

Flagellar movement in demembranated preparations of
ejaculated fowl spermatozoa

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Summary. Triton X-100 at a concentration of 0.1% in the extraction medium was optimal for demembrating the fowl spermatozoa. The most suitable conditions for reactivation were obtained when a medium composed of 0.5 mM-ATP, 25 mM-potassium glutamate, 10^{-7} M-CaCl₂, 20 mM-Tris-HCl (pH 7.9), 1 mM-MgSO₄, 1 mM-dithiothreitol and 0.2 M-sucrose was used. More than 60% motile spermatozoa were obtained under these conditions. The addition of 1 or 10 μ M-cAMP did not appreciably affect motility. Intact and demembrated spermatozoa were immotile at 40°C, whilst at 30°C motility was restored.

Introduction

In most synthetic diluents, ejaculated fowl spermatozoa become immotile at a body temperature of 40-41°C (Munro, 1938; Nevo & Schindler, 1968; Ashizawa & Nishiyama, 1978; Takeda, 1982; Wishart & Ashizawa, 1987). Motility is restored by decreasing the temperature or by the addition of calcium, caffeine or body fluids such as seminal plasma or female peritoneal fluid taken at the time of ovulation (Munro, 1938; Nevo & Schindler, 1968; Ashizawa et al., 1976; Ashizawa & Nishiyama, 1978; Takeda, 1982; Ashizawa & Okauchi, 1984; Ashizawa & Wishart, 1986, 1987; Wishart & Ashizawa, 1987). However, the mechanisms of immobilization and restoration of motility have remained unsolved.

To clarify these mechanisms, it may be necessary to

5 establish a demembrated preparation of ejaculated fowl spermatozoa. The advantage of using this preparation is that it permits the study of compounds that do not cross the cell membrane and of agents which have direct effects on the axoneme (White & Voglmayr, 1986). Furthermore, it may help to clarify whether the plasma membrane is involved in the immobilization of fowl spermatozoa at 40°C.

10 Demembrated preparations have been established with the spermatozoa of other species, including sea urchin, fish and many mammals (for reviews, see, for example, Gibbons, 1979; Mann & Lutwak-Mann, 1981; Tash & Means, 1983; Morisawa, 1985), but a little informations were available with avian spermatozoa (Woolley & Brammall, 1987).

15 We therefore attempted to determine the most suitable conditions for the demembration and reactivation of fowl spermatozoa. The effects of temperature on motility of intact and demembrated spermatozoa were also examined.

Materials and Methods

20 Animals and preparation of spermatozoa. Commercial White Leghorn roosters (Babcock strain, Akagi Poultry Breeding Farm, Miyazaki, Japan) were used throughout the study. All birds were housed in cages and fed ad libitum on a commercial breeder diet. They were exposed to 14 h light per 24 h.

Semen was collected by the method of Bogdonoff & Shaffner (1954). Samples of semen pooled from 4-6 different males were diluted approximately 10-fold in 150 mM-NaCl with 20

5 mM-TES (N- Tris- [hydroxymethyl]- methyl- 2- aminoethanesulphonic acid) at pH 7.4 and centrifuged at 700 g for 13 min at room temperature (20-25°C). The washed spermatozoa were reconstituted in the same buffer to give a final concentration of $\sim 1 \times 10^9$ cells/ml.

10 Demembration and reactivation of spermatozoa. The extraction medium and the reactivation medium were based on that used for mammalian spermatozoa (Yeung & Woolley, 1984), but modified as below; a solution consisting of 0.2 M-sucrose, 25 mM-potassium glutamate, 1 mM-MgSO₄, 1 mM-dithiothreitol (DTT) and 20 mM-Tris-HCl buffer. Various concentrations of Triton X-100 or saponin were added to the extraction medium for demembration. Various concentrations of ATP, calcium and cAMP were added to the reactivation medium, and the most suitable conditions of demembration and reactivation for fowl spermatozoa were determined.

20 To demembrate spermatozoa, 30 μ l sperm suspension were added to 0.5 ml extraction medium in a test tube. The suspension was incubated for 30 sec while stirring gently with a Pasteur pipette at 20-25°C in order to dissolve the plasma membrane. Then 30 μ l extraction medium containing demembrated spermatozoa were transferred to 0.5 ml reactivation medium. An aliquant was transferred immediately to a microscope slide chamber ~ 0.05 mm deep and a coverslip was placed on the chamber.

