A high molecular weight antifertility factor from human seminal plasma

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Summary. The presence of a high molecular weight antifertility factor in human seminal plasma was established. The factor can be precipitated by centrifugation at 104 000 g. Its activity is maximal when the protein concentration reaches 150 $\mu\text{g}/10^5$ spermatozoa using the mouse in-vitro fertilization assay as the test system. The factor is heat labile but its activity is not affected by dialysis. It prevents the penetration of the spermatozoa through the layers surrounding the egg but has no effect on the fusion of the spermatozoa with the vitelline membrane. The factor is only partly removed from spermatozoa by washing but is completely dispersed when the spermatozoa are incubated in capacitation medium. The pellet that is precipitated from the seminal plasma does not contain any particles or vesicles. However, it is significantly contaminated with low molecular weight material. This material includes the acrosin inhibitor which is present in large enough quantities to hinder fertilization. Washing the pellet twice with H_2O removes these low molecular weight compounds, as indicated by the absence of the acrosin inhibitor, but has no effect on the antifertility properties of the pellet. Therefore, before further study or purification of the factor, it is essential that the pellet is washed to remove such low molecular weight material. The washed pellet consists of at least 7 components as judged by disc gel electrophoresis.

Introduction

Capacitated, fertile spermatozoa can be rendered infertile again by incubation with seminal plasma (Chang, 1957). To date the presence of at least two seminal antifertility factors has been firmly established: (1) a proteinase inhibitor (Zaneveld, Robertson, Kessler & Williams, 1971), and (2) a high molecular weight component that can be sedimented by high-speed centrifugation (Bedford & Chang, 1962; Dukelow, Chernoff & Williams, 1967; Pinsker & Williams, 1967; Robertson, Bhalla & Williams, 1971). The high molecular weight material has been termed 'decapacitation factor', but until the factor is characterized in some detail, it will be impossible to state with certainty whether one investigator is working with the same 'decapacitation factor' as another investigator. The biochemical composition of the high molecular weight component is unknown. Some investigators consider it a glycoprotein (Dukelow *et al.*, 1966; Hunter & Nornes, 1969; Reyes, Oliphant & Brackett, 1975), whereas others have presented evidence that, at least in the rabbit, the antifertility activity is due to small membrane vesicles, approximately 50–7 nm in diameter (Davis, 1973).

Although the high molecular weight component prevents the fusion of the spermatozoon with the ovum (Gould, Srivastava, Cline & Williams, 1971; Davis & Niwa, 1974), the mechanism and site of its antifertility action is not known. Bedford (1970) suggested that the compound stabilizes the plasma and acrosomal membranes, Davis & Niwa (1974) presented evidence that the vesicle-containing fraction prevents the acrosome reaction of rat spermatozoa,

and Zaneveld & Williams (1970) showed that the lytic activity of acrosomal extracts towards the corona radiata of the egg is inhibited, presumably by the inhibition of the corona penetrating enzyme. However, detailed studies to establish at what point the union of the spermatozoon with the ovum is halted by, for instance, removing certain layers from the egg, have not been performed.

Evidence has been obtained that a similar high molecular weight component is present in human seminal plasma (Pinsker & Williams, 1968; Dukelow & Chernoff, 1969) but the nature and functional activity of this component has not been evaluated. Such a component may well influence the normal fertilization process of man and thus be of importance in clinical cases of infertility. It was therefore our objective to establish the presence or absence of a high molecular weight antifertility factor in human seminal plasma and to study some of its physiological properties. The mouse in-vitro fertilization system was used for assay because it permitted more detailed evaluations of the functional activity of the antifertility factor than in-vivo tests.

Materials and General Methods

Isolation of the high molecular weight fraction from human seminal plasma

Pooled human semen was obtained from the Washington Fertility Center (Washington D.C.) and from the Midwest Population Center (Chicago, Illinois). Spermatozoa and other particulate matter were removed by centrifugation at 800 *g* for 15 min and then at 12 000 *g* for 20 min. The supernatant was then centrifuged at 104 000 *g* for either 4 or 18 h in a Beckman L₂65B ultracentrifuge using a fixed angle rotor, type 65. The precipitates were re-suspended in distilled H₂O and dialysed at 4°C against two changes of 2 l distilled H₂O to remove any salts that might be present. The sample was freeze-dried and stored at -20°C.

