zero at 5 mM of $Na_2S_2O_4$. In spite of the drastic reduction of O_2 content, all spermatozoa exhibited full motility with constant velocity of 156–172.5 μm/sec in 0 to 40 mM $Na_2S_2O_4$.

When the semen was diluted with 100 mM NaCl solution at pH 6.0, spermatozoa moved with the velocity of 189 ± 7.5 μm/sec and their oxygen consumption was 69 ± 16.3 nmol/ml semen/sec (Fig. 5). However, when CO_2 content in the medium increased, motility, velocity and oxygen consumption decreased and became zero at 50 mM of CO_2.

DISCUSSION

Sperm motility of rainbow trout is suppressed in the sperm duct by seminal K^+, and decrease in K^+ concentration surrounding sperm at spawning into fresh water induces the initiation of sperm motility

FIG. 3. Effects of inhibitors of respiratory chain and an uncoupler of oxidative phosphorylation on the oxygen consumption and motility of rainbow trout spermatozoa. Semen was added to 100 mM NaCl containing various concentrations of NaN_3 (a), KCN (b) or CCCP (c). Vertical bars represent Means $\pm$ S.E. in 3 experiments. Sperm motility was exhibited in parentheses.

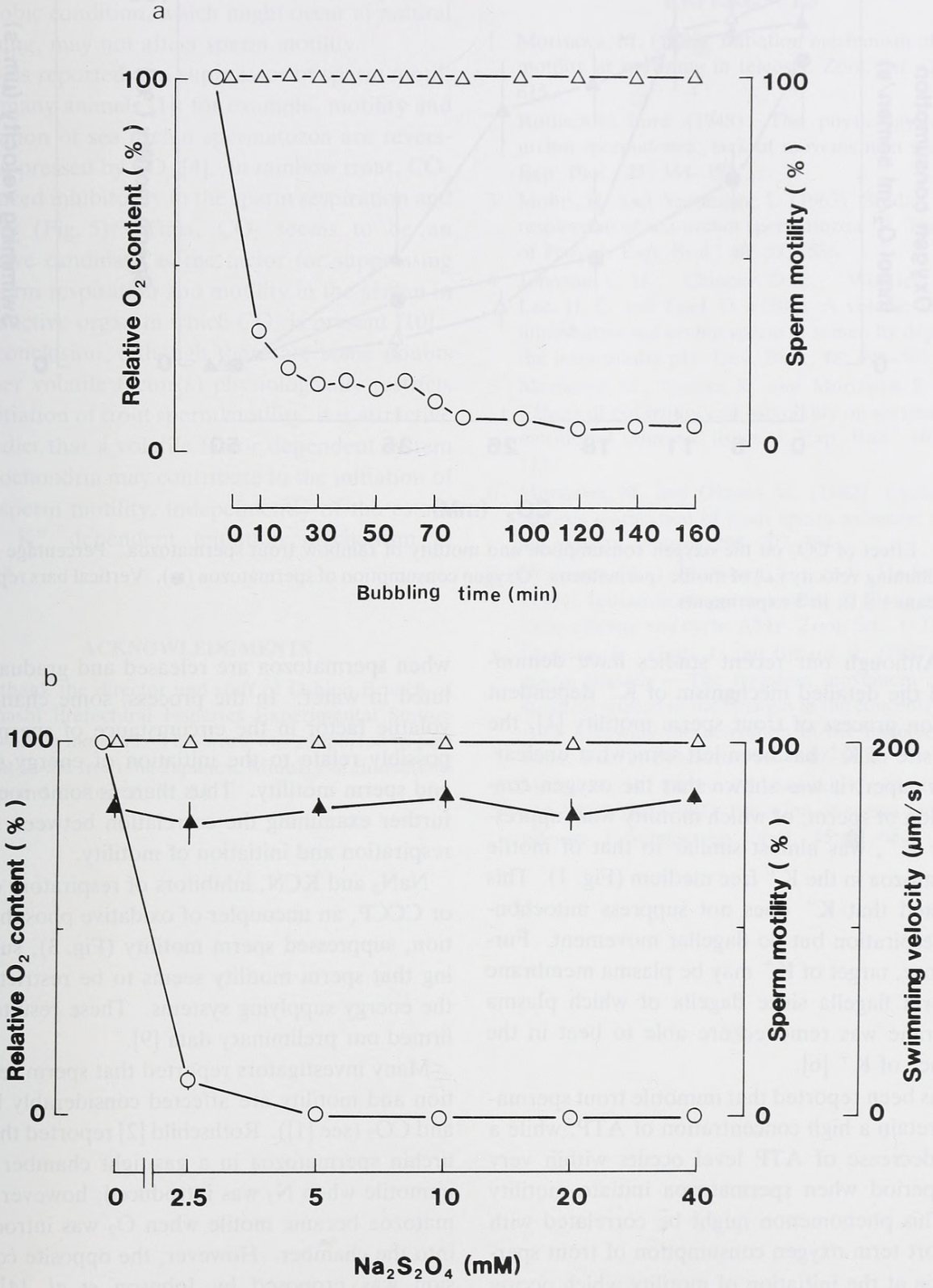


FIG. 4. Effect of O_2 on the motility of rainbow trout spermatozoa. (a), Relative percentage of dissolved oxygen (○) and motility of spermatozoa (△) in 100 mM NaCl when N_2 was bubbled for indicated time. (b), Percentage of dissolved oxygen in 100 mM NaCl containing $Na_2S_2O_4$ (○). Percentage (△) or swimming velocity (▲) of motile spermatozoa. Vertical bars represent Means ± S.E. in 20–25 spermatozoa.

