Factors from fluid of the ovarian pocket that stimulate sperm motility in domestic hens

K. Ashizawa* and G.J. Wishart

*Laboratory of Animal Reproduction,

Faculty of Agriculture, Miyazaki University,

Miyazaki 889-21, Japan and

Department of Molecular and Life Sciences

Dundee Institute of Technology,

Bell Street, Dundee DD1 1HG, UK

Short title: fowl sperm motility regulation

key words: spermatozoa, motility, domestic fowl, fertilization

Summary. About 5ml of fluid could be collected from the region of the peritoneum surrounding the ovarian pocket of domestic fowl hens only around the time of ovulation. This fluid stimulated the motility of fowl spermatozoa 6-fold at 40°C in vitro. Gel filtration of the revealed 2 peaks of motility-stimulating activity: one of these was identified as calcium; the other as a low molecular weight (Mr 200) heat-labile substance.

From evidence, which it discussed, it is suggested that this fluid is representative of the milieu which surrounds the ovum at the time of fertilization; and thus that the motility-stimulating factors may be implicated in promoting fertilization by activating spermatozoa, which are normally stored in a quiescent state within the oviduct.

Introduction

The idea that avian spermatozoa may be immotile during their sojourn in the hens reproductive tract was introduced by Munro (1938) who noted that fowl spermatozoa remained immotile *in vitro* when suspended in a simple salt solution at the avian body temperature of 40-41°C. In the uterovaginal sperm storage tubules where avian spermatozoa may remain for several weeks before fertilization (see Lake, 1975), spermatozoa do appear to be mostly immotile (Bakst, 1987). Furthermore, sperm motility does not seem to be required for the transport of spermatozoa within the oviduct from the shell gland (uterus) to the magnum, since even dead spermatozoa on inert particles such as carbon powder inserted in the shell gland can move upwards through the oviduct (Mimura, 1939, 1941). However, it has been shown that only motile spermatozoa are able to transverse from the posterior to the anterior vagina (Takeda, 1974) and it has been suggested that factors within seminal plasma, deposited along with the spermatozoa, may enhance intravaginal sperm transport (Terada *et al.* 1984). Indeed two sperm motility-stimulating factors, calcium and an unidentified regulator of low molecular weight, have been separated from fowl seminal plasma.

Like those of mammals, avian spermatozoa must penetrate the egg investments to reach and thus fertilise the ovum. In birds this involves hydrolysis of the proteinaceous perivitelline layer by sperm acrosomal enzymes (Bakst and Howarth, 1977; Okamura and Nishiyama, 1978). In some mammalian species, development of hyperactivated sperm motility is associated with capacitation and is considered to be important for zona pellucida penetration (Yanagimachi, 1981). Since penetration of the avian perivitelline layer appears to occur in an analogous manner to zona pellucida penetration, it may be presumed that fowl spermatozoa also require to be motile to locate and

penetrate the perivitelline layer. Furthermore, since these spermatozoa will have previously been immotile within the sperm-storage tubules (see Bakst, 1987), their motility will therefore require to be stimulated by factors associated with the ovum or its surrounding fluids.

At the time of ovulation, fluid collects in the peritoneal cavity beside the ovarian pocket (P.E. Lake, personal communication) which is the portion of the body cavity immediately adjacent to the ovary and the infundibulum, enclosed by the body wall, left abdominal air sac and the viscera (see Olsen and Nehrer, 1948). Fertilization occurs within this part of the peritoneum either before, or within 15-20 min of the ovulated ovum entering the infundibulum (Warren and Scott, 1934; Olsen and Nehrer, 1948; Okamura and Nishiyama, 1978). After this time the outer perivitelline layer is laid down (Bain and Hall, 1969) and is impenetrable by spermatozoa (Bakst and Howarth, 1977). Since spermatozoa can be found in ovarian pocket fluid taken at the time of ovulation (Bobr et al. 1964) and since the infundibulum actively engulfs the ovulated ovum or even surrounds the preovulatory follicle at this time (Warren and Scott, 1934; Phillips and Warren, 1937), it may be presumed that this fluid represents the milieu in which fertilization takes place.