In-vitro fertilization tests

Mature Swiss albino mice (8–10 weeks old) were injected intraperitoneally with 10 i.u. PMSG (Gestyl: Organon Inc., Holland) followed 48 h later by 10 i.u. hCG (Antuitrin "S": Parke-Davis, Detroit, Michigan, U.S.A.). The mice were killed 12–14 h after the hCG injection. All subsequent work was performed at 37°C in a temperature-controlled room. The oviducts were excised and the oocytes in their follicle cell masses were released from the ampullae into sterilized silicone oil (200 fluid: Dow Corning Corp., Michigan, U.S.A.) which was prewarmed to 37°C and gassed with a mixture of 5% CO₂, 5% O₂ and 90% N₂. Intact oocytes (i.e. surrounded by follicle cells) were transferred directly to microdishes containing 150–200 µl culture medium under silicone oil. The culture medium was a modified Krebs–Ringer bicarbonate solution (Wolf, Inoue & Stark, 1976) and contained 1 mg bovine serum albumin (BSA) (Fraction V: Sigma Chemical Company, St. Louis, Missouri, U.S.A.).

Epididymal spermatozoa were obtained by making small cuts in each cauda epididymidis of adult male mice and then placing the epididymides into 1 ml capacitating medium. This medium was essentially the same as the culture medium except that it contained 20 mg BSA/ml. After 5 min, the epididymal tissue was removed and the sperm suspension was incubated for 1 h at 37°C to capacitate the spermatozoa. The final mixture was adjusted to a sperm concentration of $3-5 \times 10^6$ /ml.

Oocytes and spermatozoa were incubated for 24 h at 37°C under an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. The ova were washed thoroughly several times in the medium to remove supplementary spermatozoa. The eggs were mounted and stained with aceto-lacmoid (Toyoda & Chang, 1974) before examination by phase-contrast microscopy for evidence of penetration and cleavage. The criteria used were the presence of a swollen (decondensing) sperm nucleus or 2 pronuclei in 1-cell eggs, or normal cleavage.

To test the antifertility activity of the high molecular weight fraction, the freeze-dried pellet was dissolved in 1 ml culture medium at a known protein concentration. The solution was added to 0.5 ml of the capacitated sperm mixture and incubated for 20 min at 37°C, and then 20–25 µl samples were added to the oocytes in the culture dish. Control cultures consisted of capacitated sperm solution and culture medium only.

Statistical analyses

The accumulated data from the individual experimental procedures were statistically compared using Student's *t* test and analyses of variance. Differences were considered to be significant at $P < 0.05$.

Detailed Methods and Results

Recovery of the high molecular weight material from human seminal plasma

After ultracentrifugation for 4 h, a pellet of 260 mg dry weight was obtained from 64 ml of human seminal plasma, from which the particulate matter had first been removed by 2 centrifugation steps at slower speed. This pellet possessed 145 mg protein (55% protein) as estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). When the same amount of seminal plasma was dialysed and subsequently freeze dried, 2.3 g solid was obtained of which 1.15 g consisted of protein.

The pellet was significantly contaminated with low molecular weight material (see next section). To remove the trapped material, the pellet was washed by re-suspension in distilled H₂O and re-centrifugation at 104 000 *g* for 4 h. This reduced the 260 mg pellet to 150 mg, but the protein content increased to 62.5%. A much larger pellet (400 mg) was obtained when 64 ml seminal plasma was ultracentrifuged for 18 h and washed twice by re-centrifugation. This pellet consisted of 90% protein and was less soluble than the unwashed pellet.

Acrosin-inhibiting activity

Since the only other antifertility compound that is definitely known to be present in human seminal plasma is an inhibitor of acrosin, assays were performed to test the inhibitory activity of the pellet towards this enzyme.