The motility of spermatozoa was recorded by videomicroscopy at 20-25°C and the percentages of motile

spermatozoa were measured by tracing the location of sperm heads on a transparent sheet according to the method of Katz & Overstreet (1981).

5 Motility of intact and demembrated spermatozoa at 30°C and 40°C. Intact spermatozoa incubated aerobically for 15 min at 40°C were demembrated at 40°C. Motility of the demembrated spermatozoa was examined at 40°C on the thermostatically-controlled warm plate according to the method described above. After that, the same samples were
10 cooled to 30°C and the motility was observed again. The motility of intact spermatozoa which were suspended in the extraction medium without detergent and transferred to the reactivation medium was also measured.

15 Electron microscopy. Sperm specimens were suspended in the above extraction medium with Triton X-100 or saponin or extraction medium without detergents for 30 sec and prefixed by adding glutaraldehyde at a final concentration of 2.5%. After 1.5 h, spermatozoa were centrifuged to a pellet and washed in 0.15 M-cacodylate buffer without resuspension.
20 The pellet was then postfixed for 1.5 h on ice with 2% osmium tetroxide in the 0.1 M-cacodylate buffer at pH 7.4, dehydrated through a graded series of alcohols and embedded in Spurr's resin. Thin sections were stained first with uranyl acetate and then with lead citrate. Electron micrographs were taken with an Hitachi H-800 electron microscope.

Chemicals. Adenosine 5'-triphosphate (ATP), adenosine 3',5'-cyclic monophosphate (cAMP), phosphodiesterase, potassium glutamate, Triton X-100, dithiothreitol and TES were purchased from Sigma Chemical Co., St Louis, MO, USA. Other chemicals were of reagent grade from Nakarai Chemicals Ltd., Kyoto, Japan.

Statistical analysis. Data were subjected to statistical analysis by the method of Duncan (1955).

Results

Effects of detergents on the motility of demembrated spermatozoa

The effects of increasing concentrations of two kinds of detergents, Triton X-100 and saponin, on the motility of demembrated spermatozoa are shown in Fig. 1. Motile spermatozoa were still observed in the extraction medium at concentrations of detergents of <0.04%. At a higher concentration (1%), the percentages of reactivation decreased. Therefore, the concentration of both detergents in the extraction medium was satisfactory at 0.1% for demembrating the fowl spermatozoa. However, the percentage of reactivation was higher with Triton X-100 treatment than with saponin treatment. Therefore, 0.1% Triton X-100 was used for demembrating the spermatozoa in the later experiments.

After extraction with 0.1% Triton X-100, the plasma membrane was completely removed (Fig. 2). In contrast, saponin-treated spermatozoa still retained the plasma membrane which appeared swollen. This could have been due to a characteristic of saponin which acts selectively on the cholesterol of the plasma membrane, producing small pores in the membrane (Gotoh & Takenaka, 1984).

Determination of optimal pH and ATP concentration

The pH of the Tris-HCl buffer for extraction and reactivation was varied between 7.0 and 9.0. Reactivation was negligible at pH 7.0. The percentages of motile spermatozoa at pH 7.5-7.9 improved significantly, but those at pH 8.5 and above were lower than that at pH 7.9 (Fig. 3a).

Optimal concentration of ATP for reactivation of the spermatozoa was between 0.1 mM and 0.5 mM. Deviation from this range resulted in a decrease in the percentage of reactivation with no reactivation of spermatozoa from 0 mM to 0.005 mM ATP (Fig. 3b).

Effects of calcium and cAMP on the motility of demembrated spermatozoa

As the concentration of calcium was increased, the proportion of motile spermatozoa increased, reaching a peak at 10^{-7} M (Fig. 3c). At concentrations of $>10^{-6}$ M, motility decreased gradually with rise in concentrations, but even at 10^{-3} M about 50% of spermatozoa were still motile.

In the absence of calcium, addition of 1 μ M- or 10 μ M-cAMP

did not stimulate motility (Table 1). In contrast, motility was slightly stimulated in the presence of 10^{-7} M calcium. Addition of phosphodiesterase (2 units), to remove endogenous cAMP, gave results similar to those of the control.