In the following work we have therefore examined this fluid for its sperm motility-stimulating properties and have attempted to separate and identify the motility-stimulating principles.

Materials and Methods

Animals. Male fowls were a Rhode Island Red-type control strain from Ross Poultry Ltd., Newbridge, Midlothian and hens were a commercial laying strain (Hi-Sex, Euribrid). All birds were caged individually, given 14h light/24h and fed a proprietary breeders ration ad libitum.

Ovarian pocket fluid collection and treatment. Hens were killed by cervical dislocation 15-20 min after oviposition and laparotomised. The viscera were carefully drawn aside to reveal the 5-10 ml of fluid lying within the region of the ovarian pocket. This was removed with a pipette. Only straw-coloured fluid was retained; blood-contaminated samples were discarded. The fluid was then centrifuged at 105,000 g for 1h at 5°C. The supernatant was stored at -20°C directly, or after concentrating 10-fold by freeze-drying. Calcium was removed from the fluid with Dowex 50x4-200 as before (Ashizawa and Wishart, 1987). The resin was converted to the sodium form with 1M NaOH and then washed and equilibrated with NaCl/TES buffer (0.15M NaCl with 20 mM TES (N-tris-[hydroxymethly]methyl-2-aminoethanesulphonic acid) adjusted to pH 7.4 with 1M-NaOH). This was then mixed well with the samples at 20% (w/v) to remove calcium. To aid characterisation of the sperm motility-stimulating factors, samples of ovarian pocket fluid were further treated by immersion in a boiling water-bath for 5 min and by organic extraction by mixing with 5 volumes of chloroform.

Gel filtration. Sephadex G-15-120 (Pharmacia Fine Chemicals, Inc.) was hydrated and equilibrated with NaCl/TES buffer in a 1.8 x 100 cm column. One ml of concentrated (x10) ovarian pocket fluid was passed through the column at a flow rate of 80 ml/h at 5°C and 80 x 5 ml samples were collected and stored at 5°C before assay.

Preparation of spermatozoa. Samples of semen pooled from 4-6 males were diluted 8-fold with NaCl/TES at room temperature mixed and centrifuged at 700g for 12 min. The pellet of spermatozoa was reconstituted gently in the same buffer to give a concentration of approximately 1 x 10^9 spermatozoa/ml. These preparations were incubated in 4 ml quantities in 25 ml Nalgene flasks in a shaking water bath at 40°C.

Assays. Sperm motility was assayed spectrophotometrically, the constant %(OD)m, which correlates with the percentage of motile spermatozoa being used to define 'motility' and sperm concentration being derived from the constant ODm (Wishart and Ross, 1985). Calcium was measured spectrophotometrically with the indicator Arsenazo 111 (Gratzer and Beaven, 1977) and protein concentration with Folin's reagent (Lowry et al. 1951). Sperm ATP concentrations were measured in boiled extracts using firefly extract luminescence as before (Wishart 1982). Osmotic pressure was measured by freezing-point depression with an Advanced Digimatic Osmometer (Advanced Instruments Inc., Needham Heights, MA, USA).

Statistical Treatment. Data were subjected to statistical analysis using student's t-test.

Results

The effect of increasing concentrations of ovarian pocket fluid on the motility of fowl spermatozoa suspended in NaCl/TES at 40°C is shown in Fig. 1a. That motility stimulation increases hyperbolically with the concentration of both native and Dowex-treated fluids is confirmed by the linear nature of the reciprocal plot (Fig. 1b), which also shows that calcium appears to account for around 70% of the motility-stimulating activity of the peritoneal fluid. The concentration of free calcium in the peritoneal fluid was 2.04±0.14 mM (mean s.e.m. of 5 different samples). The relative unresponsiveness of sperm motility to concentrations of fluid greater than 8% was not the result of reduced energy metabolism: samples of spermatozoa in the presence and absence of 10% peritoneal fluid had ATP concentrations which, at 40.3±2.4 and 38.4±1.1 (means±s.e.m., n=3)nmol/10^9 spermatozoa respectively, were not significantly different ($p > 0.01$).