Human acrosin was obtained by acid extraction of washed spermatozoa, and by Sephadex gel filtration and Sephadex CM-50 ion-exchange chromatography of the extracts (Anderson, Beyler & Zaneveld, 1978). The acrosin preparation showed a single band after polyacrylamide disc-gel electrophoresis. Esterase activity of human acrosin was measured by a minor modification of the method of Zaneveld, Dragoje & Schumacher, (1972). Hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester HCl (BAEE) (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was monitored spectrophotometrically by following the increase in absorbance at 253 nm. The reaction mixture contained acrosin (0.9–1.5 µg protein), 0.5 mM-BAEE and 50 mM-Tris-HCl buffer (pH 8.0) in a total reaction volume of 1.0 ml. When pellet preparations obtained by ultracentrifugation were evaluated for acrosin inhibitory activity, 30 µg protein in 0.05 ml Tris-HCl buffer was preincubated for 5 min in the presence of acrosin before initiating the reaction by adding substrate.

The unwashed pellet inhibited acrosin activity at a rate of 3.0 µmol BAEE hydrolysed/min/mg protein. After washing the pellet once by centrifugation, the inhibitory activity decreased to 1.1 µmol BAEE hydrolysed/min/mg protein. A pellet that was washed twice, whether originally obtained after 4 or 18 h of ultracentrifugation, did not inhibit acrosin. Such twice-washed pellets were therefore used for further analysis and for the antifertility tests.

Disc gel electrophoresis

Disc gel electrophoresis was performed as described by Ornstein (1964) and Davis (1964). The pellet, at a protein concentration of 50 µg in 25 µl, was applied to a 5% gel, 5.5 mm wide and 70 mm long, after mixing with sucrose. The reservoir buffers were Tris-glycine buffer, pH 8.8, or glycine-acetic acid buffer, pH 4.3. The gels were stained with 0.05% (w/v) Coomassie blue in 12.5% trichloroacetic acid. The unwashed pellets produced 5 major and 5 minor bands at pH 8.8. The twice-washed pellets formed 4 major and 3 minor bands at pH 8.8 and at pH 4.3, although the mobility of the bands differed.

Sodium dodecyl sulphate (SDS)-polyacrylamide disc-gel electrophoresis was performed by the technique of Weber & Osborn (1969) and Gainer (1971). The protein solution was prepared by dissolving the pellet with known protein concentration in distilled H₂O and adding 1% SDS (10 µg SDS/mg protein). After addition of β-mercaptoethanol (10 µl to 100 µl of the protein-SDS mixture), the solution was stirred overnight at room temperature. A protein concentration of 50 µg in 25 µl was applied to a 5% gel, 5.5 mm wide and 70 mm long which was stained with 500 mg Coomassie blue, 57 ml 250 propanol, 23 ml acetic acid and 145 ml H₂O. Twelve bands were formed. The molecular weights of the major bands fell within a range of 40 000 to 200 000, but there were a few bands of much higher molecular weight.

Histological and electron microscopic observations

A smear made of the pellet on a slide showed only a heavy protein precipitate when stained with Giemsa. Since light microscopy may not be sufficient to detect the vesicles described by Davis (1973), freshly collected as well as freeze-dried pellets were studied with the electron microscope. The pellets were fixed by addition of 2% glutaraldehyde in 0.1 M-cacodylate buffer. The samples were subsequently post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and examined in a Philips 200 electron microscope. Again, no particles or membrane bound vesicles could be found.