5 The addition of cAMP did not appreciably affect motility of demembranated spermatozoa for at least 15 min (Fig. 4).

Effects of temperature on the motility of intact and demembranated spermatozoa

10 Motility was measured first at 40°C, and then the same samples were observed at 30°C. Reactivation of spermatozoa was performed under the most suitable experimental condition, i.e. in a medium composed of 0.5 mM-ATP, 25 mM-potassium glutamate, 10^{-7} M-CaCl₂, 20 mM-Tris-HCl (pH 7.9), 1 mM-MgSO₄, 1 mM-DTT and 0.2 M-sucrose. The motility of intact and 15 demembranated spermatozoa at 40°C was raised by reducing the temperature to 30°C (Table 2). The addition of cAMP did not restore motility at 40°C.

Discussion

20 These experiments demonstrated that demembranated fowl spermatozoa can be reactivated under suitable conditions. Therefore, it is suggested that demembranated spermatozoa may be useful in clarifying the mechanisms of the reversible temperature-dependent immobilization of fowl spermatozoa. Before we began these experiments, we suspected that the plasma membrane was involved in the immobilization of fowl

spermatozoa at body temperature. Therefore, it was assumed that fowl spermatozoa could remain motile at 40°C after removing the plasma membrane. However, these results suggest that the mechanisms involved in the immobilization of spermatozoa at 40°C do not involve the plasma membrane, because the motility of demembrated spermatozoa was as well ^{with} intact spermatozoa almost negligible at 40°C (Table 2). Therefore, it appears that the axoneme itself is directly involved in this immobilization.

The concentrations of detergent and ATP and pH for optimal reactivation of spermatozoa have been found to vary between species and must be determined in each case. For example, the concentration of Triton X-100 for demembration of hamster and ram spermatozoa should not exceed 0.05% in order to preclude the possibility of inhibition of reactivation by ATP (Mohri & Yanagimachi, 1980; White & Voglmayr, 1986). Lower concentrations were used for dog (0.01%) and sea urchin, trout and Cinoma spermatozoa (0.04%) (Gibbons & Gibbons, 1972; Tash & Means, 1982; Morisawa & Okuno, 1982; Morisawa et al., 1984). In contrast, concentrations of 0.01-0.04% were insufficient for fowl spermatozoa, because some motile spermatozoa were still observed in the extraction medium. We therefore suggest that a concentration of 0.1% detergent is optimal for fowl spermatozoa.

The motility of the Triton-treated spermatozoa was completely dependent upon the presence of ATP, and no movement was observed when ATP was omitted from the reactivation medium (Gibbons & Gibbons, 1972). On the other

hand, Yeung (1986) demonstrated that temporary inhibition of the initiation of reactivated motility of hamster spermatozoa was observed by increasing the concentration of ATP from 1 to 3 mM. When the concentration of ATP exceeded the optimum, similar phenomena were observed in dog (Tash & Means, 1982) and ram (White & Volgmayr, 1986) spermatozoa. In this study, the optimal concentration of ATP in fowl spermatozoa was obtained between 0.1 and 0.5 mM, and a similar phenomenon was observed at 5 mM.

Regulation of sperm motility by cAMP and calcium is well known (for reviews, see, Garbers & Kopf, 1980; Tash & Means, 1983; Morisawa, 1985). Demembranated ejaculated bull spermatozoa were most motile in the presence of both ATP and cAMP (Lindemann, 1978). Mohri & Yanagimachi (1980) also reported that cAMP stimulated the ATP-induced activity of demembranated hamster spermatozoa. Cyclic AMP is also indispensable for the initiation of epididymal sperm motility at ejaculation in mammals (Ishida et al., 1987). White & Voglmayr (1986) routinely added cAMP to the reactivation medium, although the reactivation of ejaculated ram spermatozoa was usually as good with ATP alone. In the present study, cAMP did not affect the maintenance of motility (Fig. 4) and the restoration of motility at 40°C (Table 2). In addition, in the absence of calcium, addition of cAMP alone did not stimulate the motility of ejaculated fowl spermatozoa, although motility was slightly stimulated in the presence of both 10^{-7} M-calcium and 10 μ M-cAMP (Table 1).