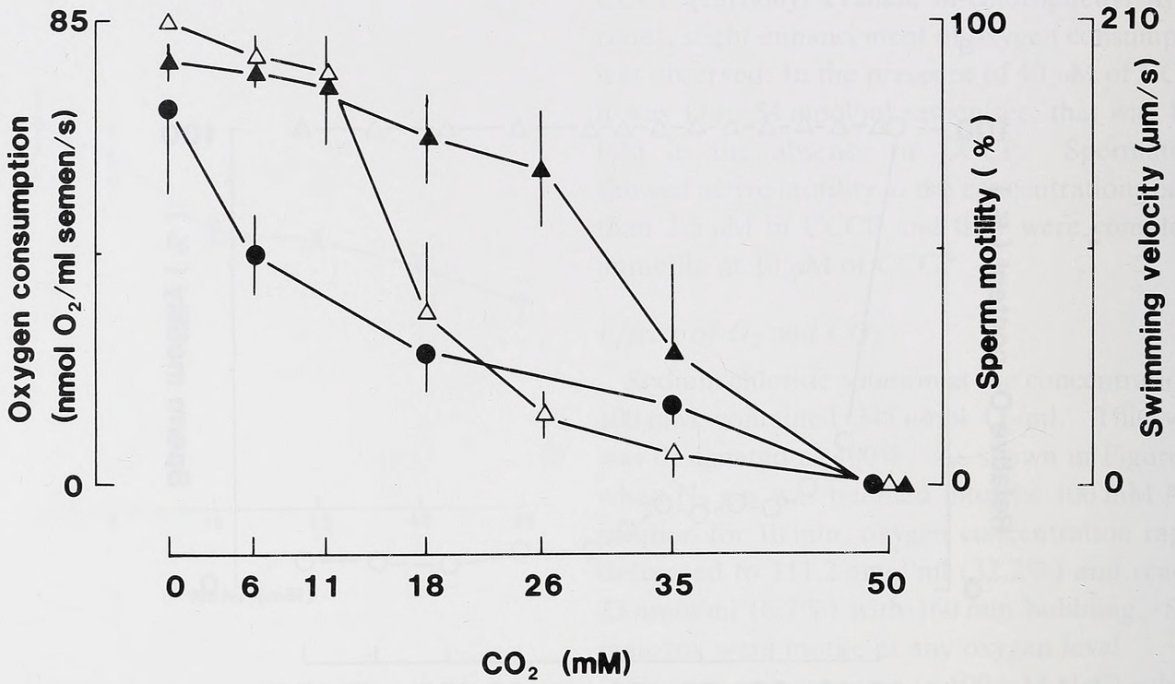


FIG. 5. Effect of CO_2 on the oxygen consumption and motility of rainbow trout spermatozoa. Percentage (△) or swimming velocity (▲) of motile spermatozoa. Oxygen consumption of spermatozoa (●). Vertical bars represent Means ± S.E. in 3 experiments.

[5]. Although our recent studies have demonstrated the detailed mechanism of K^+ dependent initiation process of trout sperm motility [1], the target site of K^+ has been left somewhat unclear. In this paper, it was shown that the oxygen consumption of sperm, of which motility was suppressed by K^+, was almost similar to that of motile spermatozoa in the K^+ free medium (Fig. 1). This suggested that K^+ does not suppress mitochondrial respiration but do flagellar movement. Furthermore, target of K^+ may be plasma membrane of sperm flagella since flagella of which plasma membrane was removed are able to beat in the presence of K^+ [6].

It has been reported that immotile trout spermatozoa retain a high concentration of ATP, while a rapid decrease of ATP level occurs within very short period when spermatozoa initiate motility [8]. This phenomenon might be correlated with the short term oxygen consumption of trout spermatozoa at the initiation of motility which occurs within a very short period. The short term oxygen consumption of trout sperm increased with increase of dilution ratio (Fig. 2). From the result, it seems to be considered that gradual activation of mitochondrial function occurs at natural spawning when spermatozoa are released and gradually diluted in water. In the process, some changes of volatile factor in the circumstance of sperm may possibly relate to the initiation of energy supply and sperm motility. Thus there is some room for further examining the correlation between sperm respiration and initiation of motility.

NaN_3 and KCN, inhibitors of respiratory chain, or CCCP, an uncoupler of oxidative phosphorylation, suppressed sperm motility (Fig. 3), suggesting that sperm motility seems to be restricted by the energy supplying systems. These results confirmed our preliminary data [9].

Many investigators reported that sperm respiration and motility are affected considerably by O_2 and CO_2 (see [1]). Rothschild [2] reported that sea urchin spermatozoa in a gas-tight chamber were immotile when N_2 was introduced, however spermatozoa became motile when O_2 was introduced into the chamber. However, the opposite conclusion was proposed by Johnson *et al.* [4]. In rainbow trout, as shown in Figure 4, spermatozoa could initiate and maintain motility in O_2 deficient medium, even in a completely anaerobic medium obtained by the addition of $Na_2S_2O_4$. This result suggests that O_2 is not a limiting factor for sperm

motility in this species. A change from anaerobic to aerobic condition, which might occur at natural spawning, may not affect sperm motility.

CO_2 is reported as a suppressor of sperm motility in many animals [1]; for example, motility and respiration of sea urchin spermatozoa are reversibly suppressed by CO_2 [4]. In rainbow trout, CO_2 influenced inhibitorily to the sperm respiration and motility (Fig. 5). Thus, CO_2 seems to be an attractive candidate as the factor for suppressing the sperm respiration and motility in the semen in reproductive organ in which CO_2 is present [10].

In conclusion, although there are some doubts whether volatile factor(s) physiologically restricts the initiation of trout sperm motility, it is attractive to predict that a volatile factor dependent system at mitochondria may contribute to the initiation of trout sperm motility, independently of the established K^+ dependent initiation mechanism at flagella.

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