Antifertility experiments

Compared to the controls, the pellet (even at the highest concentrations used in the anti-fertility experiments) had no effect on the motility of non-capacitated or capacitated mouse spermatozoa over an 8-h period. However, the pellet possessed strong antifertility activity (Table 1). At 1 mg protein/ml, a slight though significant decrease in the fertility rate was obtained. A further decrease was observed when 2 mg protein/ml was employed. Maximal antifertility

Table 1. Effect of the concentration of the pellet on the fertility *in vitro* of mouse gametes (at least 2 different experiments at each concentration)

	Total no. of oocytes examined	No. of 2-cell embryos	No. of oocytes penetrated	Total no. of oocytes fertilized	% 2-cell embryos	Total % of fertilization (mean ± s.e.m.)
Control	186	91	46	137	49	73 ± 4.9
Pellet (mg protein/ml)						
1	91	35	22	57	38	62 ± 6.8*
2	117	28	23	51	24	43 ± 7.1*
3	156	21	24	45	13	29 ± 1.6*
4	55	7	6	13	12	24 ± 9.5
5	68	9	8	17	13	25 ± 7.8

* Statistically significantly different from the value above, $P < 0.05$.

occurred when protein concentrations of ≥ 3 g/ml were used. All further assays were therefore performed with the minimal concentration of pellet that induced maximal antifertility activity, i.e. 3 mg protein/ml.

Eighteen different experiments were performed to compare the fertility of untreated, capacitated spermatozoa (control) with that of capacitated spermatozoa pretreated with pellet at 3 mg protein/ml. Of the 650 oocytes examined in the controls, 324 (50%) were in the 2-cell embryo stage and 142 (22%) had been penetrated by spermatozoa, for a total fertilization rate of 72% (s.d. ± 5.0). By contrast, of the 635 oocytes in the test experiments, 78 (12%) were in the 2-cell embryo stage and 95 (15%) were penetrated by spermatozoa, for a total fertilization rate of 27% (s.d. ± 6.0). This significant decrease ($P < 0.05$) in the fertilization percentage was consistent in all experiments performed, and ranged from 40 to 60%. It was primarily due to the 4-fold decrease in the number of 2-cell embryos since the number of penetrated oocytes remained essentially the same.

Control experiments were performed to ensure that the small amount of pellet transferred with the spermatozoa to the incubation dishes containing the eggs (see 'General Methods') did not inhibit the cleavage of the eggs. To do so, 25 μ l medium containing pellet at 3 mg protein/ml were added to oocytes that had been inseminated with untreated capacitated spermatozoa 4 h earlier. After 20 h, the oocytes were observed for cleavage. The results were compared to control eggs that were treated only with the medium. No differences between the treated and untreated eggs were observed in the formation of 2-cell embryos or the overall fertility rate.

Removal of ovum investments

Follicle cell-free oocytes were obtained by incubation of the intact oocytes in medium containing 0.1% (w/v) bovine testicular hyaluronidase (Type I: Sigma Chemical Co., St. Louis, Missouri, U.S.A.) for 5–10 min. The resultant oocytes, possessing only the zona pellucida, were washed thoroughly with medium not containing hyaluronidase and transferred to the incubation dishes. Five different experiments were performed to compare the fertility of untreated, capacitated spermatozoa (control) with that of capacitated spermatozoa pretreated with pellet at 3 mg protein/ml (test). The total number of oocytes examined, the number of 2-cell embryos, the number of oocytes penetrated by spermatozoa, and the overall fertilization percentage of the controls were, respectively: 166, 38 (23%), 68 (41%) and 64% (s.e.m. ± 1.4), and those of the test experiments were: 165, 3 (2%), 23 (14%) and 16% (s.e.m. ± 1.7). These last 3 values were significantly less than those of the controls, $P < 0.05$. The dispersion of the follicle cells did not affect the antifertility activity of the pellet. Compared to the experiments with intact ova, the controls showed a small decrease in the overall fertilization rate. A pronounced decrease in the percentage of the 2-cell embryos occurred, however, indicating that the follicle cell layer may have a beneficial effect on the development of the ovum after fertilization. Pavlok & McLaren (1972) and Gwatkin, Anderson & Williams (1974) have also presented evidence that the follicle cell layer may influence ovum development.