The effect of calcium on sperm motility is, however, less

straightforward. The complexity of the problem probably results from the fact that calcium not only affects cAMP metabolism in spermatozoa, but also has some direct effects on axonemal motility. In addition, external calcium appears to have effects different from those of internal calcium on sperm function, depending on the species (Tash & Means, 1983). Gibbons & Gibbons (1980) reported that reactivated sea urchin spermatozoa show asymmetric beating in the absence of calcium, but in the presence of 100-200 μM free calcium the cells became quiescent. The motility of reactivated dog spermatozoa could be stimulated with micromolar levels of cAMP and inhibited with micromolar levels of free calcium (Tash & Means, 1982). The addition of 10^{-3} M-calcium reduced the percentage of reactivation for ejaculated ram spermatozoa (White & Voglmayr, 1986). In contrast, fowl spermatozoa were still motile even at a concentration of 10^{-3} M free calcium (Fig. 3c).

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Table 1. Effects of cAMP on the motility of demembrated fowl spermatozoa in the absence or presence of calcium

cAMP conc. (μM)	Ca ²⁺ conc. (M)	Motility (%)
0	0	50.9 \pm 4.3 ^{ab}
	10 ⁻⁷	57.7 \pm 2.3 ^{ab}
1	0	52.8 \pm 0.7 ^{ab}
	10 ⁻⁷	59.1 \pm 3.3 ^{ab}
10	0	49.5 \pm 0.9 ^b
	10 ⁻⁷	60.5 \pm 3.1 ^a
Phosphodiesterase (2 units)	10 ⁻⁷	56.8 \pm 3.0 ^{ab}

Triton X-100 concentration in the extraction medium, ATP concentration and pH in the reactivation medium were fixed at 0.1%, 0.5 mM and 7.9, respectively.

Each value represents the mean (\pm s.e.m.) of motility of 5 samples of spermatozoa.

Values with different superscripts differ significantly ($P < 0.01$) from each other.

Table 2. Motility of intact and demembranated fowl spermatozoa at 40°C and 30°C

Treatment of spermatozoa	Motility (%)	
	40°C	30°C
Intact spermatozoa	9.9 ± 2.0 ^a	70.0 ± 4.8 ^a
Demembranated spermatozoa	2.3 ± 1.2 ^{ab}	53.0 ± 3.9 ^a
+ 1 μM-cAMP	1.8 ± 0.7 ^b	53.4 ± 5.7 ^a
+ 10 μM-cAMP	2.1 ± 1.1 ^b	53.7 ± 6.6 ^a

Each value represents the mean (±s.e.m.) motility of 5 samples of spermatozoa.

Within columns, values with different superscripts differ significantly (P<0.01) from each other.