Gel filtration of the native fluid revealed two peaks of motility-stimulating activity with peaks in fractions 18 and 27 (Fig. 2a). The effectiveness and specificity of calcium removal is shown in Fig. 2b where both calcium and the second peak of motility-stimulating activity have disappeared whilst the characteristic pattern of the other parameters remains unchanged.

In both native and Dowex-treated samples, osmolality was maximum in fractions 21, at 441 and 481 mOsm/kg respectively, against a background buffer osmolality of 310-315 mOsm/kg. The peak of A_{280} in fractions 11-14 was confirmed to be protein by the method of Lowry et al (1951). Fractions 57-61 which also had a high A_{280} did not contain protein.

The motility-stimulating activity of 10% Dowex-treated fluid was significantly ($p<0.01$) reduced from 49.3±5.8 to 15.7±0.6 by boiling for 5 min whereas boiling had a limited effect on the motility-stimulating activity of native fluid, reducing it from 68.8±4.4 to 55.5±6.7 (each figure mean % [OD]m ± s.e.m.; n=3). Extraction with chloroform also had a limited effect on the motility-stimulating activity of native (68.8±4.4 to 65.7±3.6) and of Dowex-treated (49.3±5.8 to 37.8±2.9) fluid (parameters as above). During 5 min exposure to 10% Dowex-treated fluid at 40°C, the motility of fowl spermatozoa was reduced from 30.7±4.4 to 17.1±2.7 whilst after 5 min exposure to 10% native fluid, motility at 40.9±2.3 was almost unchanged from the motility of 40.5±2.5 found after 15-20 sec of exposure (each figure mean %[OD]m± s.e.m.; n=5).

Discussion

The results show that the fluid which collects in the ovarian pocket at the time of ovulation contains two sperm motility-stimulating factors, one of which is identified as calcium and the other, which remains unidentified, is a dialysable heat-labile substance with a molecular weight of about 200. The position of the latter factor within the fractions obtained from the Sephadex G-15-200 column is exactly analogous to that of the unknown motility-stimulating factors separated from fowl seminal plasma (Ashizawa and Wishart, 1987) and may well represent the same substance. Apart from calcium, two other low molecular weight factors have been shown to stimulate fowl sperm motility: caffeine (Wishart and Ashizawa, 1987) and bicarbonate (Ashizawa et al, 1989). Although the unknown factor could not be identified as either of these, its mode of action in producing a transient stimulation of sperm motility lasting less than 5 min, was like that of caffeine rather than calcium, which produces a more sustained response (Wishart and Ashizawa, 1987).

The source of this ovulation-associated ovarian pocket fluid is not known. It is unlikely to have originated from the ovary since no liquor folliculi has beenfound in the follicles of laying hens and the follicular blood supply appears to be reduced at the time of ovulation (Phillips and Warren, 1937). And although the fluid may be truly peritoneal in origin, another potential source is the oviduct. In many mammalian species oviducal fluids flow into the peritoneal cavity with maximum production occurring at the time of ovulation (see Hamner, 1973). In fowl hens at the time of ovulation the infundibulum becomes oedematous and extremely active, particularly in the ovarian pocket adjacent to the mature follicle (Warren and Scott, 1934; Phillips and Warren, 1937) and its fluid volume is maximum. Furthermore, spermatozoa, which have originated from the oviduct can be found in greatest numbers within the infundibular (Morzenti et al, 1978) and ovarian pocket (Bobr et al, 1964) fluids at this time.

Lake and El Jack (1967) proposed that uterine fluids might contain sperm motility-stimulating factors which could influence sperm movement within the hens reproductive tract and indeed the former proposal has subsequently been demonstrated experimentally (Brillard et al, 1987). However the 'plumping' and 'oviposition' fluids from the hens uterus contained respectively 7 and 12 times more calcium (Lake and El Jack 1967) than the ovarian fluid, so direct transfer of the uterine fluid to the infundibulum and peritoneum seems unlikely.

