Inhibition of Flagellar Motility of Fowl Spermatozoa by L-Carnitine: Its Relationship with Respiration and Phosphorylation of Axonemal Proteins

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ABSTRACT The action of carnitine in regulating fowl sperm motility was investigated. As the concentration of L-carnitine was increased (0-20 mM), the motility of intact and demembranated fowl spermatozoa was reduced at 30°C. Even the presence of 1 mM $CaCl_2$ before the addition of 10 mM carnitine could not prevent the inhibition of motility at 30°C and 40°C. However, motility was restored by reducing the concentrations of carnitine. Carnitine also inhibited the oxygen consumption and ATP concentrations of intact spermatozoa, and caused a reduction in intracellular free Ca^{2+} concentrations. Phosphorylation of a 50-kDa protein and dephosphorylation of 24-kDa and 30-kDa proteins of demembranated spermatozoa were observed after the addition of carnitine. In contrast, the flagellar ATPase activity of crude dynein extract was not affected by the addition of carnitine. These results suggest that inhibitory effect of carnitine for motility may be directly on the axonemal phosphoproteins, but not directly on the dynein ATPase activity. Physiological role of carnitine for fowl spermatozoa in the ductus deferens is discussed.

Key Words: Demembranated sperm, Dynein, Calcium, Oxygen consumption

INTRODUCTION

Since high concentrations of L-carnitine were found in the mammalian epididymis (Marquis and Fritz, 1965), many investigations have been directed toward defining the functions of carnitine in sperm maturation. Epididymal spermatozoa accumulate carnitine from the epididymal plasma during maturation, attaining high intracellular concentrations (Casillas, 1972, 1973; Brooks et al., 1974; Hutson et al., 1977; Casillas and Chaipayungpan, 1979), and changes in membrane permeability to carnitine also occurs during maturation (Casillas, 1973).

Carnitine is known to be a cofactor in fatty-acid oxidation (Fritz, 1963, 1967) and will stimulate fatty-acid oxidation in isolated mitochondria, including those from sonicated bovine spermatozoa (Casillas, 1972), although Hamilton and Olson (1976) suggested that high extracellular carnitine inhibited fatty-acid oxidation of ejaculated bull spermatozoa.

With regard to motility of spermatozoa, a high concentration (approximately 19 mM) of carnitine was first found in the luminal fluid from the distal caput epididymidis of the rat, where the spermatozoa develop the potential for motility (Hinton et al., 1979). It was, therefore, suggested that carnitine might be important in the development of the potential for motility by rat spermatozoa and also for maintaining mature spermatozoa in a quiescent state, since spermatozoa are not actually motile until ejaculation (Hinton et al., 1979, 1981). On the other hand, Jeulin et al. (1987) suggest that carnitine may not be directly involved in the initiation of boar sperm motility, but the acetylation of carnitine may contribute to stimulate

the metabolic activity of epididymal spermatozoa related to modifications in the flagellar motility. When acetylcarnitine or carnitine were added to ejaculated human spermatozoa, an increase in the motility was demonstrated (Tanphaichitr, 1977), although it was suggested that carnitine was first converted to acetylcarnitine. In contrast, carnitine inhibited the progressive motility of ejaculated bovine spermatozoa (Deana et al., 1989), although no inhibition was observed in caudal epididymal spermatozoa (Carr and Acott, 1984). Carnitine has been shown to stimulate the oxygen consumption of rat, rabbit and bull spermatozoal mitochondria (Casillas, 1972; Storey and Keyhani, 1974; Hutson et al., 1977), but to inhibit the respiration of ejaculated bovine spermatozoa (Hamilton and Olson, 1976; Bøhmer and Johansen, 1978; Deana et al., 1989). Thus, the effects of carnitine on the mammalian sperm motility and metabolic activity are not straightforward, and may vary depending on the species and investigator. Moreover, the mechanisms involved in either the stimulation or inhibition of motility and metabolic activity by carnitine are not well understood.

The ductus deferens of the fowl has been considered to be equivalent to an extension of the corpus and caudal epididymis of the mammal (Lake, 1984). High levels (about 1-4 mM, 15 to 20 times higher than in the blood plasma) of carnitine have been found in the ductus deferens fluid of the fowl (Lake, 1984). However, the significance of carnitine in the functioning of fowl spermatozoa has yet to be determined.

In the following experiment, therefore, attempts were made to investigate the effects of carnitine on the motility, metabolic activity

and Ca^{2+} flux of ejaculated intact fowl spermatozoa and on the phosphorylation of axonemal proteins of demembranated spermatozoa.

MATERIALS AND METHODS

Preparation of Spermatozoa

Commercial White Leghorn roosters (Babcock strain, Akagi Poultry Breeding Farm, Miyazaki, Japan) were used throughout the study. All irds were housed in individual 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 and Shaffner (1954). Samples of semen pooled from four to six males were diluted approximately tenfold in 150 mM NaCl with 20 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 approximately 1 x 10^9 cells/ml. Samples of 3-4 ml were poured into 30 ml Erlenmeyer flasks with a screw cap.

Chemicals

Fura-2/AM was purchased from Dojindo Laboratories, Inc., Kumamoto, Japan. Adenosine 5'-triphosphate (ATP), bovine serum albumin, L-carnitine, dithiothreitol, potassium glutamate, TES, Triton X-100, Cremophor EL and desiccated firefly tails were purchased from Sigma Chemical Co., St Louis, MO, USA. Trypsin (approx. 10000 BALL A_{253} units/mg protein) was from Mochida Pharmaceutical Co., Tokyo, Japan and calyculin A was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. [γ-^{32}P]ATP was purchased from Du

Pont-New England Nuclear, Boston, MA, USA. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were obtained from Bio-Rad Laboratories, Richmond, CA, USA. Other chemicals were of reagent grade from Nacalai Tesque, Inc., Kyoto, Japan.