To ensure that the antifertility action of the pellet was directed towards the spermatozoa and not the ova, follicle cell-free oocytes were incubated for 15 min in medium containing pellet at 3 mg protein/ml (a large excess of the amount transferred to the incubation dishes during the usual procedures). The oocytes were subsequently washed by transferring them several times into incubation medium not containing any pellet material. The oocytes were then mixed with capacitated spermatozoa and observed 24 h later. Control ova were treated in the same fashion except that no pellet was added. The pretreatment of pellet had no effect on the fertilization rate of the ova; the overall rates of the control and test experiments being 63 and 70%, respectively, based on 2 different experiments involving 126 eggs. No differences in the percentages of 2-cell embryos and oocytes penetrated by spermatozoa were observed.

Zona-free oocytes were obtained by mechanical removal of the zona with hand drawn micropipettes as described by Wolf, Inoue & Stark (1976), after dispersion of the follicle cells by hyaluronidase. The oocytes were observed for sperm penetration, 4 h after addition of untreated, capacitated spermatozoa (control) or after the addition of capacitated spermatozoa pretreated with pellet at 3 mg protein/ml (test). Based on 6 different experiments, the total number of oocytes examined, the number of oocytes penetrated by spermatozoa and the total fertilization rate of the controls were, respectively: 109, 89 and 82% (s.e.m. $\pm$ 3.4), and those of the test experiments were: 123, 102 and 83% (s.e.m. $\pm$ 5.4) (not significantly different from the control). The pellet therefore had no antifertility activity. However, because observation of the eggs after 4 h may have allowed enough time for re-capacitation to occur, additional experiments were performed in which the penetrability of the spermatozoa was checked 1 h after mixing the pellet-treated male gametes with the zona-free oocytes. Again, no differences were observed in the percentage of ova penetrated by the control and test spermatozoa.

Effect of washing the pellet-treated spermatozoa or incubating them in capacitation medium

To test whether the high molecular weight material binds to the spermatozoa, the capacitated spermatozoa treated with pellet at 3 mg protein/ml were washed by centrifugation at 800 g for 10 min at 37°C. The supernatant fluid was removed, the precipitated spermatozoa were resuspended in 1 ml culture medium, and 20–25 μ l were added to the oocytes. Untreated capacitated spermatozoa were washed in the same fashion and used in the control experiments. Three different experiments were performed. The total number of oocytes examined, the number of 2-cell embryos, the number of oocytes penetrated by spermatozoa, and the overall fertilization rate of the controls were, respectively: 106, 59 (56%), 20 (18%) and 74% (s.e. $\pm$ 7.1), and those of the test experiments were: 104, 15 (14%), 37 (36%) and 50% (s.e. $\pm$ 1.2). The difference in overall fertilization rates between the test and control experiments was statistically significant ($P < 0.05$). Thus, although washing pellet-treated spermatozoa increased their fertilizing capacity compared to that of unwashed, pellet-treated spermatozoa, a significant decrease in fertility was still present. The increase in the fertility rate was primarily due to the greater number of spermatozoa that penetrated the ova. The decrease in the percentage of 2-cell embryos was almost identical to that obtained with unwashed, pellet-treated spermatozoa (compare with the previous data).

To evaluate whether the spermatozoa treated with pellet (3 mg protein/ml) could be rendered fertile again by incubation in capacitation medium, they were washed as described above, but

Table 2. Effect of incubating pellet-treated spermatozoa in capacitation medium (6 individual experiments)

	Total no. of oocytes examined	No. of 2-cell embryos	No. of oocytes penetrated	% 2-cell embryos	Total % of fertilization (mean $\pm$ s.e.m.)
Control†	165	68	32	41.2	60.6 $\pm$ 2.9
Pellet treatment (3 mg protein/ml)	160	20	15	12.5	21.2 $\pm$ 2.3*
Control‡	133	52	24	39.0	57.1 $\pm$ 2.2
Pellet-treated spermatozoa incubated in capacitation medium	160	33	18	30.0	46.3 $\pm$ 4.1

* Significantly different from the control value ($P < 0.05$).

† Employing capacitated spermatozoa.