However, whatever the source of this fluid, evidence suggests that it is indeed representative of the fluid which surrounds the ovum at the time of fertilization (see Introduction) and that the sperm motility-stimulating factors demonstrated in this work may be implicated in promoting fertilization in the domestic fowl.

Acknowledgements

We thank the Japanese Ministry of Education, Science and Culture (No. 0156022294) (KA) and the Agricultural and Food Research Council (GW) for grants. Part of this work was carried out during a sojourn by both authors at the AFRC's Poultry Research Centre.

References

Ashizawa, K. and Wishart, G.J. (1987) Resolution of the sperm motility-stimulating principle of fowl seminal plasma into Ca and an unidentified low molecular weight factor. J. Reprod. Fert. 81, 495-499.

Ashizawa, K., Masazumi, S. and Okauchi, K. (1989) Stimulation of the motility and oxygen consumption of fowl spermatozoa by bicarbonate at 40 C. Anim. Reprod. Sci. 21, 301-308

Bain, J.M. and Hall, J. (1969) Observations on the development and structure of the vitelline membrane of the hens egg: an electron microscope study. Aust. J. Biol. Sci. 22, 653-665

Bakst, M.R. (1987) Anatomical basis of sperm-storage in the avian oviduct. Scanning Microsc. 1, 1257-1266

Bakst, M.R. and Howarth, B.Jr. (1977) Hydrolysis of the hen's perivitelline layer by cock sperm in vitro. Biol. Rep. 17, 370-379.

Bobr, L.W., Ogasawara, F.X. and Lorenz, F.W. (1964) Distribution of spermatozoa in the oviduct and fertility in domestic birds, 11. Transport of spermatozoa in the fowl oviduct. J. Reprod. Fert. 8, 49-58

Brillard J.P., Galut, O. and Nys, Y. (1987) Possible causes of subfertility in hens following insemination near the time of oviposition. Brit. Poult. Sci. 28, 307-318.

El Jack, M.H. and Lake, P.E. (1967) The content of the principal inorganic ions and carbon dioxide in uterine fluids of the domestic hen. J. Reprod. Fert. 13, 127-132.

Gratzer, W.B. and Beaven, G.H. (1977) Use of the metal-ion indicator Arsenazo 111, in the measurement of calcium binding. Analyt. Biochem. 81, 118-129

Hamner, C.E. (1973) Oviducal fluid-composition and physiology. in Handbook of Physiology Section 7: Endocrinology Volume 2, Female Reproductive Systems, Part 2 pp 141-151

Lake, P.E. (1975) Gamete production and the fertile period with particular reference to domesticated birds. Symp. Zool. Soc. Lond. 35, 225-244

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.d (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275

Mimura, H. (1939) On the mechanism of travel of spermatozoa through the oviduct in the domestic fowl, with special reference to the artificial insemination. Okajimas Folia Anat. Japon. 17, 459-476.

Mimura, H. (1941) Studies on the mechanism of travel of spermatozoa through the oviduct in the domestic fowl. J. Dept. Agric. Kyushu Imperial Univ. 6, 167-259

Morzenti, A., Ogasawara, F.X. and Fuqua, C.L. (1978) The relationship of infundibular fluid to the avian sperm transport mechanisms. Poult. Sci. 57, 1173.

Munro, S.S. (1938) Fowl sperm immobilisation by a temperature-media interaction and its biological significance. Q. Jl exp. Physiol. 27, 281-291

Okamura, F. and Nishayama, H. (1978) The passage of spermatozoa through the vitelline membrane in the domestic fowl, Gallus gallus. Cell. Tiss. Res. 188, 497-508

Olsen, M.W. and Nehrer, B.H. (1948) The site of fertilization in the domestic fowl. J. Exptl. Zoo. 109, 355-366

Phillips, R.E. and Warren, D.C. (1937) Observations concerning the mechanics of ovulation in the fowl. J. Exptl. Zoo. 76, 117-136

Takeda, A. (1974) The transport of spermatozoa in the vagina of the hen. Jpn. Poult. Sci. 11, 45-54

Terada, T., Watanabe, M. and Tsutsumi, Y. (1984) Possible significance of accessory reproductive fluid in the exhibition of fertilizing ability of spermatozoa in the domestic fowl. Jpn. J. Zootech. Sci. 55, 52-58.