Analysis of Intact and Demembranated Sperm Motility

Sperm samples were pre-incubated aerobically in a shaking water bath at 30°C or 40°C for 10 min. After the pre-incubation, the dose-response and time-course of motility of intact and demembranated spermatozoa were measured at 30°C or 40°C after addition of various concentrations of carnitine. The effects of the addition of $CaCl_2$ before the addition of carnitine were also examined to be compared with those of carnitine alone. Diluent for the measurement of intact sperm motility used was TES/NaCl buffer described above. Demembranation and reactivation of spermatozoa were performed according to the method described previously (Ashizawa et al., 1989). The extraction medium used consisted of 0.1% Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM $MgSO_4$, 1 mM dithiothreitol and 20 mM Tris-HCl buffer (pH 7.9). The reactivation medium consisted of 0.5 mM ATP, 200 mM sucrose, 25 mM potassium glutamate, 1.5 mM $MgSO_4$, 1 mM dithiothreitol and 20 mM Tris-HCl buffer (pH 7.9). Various concentrations of carnitine were added to the reactivation medium. Addition of trypsin or calyculin A, inhibitor of protein serine/threonine phosphatases, to carnitine-treated spermatozoa was also performed. The suspension of intact or demembranated

spermatozoa was viewed in a microscope slide chamber (Sekisui Chemical Co., Ltd., UR-157 type, Tokyo, Japan) on a thermostatically-controlled warm plate, and the motility of spermatozoa was recorded by videomicroscopy at 30°C or 40°C (Katz and Overstreet, 1981).

Analysis of Oxygen Consumption and ATP concentrations

Oxygen consumption of spermatozoa was determined polarographically with a Clark electrode by the method of Kielley (1963), using a YSI model 53 biological oxygen monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, USA). The rate of oxygen consumption was expressed in terms of μl O_2 consumption/10^8 spermatozoa/h. Numbers of spermatozoa were estimated by the method of Wishart and Ross (1985), using a double-beam spectrophotometer (Shimadzu, Model UV-150-02, Kyoto, Japan).

ATP concentration in spermatozoa was assayed by firefly bioluminescence in a boiled extract (Wishart, 1982).

Intracellular Free Ca^{2+} Concentrations

Determination of the intracellular free Ca^{2+} concentration was carried out with the fluorescent indicator fura-2, essentially according to Grynkiewicz et al. (1985), but with some modifications (Ashizawa et al., 1992). Briefly, stock solutions of fura-2 acetoxymethyl ester (fura-2/AM) were made up to 1 mM in dry dimethylsulphoxide (DMSO). For the complete emulsification of fura-2/AM, 2 μl of 10% Cremophor EL was added to 5 μl of fura-2/AM, and then 2 ml of NaCl/TES buffer

was added and sonication of this solution was performed. One ml of sperm suspension was added to fura-2/AM solution and shaken for 45 min at 30°C in the dark. During this incubation, final concentrations of fura-2/AM and spermatozoa were 1.7 μM and about 3.3 x 10^8/ml, respectively. After the incubation, the spermatozoa were washed twice and resuspended in NaCl/TES buffer and sperm concentrations were adjusted to 1 x 10^8/ml. Fluorescence intensity was measured with a dual-wavelength spectrofluorimeter (Shimadzu, Model RF-5000, Kyoto, Japan) with the sample chamber thermostatically controlled at 30°C or 40°C and the sperm suspension was mixed continuously with a magnetic stirring bar. For the measurement of intracellular free Ca^{2+} concentrations, the excitation wavelength was set to 340 and 380 nm, respectively and the emission wavelength was set to 500 nm. The ratios (R: 340:380nm) of fluorescence intensities were monitored continuously. Calculation of intracellular free Ca^{2+} was based upon the equation described by Grynkiewicz et al. (1985) where a dissociation constant (K_d) was 224 nM.

Crude Dynein Preparation and ATPase Assay

The method of McConnell et al. (1987) for the isolation of crude dynein from bovine spermatozoa was modified for use in fowl spermatozoa. Approximately 9 x 10^9 spermatozoa washed with NaCl/TES buffer (pH 7.4) were demembranated by resuspending the sperm pellet in 30 ml of cold extraction medium described above. After centrifugation at 700 g for 10 min at 4°C, demembranated spermatozoa were washed once with cold extraction medium excluding Triton X-100

and recentrifuged. Axonemal ATPase was solubilized by resuspending the demembranated spermatozoa in 0.6 M NaCl, 20 mM Tris-HCl buffer (pH 7.9), 1 mM dithiothreitol, 4 mM $MgCl_2$ for 10 min at 4°C. The supernatant obtained after centrifugation of the axonemal extract at 100000 g for 30 min at 4°C was designated as crude dynein extract.

ATPase activities were assayed by the reduction of ATP concentrations determined by firefly bioluminescence. The assay solution consisted of 20 mM Tris-Hcl buffer (pH 7.9), 4 mM $MgSO_4$ and 0.1 mM ATP. Carnitine and/or $CaCl_2$, when incorporated, was present at final concentration of 10 mM or 1 mM, respectively. 150 μl of assay solution and 15 μl of crude dynein extract were mixed and the reaction was initiated. After a 30 min incubation period at 30°C, the reaction was stopped by boiling the mixture. ATP concentrations remained were assayed by the method of Wishart (1982). Protein concentrations of crude dynein extract were determined by using the method of Bradford (1976), with bovine serum albumin as a standard. The rate of ATPase activity was expressed in terms of nmol ATP hydrolysis/mg protein/min.

Protein Phosphorylation and Electrophoresis

Demembranation of spermatozoa was carried out by the same method described above. Approximately 7000 cpm/pmol [γ-^{32}P]ATP and 0.1 mM ATP were added in the reactivation medium. Sperm samples were incubated for 7 min at 30°C. Carnitine and/or $CaCl_2$, when incorporated, was present at final concentration of 10 mM or 1 mM, respectively. The phosphorylation reaction was terminated by the

addition of equal volumes of concentrated (twofold) Laemmli (1970) sample buffer and boiling for 5 min. Samples containing protein from approximately 1.0 x 10^6 spermatozoa were loaded on 10% SDS-polyacrylamide slab gels, and electrophoresed. Autoradiography was performed at -80°C for 2-4 days exposure to X-ray film with an intensifying screen (Lightning plus, Du Pont, Wilmington, DE, USA).