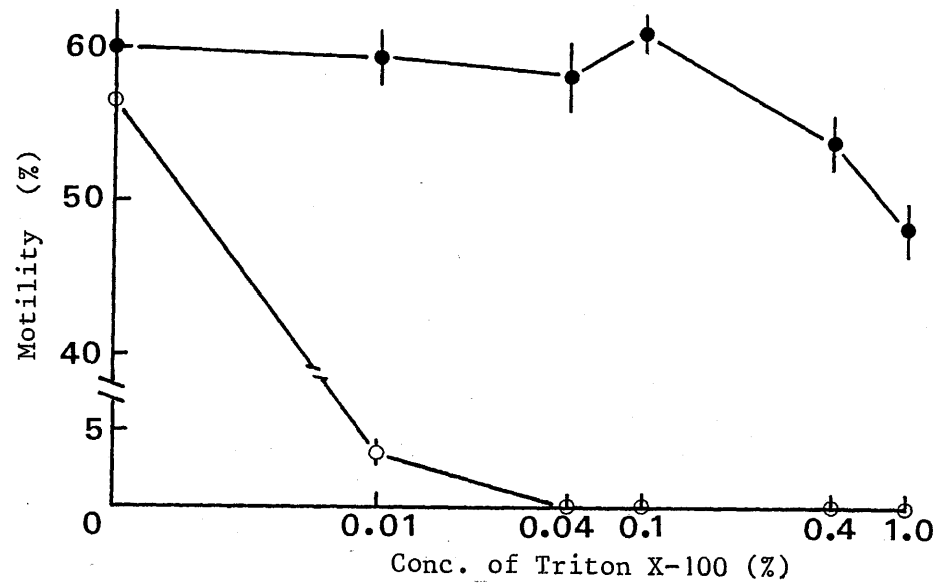


Fig. 1. Motility of the demembrated fowl spermatozoa in the extraction medium(O) or reactivation medium(●). Spermatozoa were demembrated at various concentrations of Triton X-100 in the extraction medium. ATP concentration and pH in the reactivation medium were fixed at 0.5mM and 7.9, respectively. Calcium and cAMP were not added. Each point represents the mean (\pm s.e.m.) motility of five samples of spermatozoa.

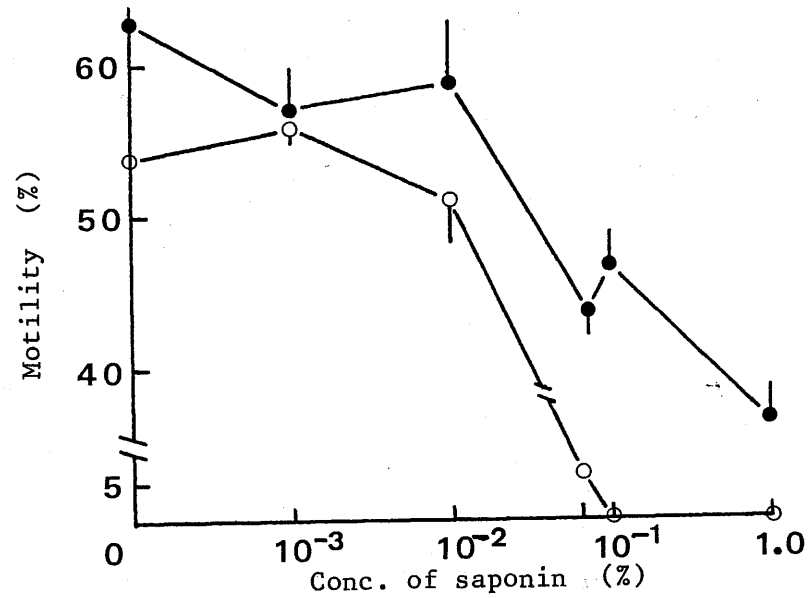


Fig. 2. Motility of the demembrated fowl spermatozoa in the extraction medium(O) or reactivation medium(●). Spermatozoa were demembrated at various concentrations of saponin in the extraction medium. ATP concentration and pH in the reactivation medium were fixed at 0.5mM and 7.9, respectively. Calcium and cAMP were not added. Each point represents the mean (\pm s.e.m.) motility of five samples of spermatozoa.