‡ The capacitated spermatozoa were centrifuged and re-suspended in capacitation medium.

afterwards re-suspended in 1 ml capacitation medium (instead of the culture medium) for 1 h at 37°C; 20–25 μ l were subsequently added to the oocytes. Parallel control experiments were performed to ensure that the centrifugation and incubation procedures did not affect the fertility of the spermatozoa (Table 2). The pellet-treated spermatozoa incubated in capacitation medium possessed a fertilization rate that was almost identical to that of untreated control spermatozoa and more than twice that of the pellet-treated spermatozoa that were not incubated in capacitation medium. In contrast to the spermatozoa that were only washed after pellet treatment (see before), fertilization with pellet-treated spermatozoa incubated in capacitation medium produced essentially the same percentage of 2-cell embryos as did the untreated, control spermatozoa.

Heat stability

The heat stability of the pellet was tested by dissolving the freeze-dried material in distilled H₂O and placing it in a test tube which was kept for 1 h in a boiling water bath. The heated solution was subsequently freeze dried and tested for activity (Table 3). Boiling the pellet for 1 h destroyed its antifertility activity. No significant difference could be observed in the numbers of penetrated or cleaved ova between the control spermatozoa and those treated with the boiled pellet.

Table 3. Effect of heating on the antifertility activity of the pellet (3 individual experiments)

	Total no. of oocytes examined	No. of 2-cell embryos	No. of oocytes penetrated	Total no. of oocytes fertilized	% 2-cell embryos	Total % of fertilization (mean $\pm$ s.e.m.)
Control	110	44	29	73	40	66 $\pm$ 4.2
Pellet (not heat-treated)†	142	19	14	33	13	23 $\pm$ 0.9*
Heat-treated pellet†	96	38	31	69	40	72 $\pm$ 1.0

* Significantly different from the control value ($P < 0.05$).

† Concentration of pellet used was 3 mg protein/ml.

Discussion

The results clearly establish the presence of one or more high molecular weight antifertility agents in human seminal plasma. The activity is not due to particles or vesicles, as is at least partly so in some animal species (Davis, 1973; Davis & Niwa, 1974). However, since the particulate matter was removed from our preparation before ultracentrifugation, the possibility that vesicles in human seminal plasma may also have antifertility activity cannot be excluded. The active material is non-dialysable and heat labile. This eliminates the possibility that the antifertility effect is obtained by some trapped inorganic ions or other heat-stable compounds.

The human material was active at 150 μ g protein/ 10^5 mouse spermatozoa. This compares favourably with the results obtained by Pinsker & Williams (1968), who used 5 mg human pellet/ 10^5 rabbit spermatozoa, and by Dukelow & Chernoff (1969), who employed 1 mg human pellet/ 10^5 rabbit spermatozoa. A pellet obtained from bull seminal plasma was effective at 1 mg protein/ 10^5 rabbit spermatozoa (Dukelow *et al.*, 1966) and a pellet from rabbit seminal plasma

was active at 1 mg dry weight/ 10^5 rabbit spermatozoa (Weinman, 1966). The higher specific activity of our preparation is probably caused by the fact that previous investigators did not use two low-speed centrifugation steps before ultracentrifugation or wash their pellets.

The material acts by preventing the passage of the spermatozoa through the outer investments of the ovum but does not hinder the fusion of the spermatozoon with the vitelline membrane. The latter further emphasizes that the material has a specific effect on the spermatozoa and does not generally disturb its fertilizing capacity, e.g. by affecting sperm motility. At present, it is not known how the factor(s) act(s). Two mechanisms of action appear most plausible: (1) prevention of the acrosome reaction (the vesiculation of the plasma and outer acrosomal membranes after the spermatozoa have become capacitated and come into contact with the follicle cell layer of the ovum; without the acrosome reaction, fertilization will not occur); and/or (2) inhibition of one or more enzymes, such as the corona-penetrating enzyme but not acrosin, which are involved in the penetration of the spermatozoon through the ovum investments.