Statistical Analysis

Statistical comparisons were performed using Student's t test.

RESULTS

Effect of Carnitine on the Motility of Intact and Demembranated Fowl Spermatozoa

The motility of both intact and demembranated spermatozoa was markedly affected by the addition of carnitine at 30°C: as the concentrations of carnitine were increased, the motility of intact spermatozoa was reduced gradually and demembranated spermatozoa became almost immotile by the addition of more than 10 mM carnitine (Fig. 1).

Fig 1 shows the inhibition of motility at 5 min after the addition of carnitine. The effect of more prolonged exposure is shown in Fig. 2. Inhibition by carnitine was not irreversible, but the motility of intact and demembranated spermatozoa could be restored by the dilution (8 fold) in carnitine free assay medium to decrease carnitine concentrations after 9 min of exposure. However, even the presence of Ca^{2+} before the addition of carnitine could not prevent the inhibition of motility of intact and demembranated spermatozoa at 30°C (Fig. 3a, b). At 40°C, the motility of intact spermatozoa was almost negligible, but was restored instantly after the addition of Ca^{2+}. However, the subsequent addition of carnitine inhibited the motility again (Fig. 3a). Demembranated sperm motility remained negligible during incubation with or without Ca^{2+} and carnitine at 40°C (Fig. 3b).

The presence of trypsin or calyculin A alone permitted reactivation of demembranated spermatozoa at 40°C, but this effect was inhibited by the addition of carnitine (Table 1).

Effect of Carnitine on the Oxygen Consumption, ATP Concentration and Intracellular Free Ca^{2+} Concentration of Fowl Spermatozoa

As with the inhibition of motility, oxygen consumption and ATP concentrations also decreased with increasing concentrations of carnitine at 30°C (Fig. 4).

The rate of oxygen consumption increased slightly after the addition of Ca^{2+} at 30°C and 40°C, but decreased following the addition of carnitine (Fig. 5).

Sperm ATP concentrations decreased significantly ($P<0.05$) after the addition of carnitine at 40°C compared with the value of no addition of carnitine, but there was no significant difference between the two treatments at 30°C (Fig. 6).

The time course of the effect of carnitine on the intracellular free Ca^{2+} concentrations at 30°C is shown in Fig. 7. Intracellular free Ca^{2+} concentrations increased rapidly after the addition of Ca^{2+}, and then maintained a constant value at approximately 150 nM. Subsequently, the addition of carnitine caused a reduction in the intracellular free Ca^{2+} concentrations. However, spermatozoa could still maintain a constant value approximately at 70 nM. In contrast, the Ca^{2+} concentrations did not change in the control (0 mM carnitine). Similar results were obtained at 40°C (data not shown).

Effect of Carnitine on Dynein ATPase Activity and the Phosphorylation State of Demembranated Fowl Sperm Proteins

The flagellar ATPase activity of crude dynein extract with or without Ca^{2+} was approximately 24 nmol ATP hydrolysis/mg protein/min. This activity did not decrease by the addition of carnitine, even though the motility of demembranated spermatozoa was inhibited (Table 2).

A marked difference in the phosphorylation status of demembranated sperm proteins was not obtained between control (no addition, lane 1) and Ca^{2+} treatment (lane 3): five major phosphorylated protein bands of molecular weights of 41-kDa, 34-kDa, 30-kDa, 27-kDa and 24-kDa were identified together with several minor phosphorylated proteins (Fig. 8). In contrast, phosphorylation of a 50-kDa protein and dephosphorylation of both 24-kDa and 30-kDa proteins was observed after the addition of carnitine alone (lane 2). These results were the same as those obtained even in the presence of Ca^{2+} (lane 4) (Fig. 8, denoted by arrows).

DISCUSSION

In bulls, spermatozoa from the caput epididymis accumulate isotopically labeled carnitine in vitro, whereas spermatozoa isolated from the cauda do not (Casillas, 1973; Hutson et al., 1977; Johansen and Bøhmer, 1979). Therefore, it has been suggested that sperm permeability to carnitine decrease during epididymal maturation, possibly because of changes in plasma membrane and that free carnitine cannot permeate the plasma membrane of the ejaculated bull spermatozoa (Hamilton and Olson, 1976). On the other hand, Deana et al. (1989) reported that [^{3}H] labeled carnitine was rapidly taken up by the ejaculated bovine spermatozoa by a process strongly dependent on temperature and extracellular concentrations of carnitine. In the present work, we have found that the addition of carnitine inhibits the motility of intact fowl spermatozoa and furthermore, the motility of demembranated spermatozoa (Fig. 1). From these results, it is possible that extracellular carnitine could permeate the plasma membrane of the ejaculated fowl spermatozoa and could act directly on the flagellar axoneme.

Several lines of evidence exist to indicate that carnitine is involved in the control of sperm motility, although the effect of carnitine is less straightforward. Depending on the species, carnitine may stimulate (Tanphaichitr, 1977; Hinton et al., 1981), inhibit (Deana et al., 1989) or have little effect (Jeulin et al., 1981; Carr and Acott, 1984). However, it is not well understood the mechanisms of regulation by carnitine for sperm motility. Carnitine has been shown to inhibit the respiration of ejaculated bovine spermatozoa (Hamilton

and Olson, 1976; Bøhmer and Johansen, 1978). Deana et al. (1989) demonstrated that high concentration (20 mM) of carnitine inhibited the progressive motility and the oxygen consumption of ejaculated bovine spermatozoa. Our results also have shown that the oxygen consumption of ejaculated fowl spermatozoa decreased with increasing concentrations of carnitine (Fig. 4). Therefore, it is assumed that the inhibition of motility by carnitine is probably due to the reduction of respiration. However, as mentioned above, carnitine also inhibited the motility of demembranated fowl spermatozoa reversiblly. Thus, it is suggested that inhibitory effect of carnitine for motility may be directly on the axoneme as well as it's effects on respiration.