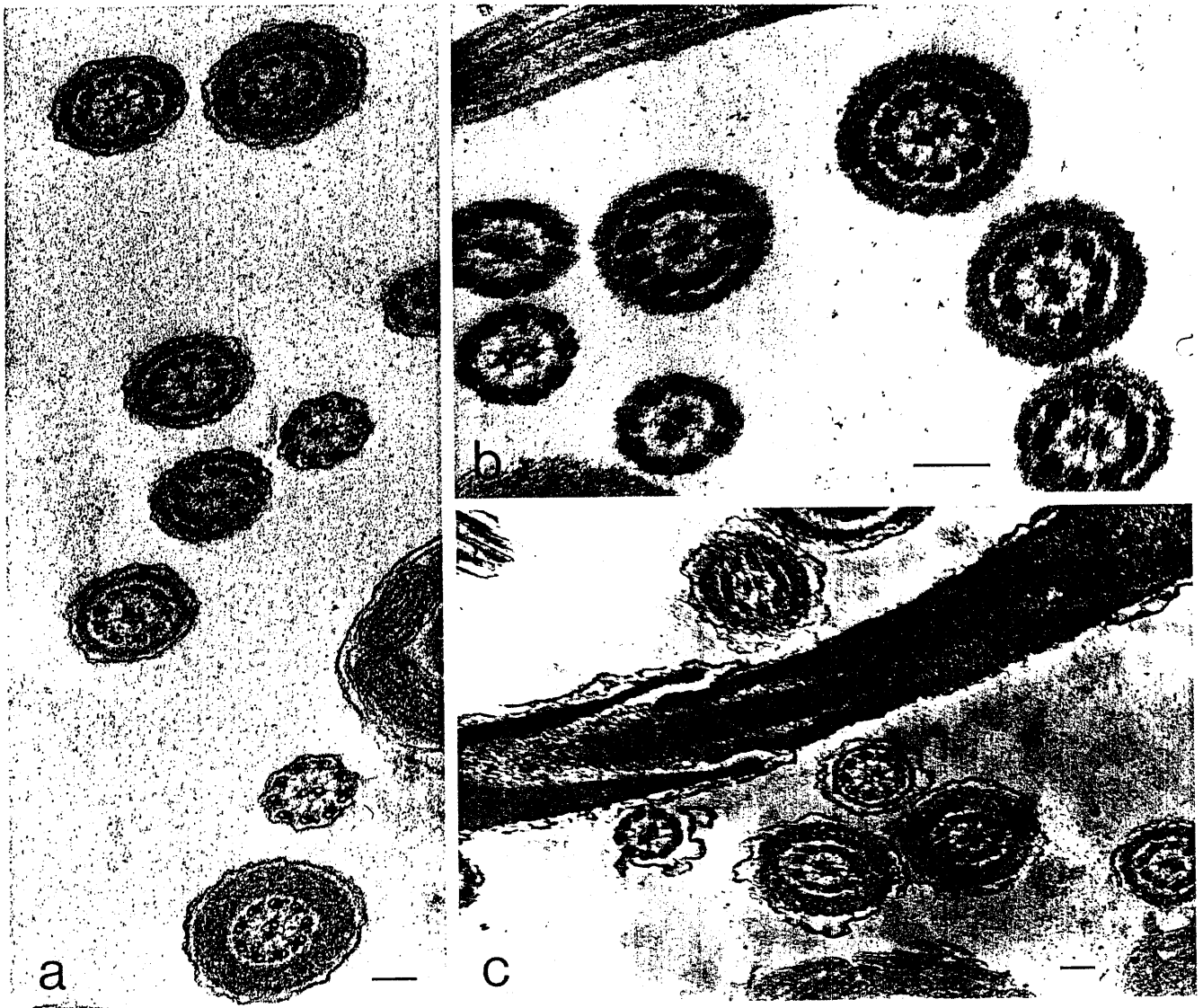


Fig. 3. Electron micrographs of fowl spermatozoa. Spermatozoa extracted for 30 sec with extraction medium containing (a) no detergent, (b) 0.1% Triton X-100 and (c) 0.1% saponin. Bar; 0.1 μ m.