Warren, D.C. and Scott, H.M. (1934) Ovulation in the domestic hen. Science 80, 461-462

Wishart, G.J. (1982) Maintenance of ATP concentrations in and of fertilizing ability of fowl and turkey spermatozoa in vitro. J. Reprod. Ferrt. 66, 457-462.

Wishart, G.J. and Ashizawa, K. (1987) Regulation of the motility of fowl spermatozoa by calcium and cAMP. J. Reprod. Fert. 80, 607-611

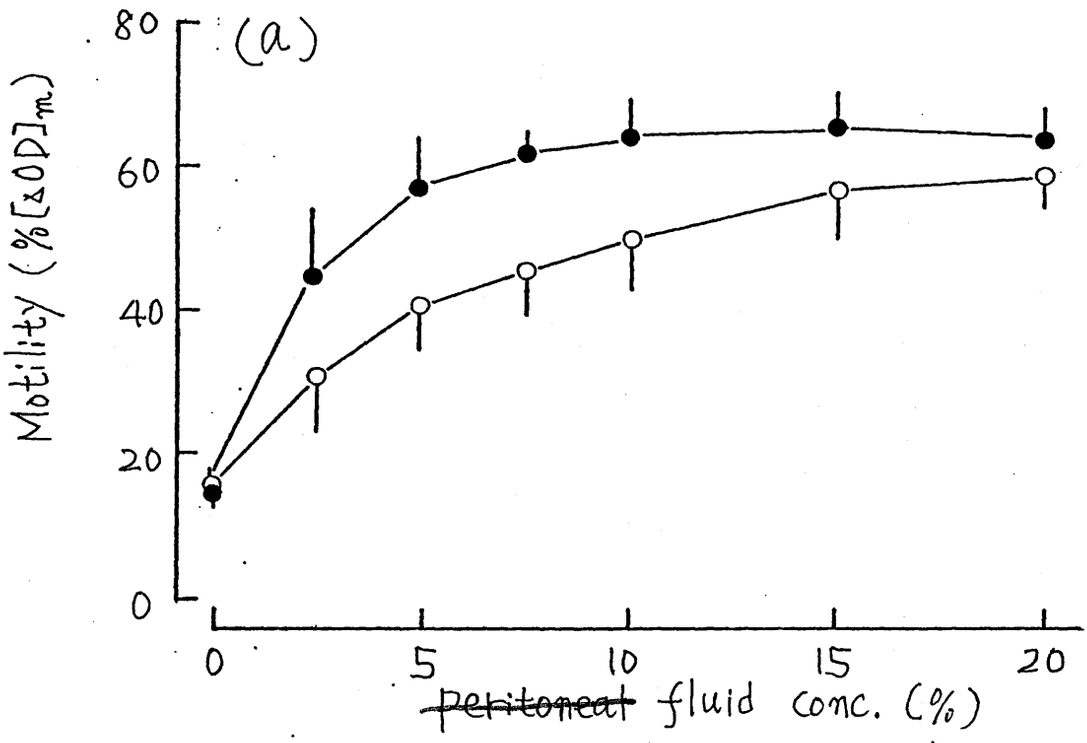
Wishart, G.J.d and Ross, F.H. (1985) Characterisation of a spectrophotometric technique for the estimation of fowl and turkey sperm motility. Gamete Res. 11, 169-178

Yanagimachi, R. (1981) Mechanisms of fertilization in mammals, in 'Fertilization and embryonic development in vitro, eds. L. Mastroianni and D.J. Biggers, Plenum Press, pp 81-182

Fig. 1. The potency of motility-stimulating activity of fowl hen ovarian pocket fluid before (●) and after (○) Dowex treatment. Each point in (a) represents the mean ± s.e.m. of motility of 3 samples of spermatozoa after 30 sec exposure to varying concentrations of a different fluid sample, before and after Dowex treatment. Graph (b) is formed using the mean estimations from the same data.