If carnitine affects the axoneme directly, then the question of its locus of action is raised. Current evidence suggest that an endogenous protease activity is required for fowl sperm motility, since demembranated sperm motility could be restored at 40°C by the addition of trypsin and was inhibited by protease inhibitors at 30°C (Ashizawa et al., 1993). Also, phosphorylation-dephosphorylation of axonemal proteins is likely to be a regulatory step in fowl sperm motility, since the addition of calyculin A, specific inhibitor of protein serine/threonine phosphatases, stimulated the motility of demembranated fowl spermatozoa at 40°C (Ashizawa et al., unpublished data). In the present study, the presence of trypsin or calyculin A permitted reactivation of demembranated spermatozoa at 40°C (Table 1). However, stimulation for motility by such effectors was not observed by the addition of carnitine. Furthermore, the present results also showed that ATPase activity of dynein extract of fowl spermatozoa was not inhibited by the addition of carnitine (Table 2), suggesting that

carnitine might not act directly on dynein ATPase activity. Therefore, it is proposed that the site of action of carnitine may be a 24-kDa, 30-kDa or 50-kDa protein and that the phosphorylation or dephosphorylation of these proteins may inhibit the motility of fowl spermatozoa (Fig. 8).

The motility and respiration of fowl spermatozoa are strongly influenced by their intracellular Ca^{2+} concentrations (Thomson and Wishart, 1989, 1991; Ashizawa et al., 1992). The effect of carnitine on Ca^{2+} transport of spermatozoa was first observed by Deana et al. (1989). They reported that high concentrations of carnitine caused an increase of cellular Ca^{2+} transport of ejaculated bovine spermatozoa, i.e., $^{45}CaCl_2$ uptake of spermatozoa increased after the addition of 20 mM carnitine, whereas the cytosolic free Ca^{2+} concentrations were not significantly changed, indicating that carnitine activated cellular Ca^{2+} cycling (Deana et al., 1989). In contrast, the present study showed that the intracellular free Ca^{2+} concentrations increased rapidly after the addition of Ca^{2+} and then the subsequent addition of carnitine caused a reduction in Ca^{2+} concentrations. However, intracellular free Ca^{2+} concentrations were maintained still higher than those of before the addition of Ca^{2+} (Fig. 7). During this time, spermatozoa could not maintain their motility at 30°C and 40°C (Fig. 3a). Therefore, it may be proposed that carnitine inhibits the motility in Ca^{2+}-independent manner. This hypothesis might explain a physiological role of carnitine in the ductus deferens of the fowl: the presence of Ca^{2+} alone stimulates the motility of fowl spermatozoa at the avian body temperature of 40°C (Wishart and Ashizawa, 1987), but the high concentrations of carnitine in the ductus deferens (Lake, 1984) may

act to maintain spermatozoa in a quiescent state in spite of the presence of high (1–2.5 mM) concentrations of Ca^{2+} (Lake, 1971).

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TABLE 1. The Effects of Trypsin and Calyculin A on the Motility of Carnitine-Treated Demembranated Fowl Spermatozoa

Substances (conc.)	Motility (%)	
	30°C	40°C
None (control)	77.3± 5.2[a]	0.7± 0.7[a]
Carnitine (10 mM)	0[b]	0[a]
Trypsin (50 ng/ml)	84.8± 4.1[a]	72.5± 2.6[b]
Trypsin (50 ng/ml) + carnitine (10 mM)	0[b]	0[a]
Calyculin A (100 nM)	79.9± 3.3[a]	59.5± 4.0[b]
Calyculin A (100 nM) + carnitine (10 mM)	0[b]	0[a]

Demembranation and reactivation of spermatozoa were performed as described in Materials and Methods. Carnitine, trypsin and calyculin A were added to the reactivation medium. The motility was analyzed by videomicroscopy at 30°C and 40°C on a thermostaticaly-controlled warm plate. Each value represents the mean (± SEM) of five samples of spermatozoa.

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

TABLE 2. The Effects of Carnitine and Ca^{2+} on the ATPase Activity of Crude Dynein Extract of Fowl Spermatozoa at 30°C

Substances (conc.)	ATPase activity (nmol ATP hydrolysis/mg protein/min)
None (control)	23.8 ± 3.1[a]
$CaCl_2$ (1 mM)	23.6 ± 3.9[a]
Carnitine (10 mM)	21.8 ± 3.7[a]
Carnitine (10 mM) + $CaCl_2$ (1 mM)	22.4 ± 3.4[a]

Axonemal crude dynein ATPase was solubilized by resuspending the demembranated spermatozoa in 0.6 M NaCl, 20 mM Tris-HCl (pH 7.9), 1mM dithiothreitol and 4 mM $MgCl_2$ for 10 min at 4°C. ATPase activities were assayed by the reduction of ATP concentrations determined by firefly bioluminescence. $CaCl_2$ and carnitine were added to the assay solution consisted of 20 mM Tris-HCl (pH 7.9), 4 mM $MgSO_4$ and 0.1 mM ATP.

Each value represents the mean (± SEM) of five samples of spermatozoa.

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

Legends for figures

Fig. 1. Motility of intact (○) and demembranated (●) fowl spermatozoa after addition of various concentrations of carnitine at 30°C. Demembranation and reactivation of spermatozoa were performed as described in Materials and Methods. The motility was analyzed by videomicroscopy on a thermostaticaly-controlled warm plate. Spermatozoa were assayed for motility 5 min after the addition of carnitine at each concentration. Each point represents the mean (± SEM) of five samples of spermatozoa. **P<0.01 compared with value at 0 mM.

Fig. 2. The time course of motility of intact (○−●) and demembranated (□−■) fowl spermatozoa at 30°C after addition of 10 mM carnitine. At arrow, sperm samples were diluted (8 fold) in carnitine free assay medium to decrease extracellular carnitine concentrations. Each point represents the mean (±SEM) of five samples of spermatozoa. **P<0.01 compared with value at 0 min.

