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Resolution of the sperm motility-stimulating principle of fowl seminal plasma into Ca^{2+} and an unidentified low molecular weight factor*

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Summary. It was confirmed, using an objective assay of motility, that fowl seminal plasma restores and stimulates the motility of fowl spermatozoa at 40°C in a dose-dependent manner. By separation of a 100 000 *g* supernatant of fowl seminal plasma with Sephadex G-15, two peaks of motility-stimulating activity were distinguished. One peak coincided with that of calcium and was absent when calcium was removed from the seminal plasma with Dowex 50. The other peak, which accounted for 44% of motility-stimulating activity, contained a low molecular weight, dialysable factor which remains to be identified.

Introduction

In most simple salt solutions, fowl spermatozoa become immotile as the temperature is raised from 30 to 40°C (Munro, 1938; Nevo & Schindler, 1968; Ashizawa & Nishiyama, 1978; Takeda, 1982). Motility at this temperature can be restored by the addition of seminal plasma (Munro, 1938; Nevo & Schindler, 1968; Takeda, 1982). The factor(s) responsible stimulate motility and oxygen consumption in a dose-dependent manner and have been shown to be of low (M_r < 1000) molecular weight (Ashizawa & Okauchi, 1984).

The following work was designed to quantitate the potency of motility-stimulating factor(s) using an objective assay of motility (Wishart & Ross, 1985) and to attempt to identify the factor(s) after gel filtration of the seminal plasma.

Materials and Methods

Animals. Birds were a Rhode Island Red-type control strain from Ross Poultry Ltd, Newbridge, Midlothian. They were caged individually, given 14 h light per 24 h and fed a commercial breeders ration *ad libitum*. Semen was collected (Lake, 1957) from these birds 3 times every week.

Seminal plasma preparation and gel filtration. Pooled semen was centrifuged at 700 *g* for 12 min at room temperature to remove spermatozoa. The supernatant was then recentrifuged at 100 000 *g* for 1 h at 5°C to produce a pellet of vesicular material (Servouse *et al.*, 1976) and a clear supernatant. The supernatant (referred to subsequently as 'seminal plasma') was stored at −20°C directly or after concentrating 10-fold by freeze drying. For gel filtration, Sephadex G-15-120 (Pharmacia Fine Chemicals, Inc., supplied by Sigma Chemical Co., Poole, Dorset, U.K.) was hydrated and equilibrated in 0·15 M-NaCl with 20 mM-TES (*N*-tris-[hydroxymethyl]methyl-2-aminoethanesulphonic acid), adjusted to pH 7·4 with 1 M-NaOH, in a 1·8 × 100 cm column. The × 10 concentrated seminal plasma (1 ml) was passed through the column at a flow rate of 80 ml/h at 5°C and 80 × 5 ml samples were collected and stored at 5°C before assay.

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Calcium was removed from seminal plasma or column fractions with Dowex 50 × 4-200 (Sigma Chemical Co.). The resin was converted to the sodium form with 1 M-NaOH and then washed and equilibrated with NaCl/TES solution (see above). This was mixed well with seminal plasma samples at 20% (w/v) to remove calcium.

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

Assays. The motility of spermatozoa was assayed spectrophotometrically: the constant %(ΔOD), which correlates with the percentage of motile spermatozoa (Wishart & Ross, 1985), was used to define 'motility'. Sperm concentration was estimated by correlation with optical density (Wishart & Palmer, 1986).

ATP concentrations were measured in extracts of boiled spermatozoa, with firefly luciferase as described previously (Wishart, 1982). Cyclic AMP concentrations in the range 0·2–1·6 pmol were measured in similarly-prepared extracts by a protein binding assay using [^{3}H]cAMP (kit from Amersham International plc, Amersham, U.K.).

Osmotic pressure was measured by freezing-point depression with an Advanced Digimatic Osmometer (Advanced Instruments Inc., Needham Heights, MA, U.S.A.). Calcium was measured spectrophotometrically with the indicator Arsenazo 111 (Gratzer & Beaven, 1977) and protein concentration with Folin's reagent (Lowry *et al.*, 1951).