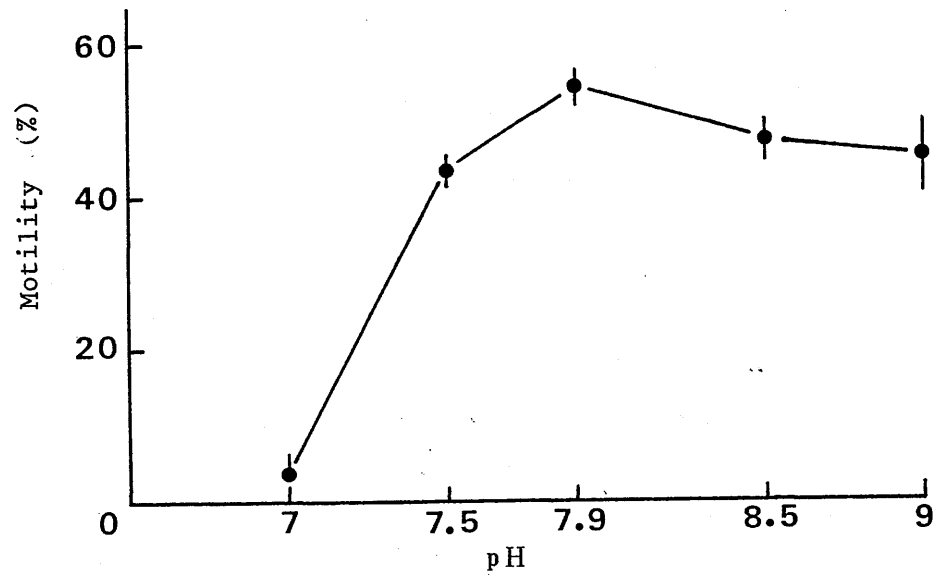


Fig. 4. Motility of the demembrated fowl spermatozoa at various pHs in the reactivation medium. Triton X-100 concentration in the extraction medium and ATP concentration in the reactivation medium were fixed at 0.1% and 0.5mM, respectively. Calcium and cAMP were not added. Each point represents the mean (\pm s.e.m.) motility of five samples of spermatozoa.

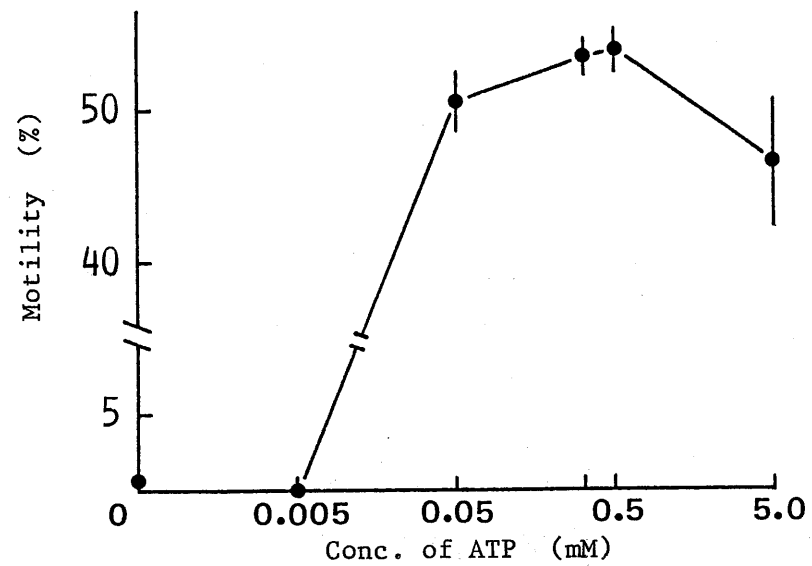


Fig. 5. Motility of the demembrated fowl spermatozoa at various concentrations of ATP in the reactivation medium. Tritox X-100 in the extraction medium and pH in the reactivation medium were fixed at 0.1% and 7.9, respectively. Calcium and cAMP were not added. Each point represents the mean (\pm s.e.m.) motility of five samples of spermatozoa.

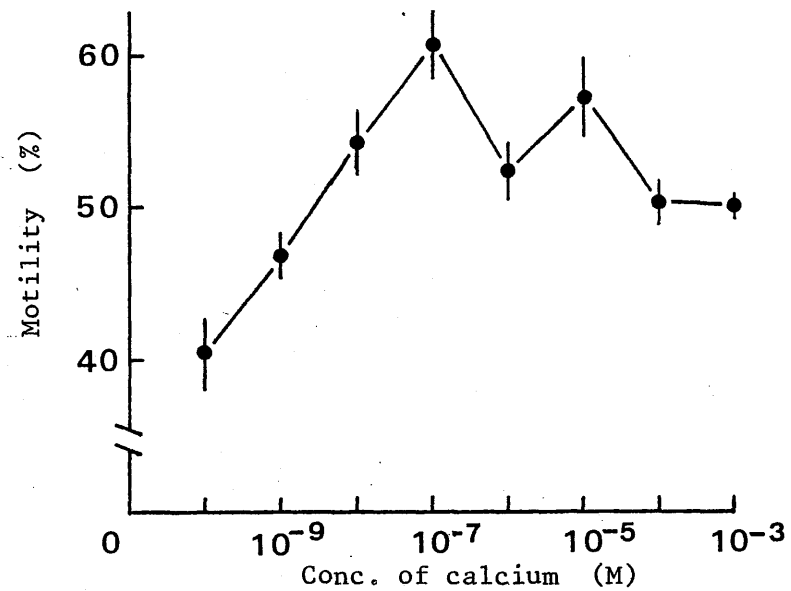


Fig. 6. Motility of the demembrated fowl spermatozoa at various concentrations of calcium in the reactivation medium. Calcium concentrations were adjusted by the addition of various concentrations of EGTA. Triton X-100 concentration in the extraction medium, ATP concentration and pH in the reactivation medium were fixed at 0.1%, 0.5mM and 7.9, respectively. Each point represents the mean (\pm s.e.m.) motility of five samples of spermatozoa.