Fig. 3. Motility of (A) intact fowl spermatozoa at 30°C (○,●) and 40°C (□,■); or (B) demembranated fowl spermatozoa at 30°C (○,●) and 40°C (□,■) after addition (↓) of 1 mM $CaCl_2$ (○,□) and 10 mM carnitine (●, ■). Each point represents the mean (±SEM) of five samples of spermatozoa. **P<0.01 compared with value of no addition of carnitine at each point.

Fig. 4. Oxygen consumption (●) and ATP concentrations (○) of intact

fowl spermatozoa after addition of various concentrations of carnitine at 30°C. Oxygen consumption was determined polarographically with a Clark electrode. ATP concentration was assayed by firefly bioluminescence in a boiled extract. Spermatozoa were assayed 5 min after the addition of carnitine at each concentration. Each point represents the mean (±SEM) of five samples of spermatozoa. **P<0.01, *P<0.05 compared with value at 0 mM.

Fig. 5. Oxygen consumption (μl $O_2/10^8$ spermatozoa/h) of intact fowl spermatozoa after addition of 1 mM $CaCl_2$ and 10 mM carnitine at 30°C and 40°C. Each value represents the mean (±SEM) of five samples of spermatozoa. **P<0.01, *P<0.05 compared with value of no additon of carnitine.

Fig. 6. ATP concentration of intact fowl spermatozoa at 30°C (○,●) and 40°C (□,■) after addition (↓) of 1 mM $CaCl_2$ (○,□) and 10 mM carnitine (●,■). Each point represents the mean (±SEM) of five samples of spermatozoa. *P<0.05 compared with value of no addition of carnitine at each point.

Fig. 7. Changes of intracellular free Ca^{2+} concentrations in fowl spermatozoa at 30°C after addition (↓) of 1 mM $CaCl_2$ and carnitine (0 and 10 mM). The intracellular free Ca^{2+} concentration was analyzed with the fluorescent indicator fura-2, as described in Materials and Methods. The excitation wavelength was set to 340 and 380 nm, respectively and the emission wavelength was set to 500 nm. The ratios (R: 340:380nm) of fluorescence intensities were monitored

continuously. Calculation of intracellular free Ca^{2+} was based upon the equation described by Grynkiewicz et al. (1985) where a dissociation constant (K_d) was 224 nM.

Fig. 8. 10% SDS-PAGE profile of demembranated fowl sperm proteins stained with the Coomassie blue (lane 5) and corresponding autoradiography of phosphorylated proteins (lanes 1-4). Triton X-100 extracted and reactivated samples were incubated at 30°C for 7 min. Approximately 7000 cpm/pmol [γ-^{32}P]ATP and 0.1 mM ATP were added in the reactivation medium. Samples containing protein from approximately 1.0 x 10^6 spermatozoa were loaded. Lane 1, no treatment (control); lane 2, presence of 10 mM carnitine; lane 3, presence of 1 mM $CaCl_2$; lane 4, presence of 10 mM carnitine and 1 mM $CaCl_2$.

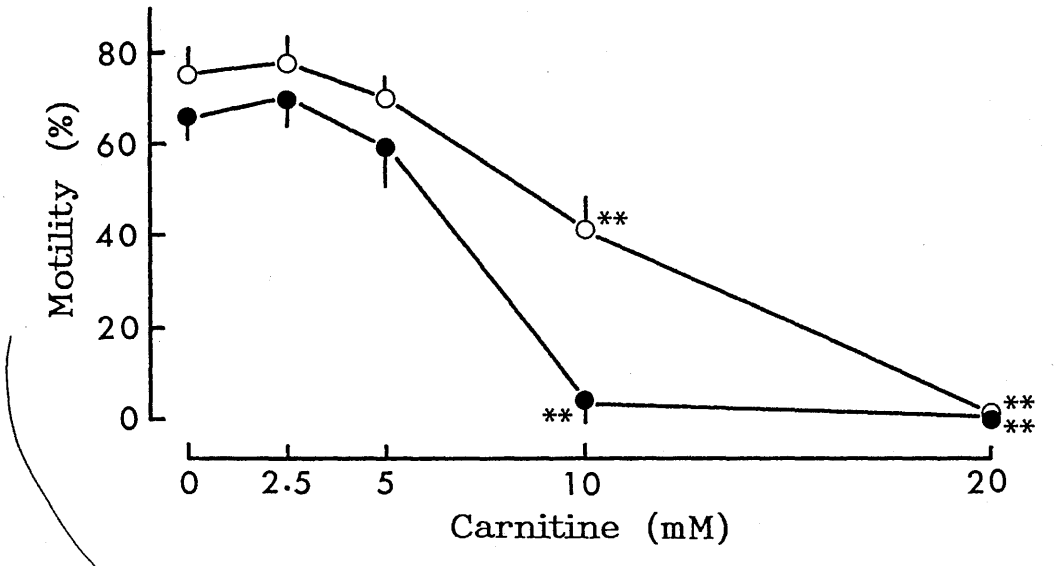

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Fig 1

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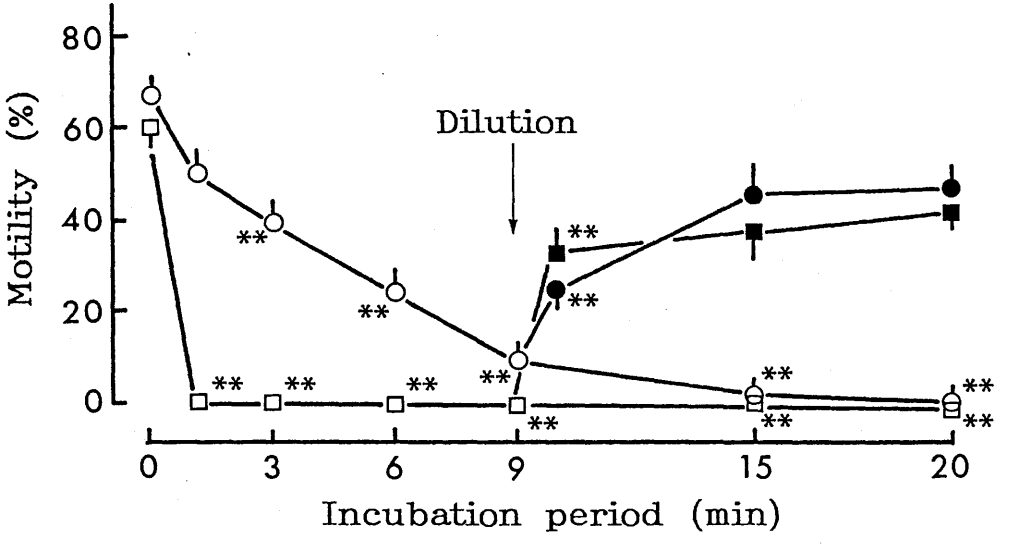