Results

The effect of increasing concentrations of seminal plasma on the motility of fowl spermatozoa is shown in Fig. 1. Stimulation increased in a hyperbolic manner, reaching a maximum at 75%. The relative unresponsiveness of sperm motility to concentrations of seminal plasma greater than 8% was not the result of reduced energy metabolism: samples of spermatozoa in the presence and absence of 10% seminal plasma had ATP concentrations which, at 32·4 ± 2·2 and 34·4 ± 1·8 (mean ± s.e.m., $n = 3$) nmol/10^9 spermatozoa respectively, were not significantly different ($P > 0{\cdot}05$). The same samples of spermatozoa in the presence of 8 mM-calcium, which also stimulates motility maximally (Wishart & Ashizawa, 1987), showed a significant reduction in ATP concentrations (25·8 ± 0·5 nmol/10^9 spermatozoa), suggesting that the seminal plasma also contians oxidizable subtrates.

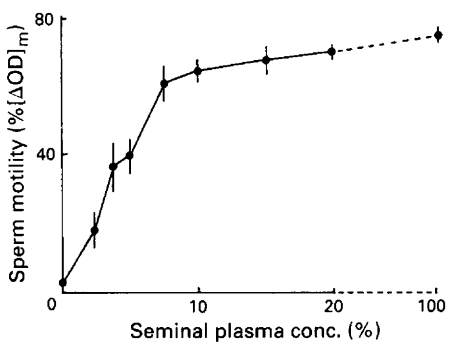

Fig. 1. The effect of seminal plasma on fowl sperm motility at 40°C. Washed samples of spermatozoa were incubated at 40°C in NaCl/TES buffer (see 'Materials and Methods') for 15–60 min before assaying motility 20 sec after further dilution in the same buffer containing seminal plasma at various concentrations. Each point represents the mean ± s.e.m. of 3 estimations.

Separation of two motility-stimulating factors is shown in Fig. 2. The first peak of absorbance at 280 nm, in Fractions 11 and 12, was confirmed to be protein by the Lowry *et al.* (1951) method. The second peak, between Fractions 54 and 65, showed spectral properties similar to those of tyrosine. The osmolarity was raised above that of the NaCl/TES buffer (310 mosmol/kg) in Fractions 16 to 24. With native seminal plasma two peaks in Fractions 18 (351 mosmol/kg) and 22 (397 mosmol/kg) were found. Dowex-treated seminal plasma showed peaks of osmolarity at the

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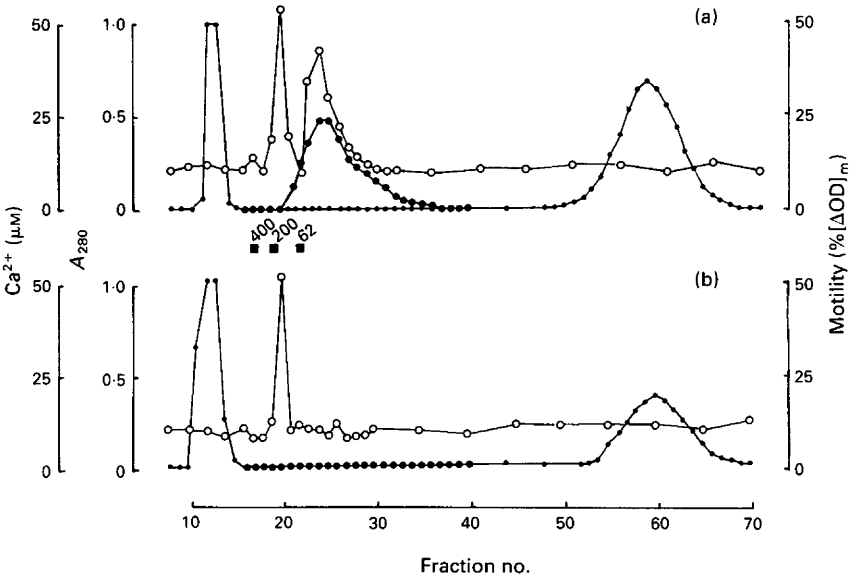

Fig. 2. Separation of motility-stimulating activities of fowl seminal plasma using Sephadex G15. The elution profiles represent fractions from the same sample before (a) and after (b) Dowex treatment. Calcium concentrations (●) and absorbance at 280 nm (•) of samples are shown with motility-stimulating activity (○) measured as the motility of a standard sample of spermatozoa suspended in a portion of each fraction. The molecular weight 'markers' were ethanediol (M_r 62) and polyethylene glycol (M_r 200 and 400), respectively. Their positions are shown by closed squares.