Fig. 2. Separation of motility-stimulating activities of fowl hen ovarian pocket fluid using Sephadex G-15-200. The elution profiles represent fractions from the same sample before (a) and after (b) Dowex treatment. Calcium concentrations (▲) and absorbance at 280 nm (o) of samples are shown with motility-stimulating activity (●) measured as the motility of a standard sample of spermatozoa suspended in a portion of each fraction. The molecular weight 'markers' were ethanediol (Mr62) and polyethylene glycol (Mr200 and 400). Their positions are shown by closed squares.

Fig. 1.


(a)
Motility (%[ΔOD]m)
80
60
40
20
0
0
5
10
15
20
~~peritoneal~~ fluid conc. (%)

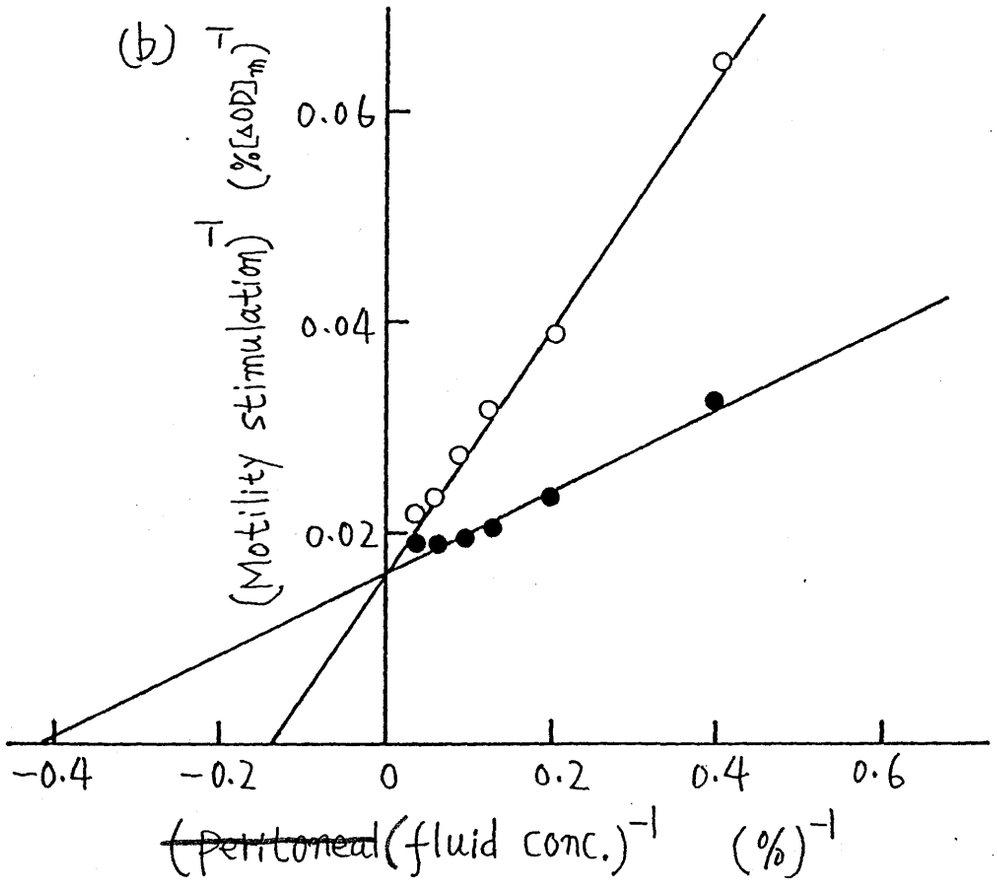
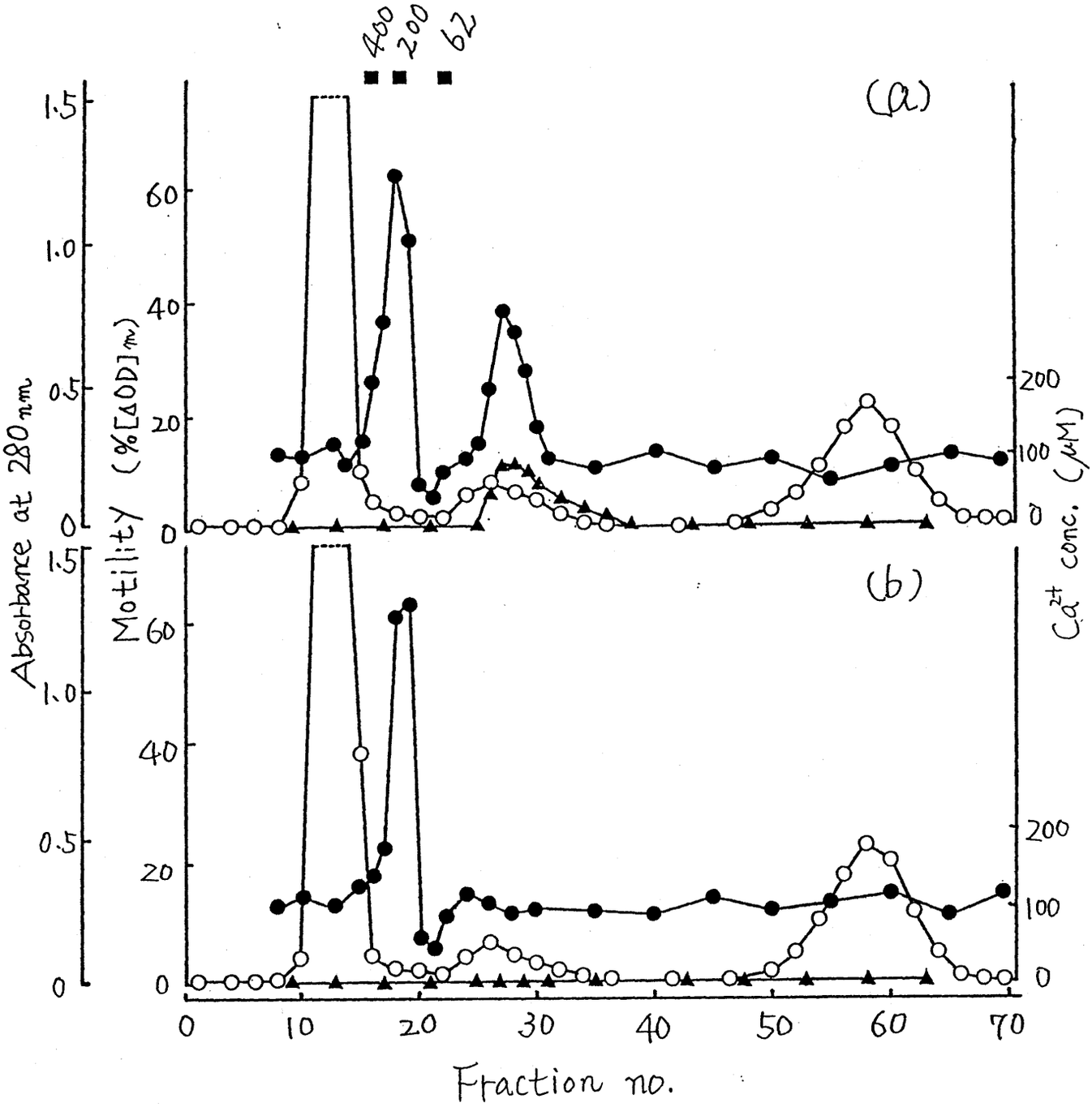

(b)
(Motility stimulation)⁻¹ (%[ΔOD]m)⁻¹
0.06
0.04
0.02
-0.4
-0.2
0
0.2
0.4
0.6
~~(peritoneal~~(fluid conc.)⁻¹ (%)⁻¹

Fig. 2.


400
200
62
(a)
(b)
Absorbance at 280 nm
Motility (%[ΔOD]/m)
Ca2+ conc. (μM)
Fraction no.
0
10
20
30
40
50
60
70
0.5
1.0
1.5
20
40
60
100
200