Fig 2

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(a)

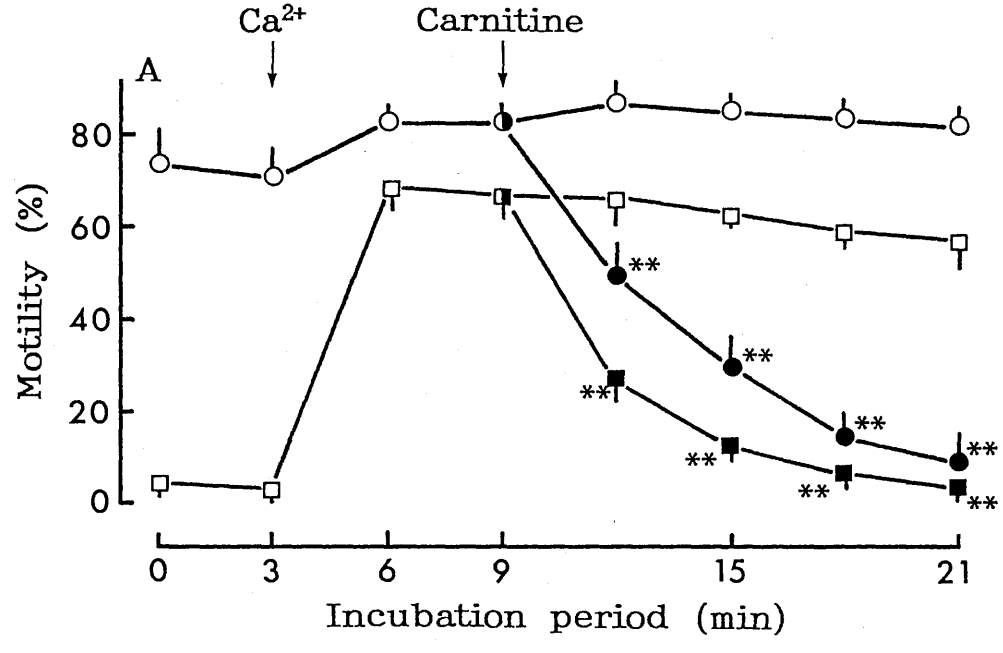

Fig 3a

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(b)