It is fairly well established that spermatozoa have to undergo the acrosome reaction before they can fuse with the vitelline membrane (Yanagamachi & Noda, 1970; Yanagamachi, 1972; Wolf *et al.*, 1976). Our preparation did not prevent the fusion of spermatozoa with zona-free ova, suggesting that the acrosome reaction was not prevented. In an attempt to show this directly, capacitated mouse spermatozoa were stained. Like the results by most other investigators, such attempts were unsuccessful. It is extremely difficult to observe the acrosome reaction in mouse spermatozoa (Stefanini, Oura & Zamboni, 1969). Other species, such as the guinea-pig and hamster which are more suitable for this purpose will be used in the future.

The overall fertility rate of the pellet-treated spermatozoa increased after washing the spermatozoa. However, this was almost entirely due to the increase in the number of penetrated ova rather than the number of cleaved ova. By contrast, after incubating the pellet-treated spermatozoa with capacitation medium (containing 20 mg/BSA ml), the percentage of the 2-cell embryos was similar in the test and control experiments. The antifertility factor(s) is therefore at the most partly removed by washing but is dissipated completely by incubating the spermatozoa in the capacitating medium. The greater number of penetrated ova after washing the pellet-treated spermatozoa is most probably due to the partial removal of the antifertility factor while the spermatozoa are with the ova in the culture medium. Mouse spermatozoa require only 1 h for capacitation compared to the rabbit for which 6–12 h are required. Although the culture medium possesses only 1 mg BSA/ml, Miyamota & Chang (1973) reported a 32.9% fertilization at this concentration, showing that capacitation and, presumably, removal of the antifertility factor, can occur. However, more time is required than in capacitating medium because the BSA of the latter is 20-fold higher. We have confirmed these results (unpublished). Thus when ova were observed after 24 h, washed pellet-treated spermatozoa had had the chance to penetrate the eggs but ovum cleavage had not occurred, although normal cleavage rates were obtained with capacitated, washed pellet-treated spermatozoa.

It is likely that the small amount of the pellet that was added to the culture medium when the spermatozoa were not washed decreased the ability of the culture medium to remove the antifertility factor(s) even more, so that the penetration of the spermatozoa into the oocytes was further delayed. Large differences were therefore seen in the overall fertility rate between the test and the control when unwashed spermatozoa were used. Because of the rapid and fairly easy capacitation of mouse spermatozoa, complete absence of fertilization was never observed in our system, in contrast to the results of others using the rabbit in-vivo capacitation test. In addition, a much smaller number of ova is evaluated at one time when the rabbit in-vivo test is performed. Some fertility was therefore seen even under saturating conditions (incubating the spermatozoa with pellet at a concentration of 3 mg protein/ml or more).

The molecular weights found for the material in the pellet by SDS electrophoresis are of interest. Although at 104 000 g only extremely high molecular weight compounds are

precipitated, the major bands possessed molecular weights ranging from 40 000 to 200 000. This indicates that the components of the pellet either consist of several subunits or of lower molecular weight compounds tightly adhered to higher molecular weight material which can be reduced to their individual forms by treatment with mercaptoethanol and SDS.

Before purifying, or studying the biochemical characteristics of the pellet, it is extremely important that it is washed several times to remove contaminating compounds of low molecular weight. This has not been reported by other investigators in any species, and may explain some of the controversial results obtained. Since the supernatant solution after ultracentrifugation, at least that of bull seminal plasma (Pinsker & Williams, 1967), possesses antifertility properties, and because a compound such as the acrosin inhibitor which has a molecular weight of about 5600 can become trapped in the pellet, it is essential that the pellet is washed before further studies are performed. For example, the acrosin-inhibiting activity of 150 µg unwashed pellet (the amount used to inhibit the fertility of 1×10^5 spermatozoa) is approximately equal to 30 µg human seminal plasma acrosin inhibitor (Zaneveld, Schumacher, Fritz, Fink & Jaumann, 1973), an amount that could be sufficient to induce antifertility activity (Yang, Zaneveld & Schumacher, 1976). The twice-washed pellet had no such acrosin inhibitory activity and possessed fewer bands after electrophoresis, indicating that most of the trapped, low molecular weight material was removed. It still possessed high antifertility activity and should thus form the starting material for further biochemical study.

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