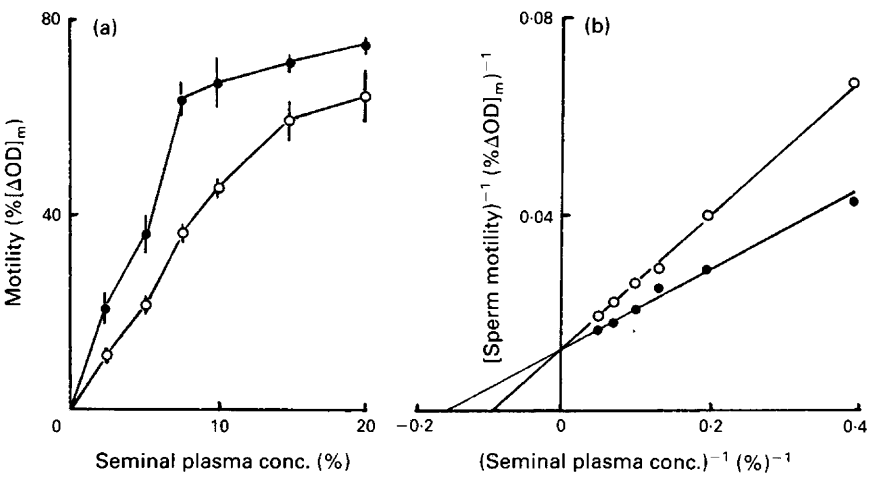

Fig. 3. The potency of motility-stimulating activity of seminal plasma before (●) and after (○) Dowex treatment. Each point in (a) represents the mean ± s.e.m. of motility of 3 samples of spermatozoa, each treated with a different seminal plasma sample before and after Dowex treatment. The inverse of the same mean estimations are shown in (b).

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same locations and similar concentrations: 351 and 415 mosmol/kg in Fractions 18 and 22, respectively. With native seminal plasma two peaks of motility-stimulating activity were clearly distinguished in Fractions 19 and 24. The latter peak coincided with the maximum concentration of calcium in the fractions. When Dowex-treated seminal plasma samples were separated, both the motility-stimulating activity and calcium peak were absent from Fraction 24, leaving a single motility-stimulating peak in Fraction 19, containing a factor of molecular weight between 200 and 400.

A comparison of the motility-stimulating ability of native and Dowex-treated seminal plasma is shown in Fig. 3. The hyperbolic nature of the relationship between stimulation and motility and concentration of seminal plasma is confirmed by the linearity of the reciprocal plot in Fig. 3(b). By comparing the slopes of the two plots in Fig. 3(b), it may be shown that calcium accounts for 56% of the motility-stimulating activity of fowl seminal plasma.

Stimulation of motility by seminal plasma was not accompanied by an increase in sperm cyclic AMP concentrations: the mean cyclic AMP concentrations of 3 samples of washed spermatozoa in the presence of 5 and 10% seminal plasma were 116 ± 15 and 113 ± 11 pmol/10^9 spermatozoa, compared with 114 ± 11 pmol/10^9 spermatozoa for untreated samples.

Discussion

Fowl seminal plasma has been shown to contain a factor(s) which restores and stimulates the motility of fowl spermatozoa (Fig. 1) when measured at 40°C by a spectrophotometric method (Wishart & Ross, 1985), confirming previous subjective observations (Munro, 1938; Nevo & Schindler, 1968; Takeda, 1982). As shown by Ashizawa & Okauchi (1984) this activity is displayed by factors of low molecular weight. The present work extends these findings and shows that motility is stimulated by two factors separable by gel filtration. One factor has been identified as calcium and accounts for 56% of the motility-stimulating activity of seminal plasma. The other unidentified factor has a molecular weight to about 200.

Calcium and caffeine have been shown to stimulate fowl sperm motility at 40°C, the effect of the latter being associated with a rise in sperm cyclic AMP concentrations (Wishart & Ashizawa, 1987). The unidentified factor in the present work was not calcium, from which it was clearly separable, and did not elicit its effect *via* sperm cyclic AMP metabolism.

Studies of motility-stimulating activity in the male reproductive fluids of other species have dealt mainly with proteinaceous factors (see Garbers & Kopf, 1980). However, other low molecular weight factors have also been found in hamster (Bavister & Yanagimachi, 1977) and pig (Okamura & Sugita, 1983) seminal plasma. These factors have been identified as taurine (Mrsny *et al.*, 1979) and bicarbonate (Okamura *et al.*, 1985), respectively. It is not clear whether the unknown factor described in this work relates to either of these: taurine does not stimulate fowl sperm motility at 40°C (G. J. Wishart & K. Ashizawa, unpublished observations); and the 'unknown' factor did not stimulate an increase in sperm cyclic AMP levels, the mechanism by which bicarbonate has been shown to act (Garbers, *et al.*, 1982; Okamura & Sugita, 1983; Okamura *et al.*, 1985).

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