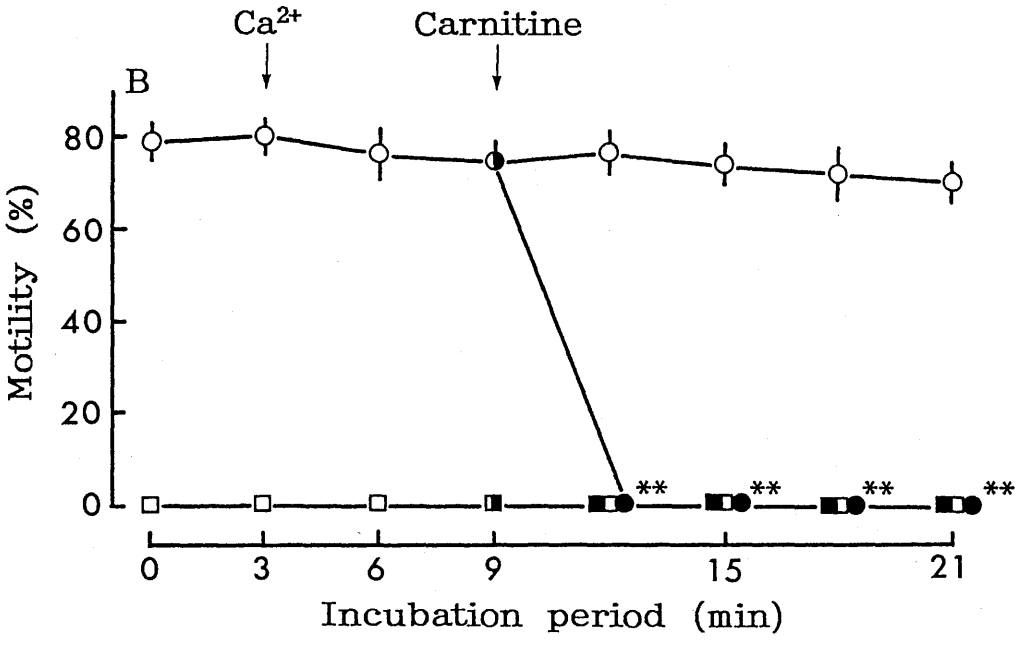

Fig 3b

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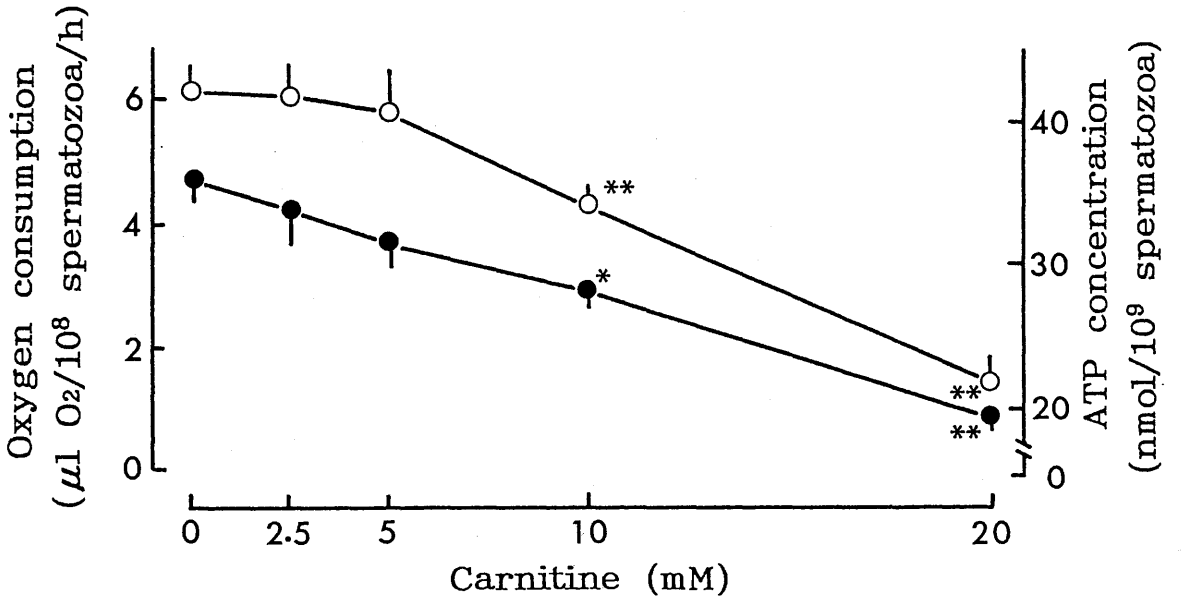

Fig 4

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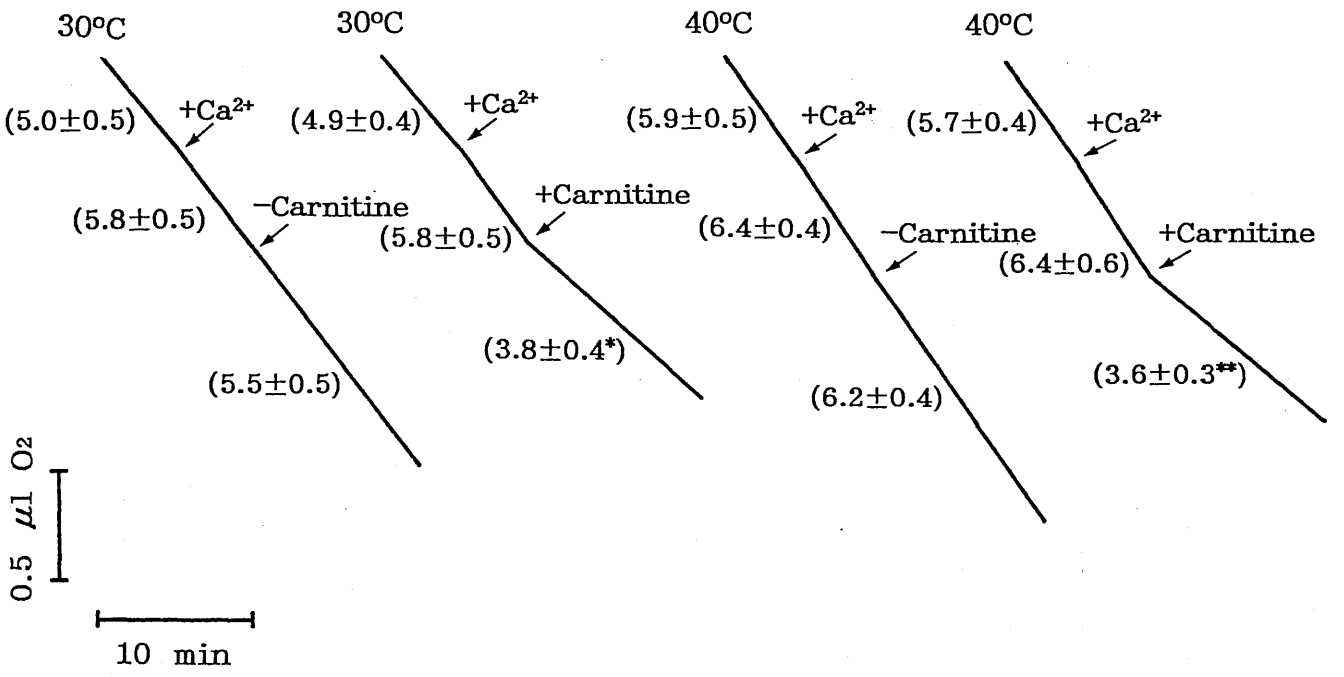

Fig 5

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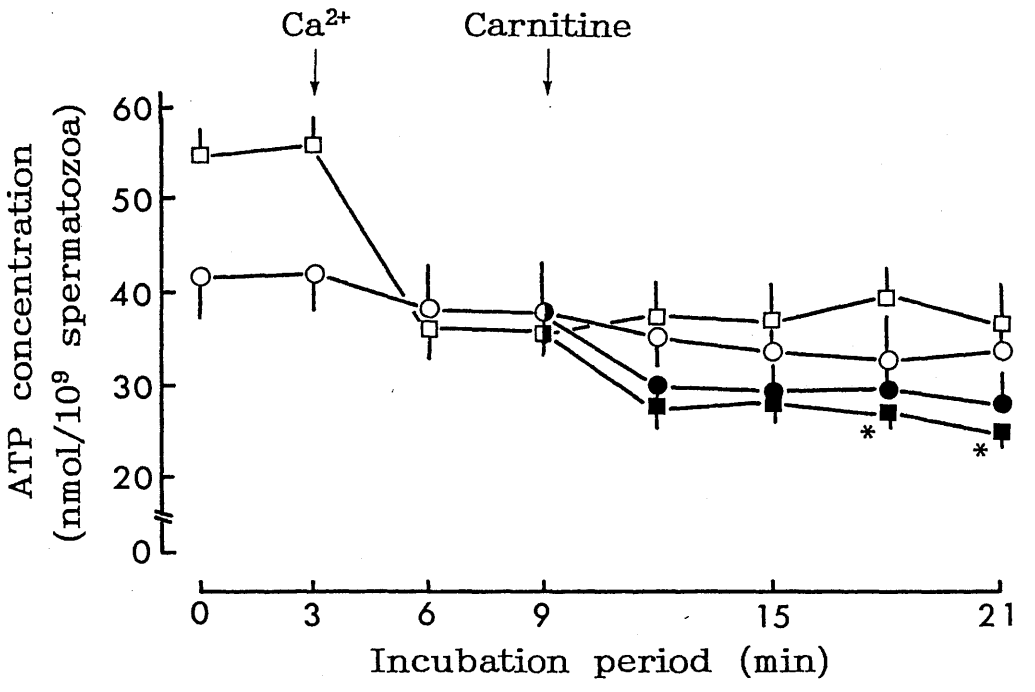