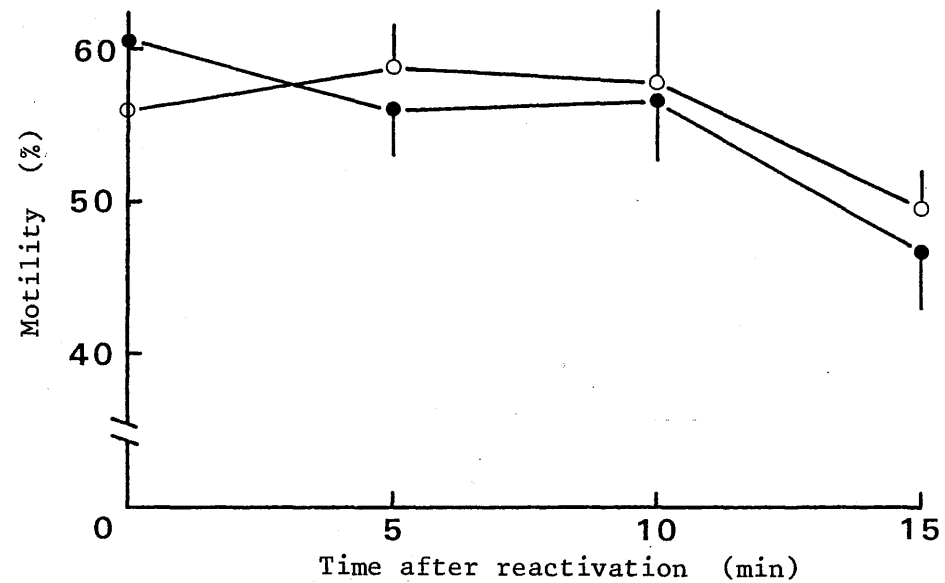


Fig. 7. The effect of cAMP on the maintenance of motility of the demembrated fowl spermatozoa. Triton X-100 concentration in the extraction medium, ATP and calcium concentrations and pH in the reactivation medium were fixed at 0.1%, 0.5mM, 10^{-7} M and 7.9, respectively. Each point represents the mean (\pm s.e.m.) motility of five samples of spermatozoa after the addition of 10µM cAMP(○) or no addition(●).

Table 1. Effects of cAMP on the motility of demembrated fowl spermatozoa in the absence or presence of calcium

Conc. of cAMP (μM)	Conc. of Ca^{2+} (M)	Motility (%)
0	0	50.9 ± 4.3^{ab}
	10^{-7}	57.7 ± 2.3^{ab}
1	0	52.8 ± 0.7^{ab}
	10^{-7}	59.1 ± 3.3^{ab}
10	0	49.5 ± 0.9^b
	10^{-7}	60.5 ± 3.1^a
Phosphodiesterase (2 units)	10^{-7}	56.8 ± 3.0^{ab}

Triton X-100 concentration in the extraction medium, ATP concentration and pH in the reactivation medium were fixed at 0.1%, 0.5mM and 7.9, respectively. Each figure represents the mean (\pm s.e.m.) of motility of five samples of spermatozoa. Values with different superscripts differ significantly ($P < 0.01$) from each other.

Table 2. Motility of intact and demembranated fowl spermatozoa at 40°C and 30°C

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Demembranated spermatozoa	2.3 ± 1.2 ^{ab}	53.0 ± 3.9 ^a
+ 1µM cAMP	1.8 ± 0.7 ^b	53.4 ± 5.7 ^a
+ 10µM cAMP	2.1 ± 1.1 ^b	53.7 ± 6.6 ^a

The condition for demembranation of spermatozoa is shown in the Result. Each figure represents the mean (\pm s.e.m.) motility of five samples of spermatozoa. Within columns, values with different superscripts differ significantly ($P < 0.01$) from each other.