Fig 6

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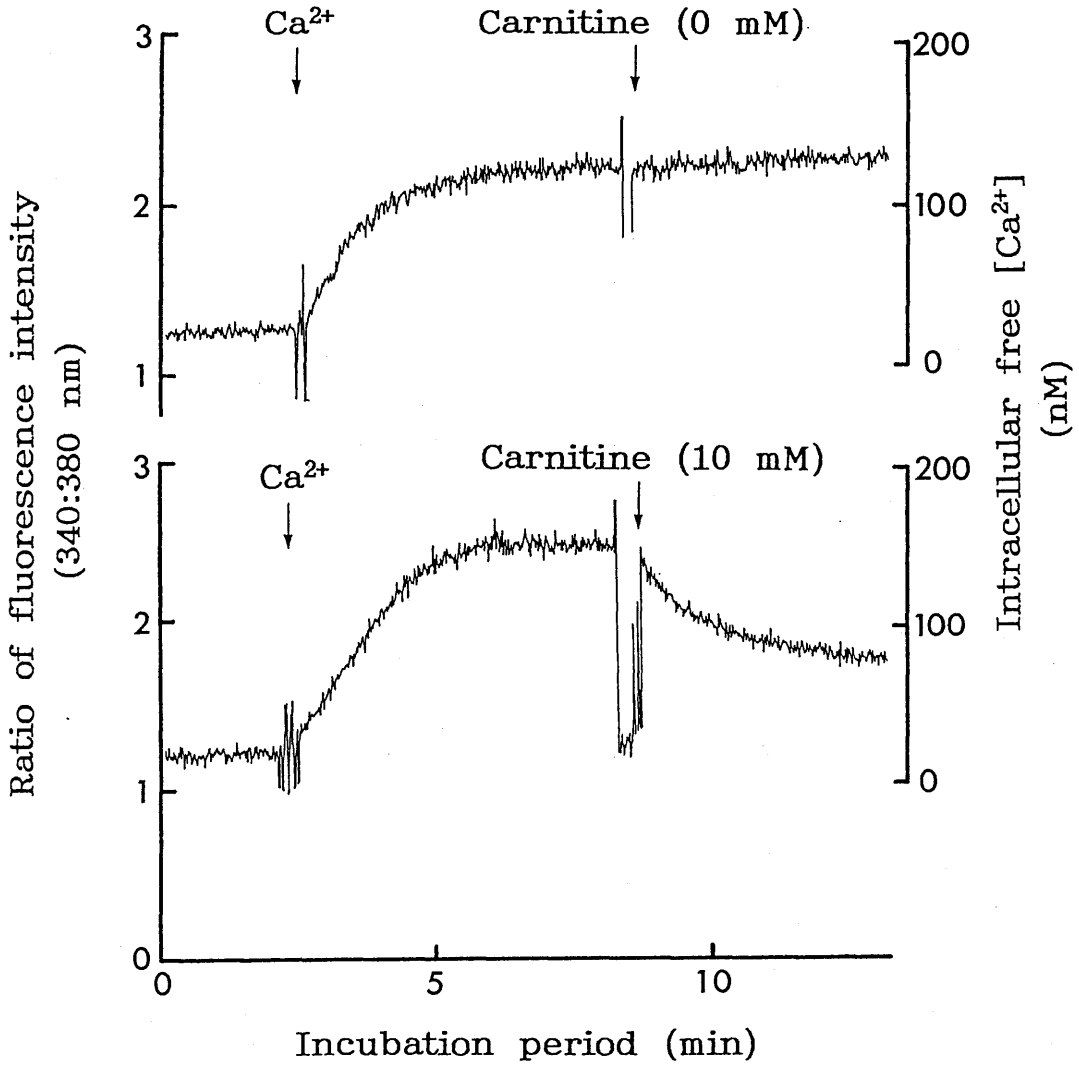

Fig 7

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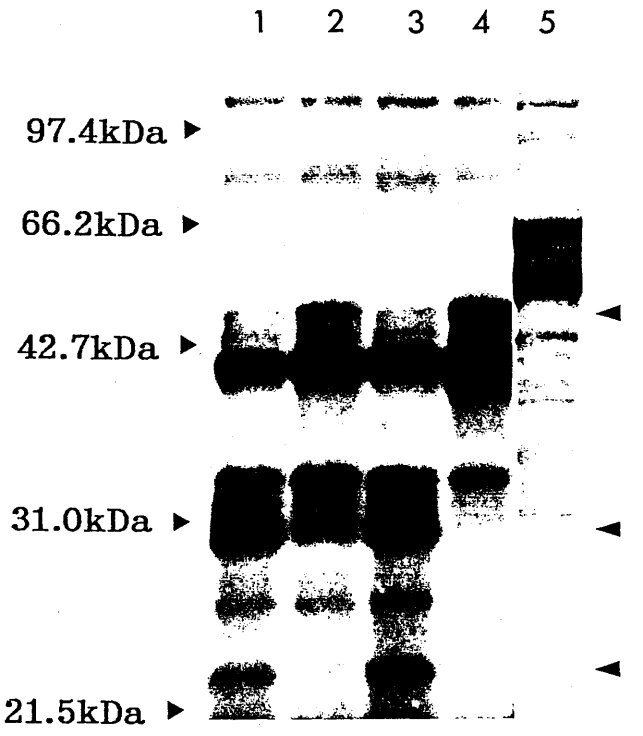

Fig. 8
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