IMMUNOLOGICAL AND BIOCHEMICAL STUDIES ON FRACTIONATED BULL SPERMATOZOA

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Summary. Washed, ejaculated bull spermatozoa were separated by sonication and sucrose density gradient centrifugation into three fractions, one containing a protein mixture (Fraction P), another only sperm tails (Fraction T), and the third only sperm heads (Fraction H). Detergent extracts containing mostly acrosomal and plasma membrane material were prepared from whole bull spermatozoa (Extract S) and the isolated sperm heads (Extract E). Extracts S and E and Fraction P contained acrosin (acrosomal proteinase) that appeared to be associated with an inhibitor in Extract S and Fraction P. Fraction P and Extract S, but not Extract E, possessed hyaluronidase. Extract P was absent if detergent-treated spermatozoa were sonicated and centrifuged using a sucrose density gradient.

Heteroantisera against Fractions P, T and H, and Extract S were prepared by immunizing rabbits. Immunodiffusion experiments revealed the formation of precipitin bands between bull seminal plasma and Fraction H or Extract S antisera but not with antisera of Fractions P and T. These bands did not occur after absorption of the antisera with bull seminal plasma. Cross-reactions occurred among all sperm preparations. The precipitating antibodies appeared to be species specific. The Extract S antisera caused the highest degree of sperm agglutination, followed in decreasing order by the antisera against Fractions H, T and P. Extract S antisera also produced the most rapid sperm immobilization. No difference among the immobilization rates of the other antisera was observed. Removal of the seminal plasma specific antibodies by absorption with seminal plasma resulted in a significant decrease in sperm agglutination but had no effect on sperm immobilization. Thus, sperm agglutinating antigens are present in both spermatozoa and seminal plasma, whereas the sperm immobilizing antigens are only associated with spermatozoa. Complement was shown to be a necessary part of the sperm immobilization process.

Extract S antisera inhibited the activity of testicular and acrosomal hyaluronidase. Immunoglobulins prepared from Extract S antisera by...
was somal, higher plasma, serum, prepared between were hyaluronic spermatozoa separated tails antigen^-globulin, bers Protzman, affecting by iso- seminal and Behrman, 1968; Edwards, 1969; Shulman, 1971; Metz, 1972. Spermatozoa, seminal plasma, and testis antigens are able to induce the formation of hetero-, iso- and autoantibodies that can affect the fertilizing capacity of both female and male animals. Most investigators used whole semen or testis extracts, however, immunizing females and males in heterologous species or in the same species with varying and partly contradictory results. As Katsh & Katsh (1961) stated: ‘Any concerted attempt to control fertility (as in population problems) by immune methods will be suspended until the antigenic materials can be identified’. Although many antigenic components are present in semen, those affecting fertility appear to be associated with spermatozoa only (Menge & Protzman, 1967) and are therefore of special interest. Only a few authors have attempted to characterize the sperm-specific antigens. Henle, Henle & Chambers (1938) showed that bull spermatozoa contain heat-labile and heat-stable antigens that were specific for either the sperm head or sperm tail, and one antigen that could only be obtained after rupture of spermatozoa. Pernot (1956) separated heads and tails of guinea-pig spermatozoa and found that sperm tails have at least five antigens in common with seminal plasma and one, a $\beta$-globulin, with sperm heads. Katsh & Katsh (1961) fractionated guinea-pig spermatozoa and obtained four antigenically separate fractions. One hydrolysed hyaluronic acid, another was associated with nucleic acids since its antigenicity was destroyed by treatment with RNase and DNase, the third was a protein with a very slow electrophoretic mobility and the fourth a polysaccharide. The latter may have been part of the acrosomal envelope since polysaccharides are elements of this structure (Hartree & Srivastava, 1965). The effects of antisera prepared against these four fractions on sperm agglutination and immobilization were not reported. Hathaway & Hartree (1963) obtained four precipitin lines between ram acrosomal extract, prepared by NaOH treatment, and its antiserum. These lines were continuous with precipitin bands formed with seminal plasma. The antiserum had a sperm agglutination titre that was significantly higher than that of control sera whereas the sperm immobilization titre was the same as that of control sera. Metz (1972), Metz, Seiguer & Castro (1972) and Zaneveld, Polakoski & Schumacher (1973) reported that antibodies prepared against whole semen or detergent (acrosomal) extracts inhibited sperm acrosomal hyaluronidase activity. This inhibition was species specific. Similarly it was shown by Zaneveld, Schumacher & Travis (1973) that detergent extracts 

INTRODUCTION

The immunological aspects of fertility and infertility have been studied extensively and excellent reviews are available (Mann, 1965; Katsh, 1967; Behrman, 1968; Edwards, 1969; Shulman, 1971; Metz, 1972). Spermatozoa, seminal plasma, and testis antigens are able to induce the formation of hetero-, iso- and autoantibodies that can affect the fertilizing capacity of both female and male animals. Most investigators used whole semen or testis extracts, however, immunizing females and males in heterologous species or in the same species with varying and partly contradictory results. As Katsh & Katsh (1961) stated: ‘Any concerted attempt to control fertility (as in population problems) by immune methods will be suspended until the antigenic materials can be identified’. Although many antigenic components are present in semen, those affecting fertility appear to be associated with spermatozoa only (Menge & Protzman, 1967) and are therefore of special interest. Only a few authors have attempted to characterize the sperm-specific antigens. Henle, Henle & Chambers (1938) showed that bull spermatozoa contain heat-labile and heat-stable antigens that were specific for either the sperm head or sperm tail, and one antigen that could only be obtained after rupture of spermatozoa. Pernot (1956) separated heads and tails of guinea-pig spermatozoa and found that sperm tails have at least five antigens in common with seminal plasma and one, a $\beta$-globulin, with sperm heads. Katsh & Katsh (1961) fractionated guinea-pig spermatozoa and obtained four antigenically separate fractions. One hydrolysed hyaluronic acid, another was associated with nucleic acids since its antigenicity was destroyed by treatment with RNase and DNase, the third was a protein with a very slow electrophoretic mobility and the fourth a polysaccharide. The latter may have been part of the acrosomal envelope since polysaccharides are elements of this structure (Hartree & Srivastava, 1965). The effects of antisera prepared against these four fractions on sperm agglutination and immobilization were not reported. Hathaway & Hartree (1963) obtained four precipitin lines between ram acrosomal extract, prepared by NaOH treatment, and its antiserum. These lines were continuous with precipitin bands formed with seminal plasma. The antiserum had a sperm agglutination titre that was significantly higher than that of control sera whereas the sperm immobilization titre was the same as that of control sera. Metz (1972), Metz, Seiguer & Castro (1972) and Zaneveld, Polakoski & Schumacher (1973) reported that antibodies prepared against whole semen or detergent (acrosomal) extracts inhibited sperm acrosomal hyaluronidase activity. This inhibition was species specific. Similarly it was shown by Zaneveld, Schumacher & Travis (1973) that detergent extracts

$$\text{(NH}_4\text{)}_2\text{SO}_4 \text{ precipitation inhibited the activity of acrosin but only if a high molecular weight substrate was used. Such immunoglobulins did not inhibit bovine pancreatic trypsin.}$$

The detergent extraction of spermatozoa seems to be the most suitable method to prepare surface and acrosomal antigens that induce sperm-agglutinating, sperm-immobilizing and enzyme-inhibiting antibodies after injection into animals of a heterologous species.
from human spermatozoa induced antibodies that completely inhibited human acrosin, the acrosomal proteinase that at neutral pH readily hydrolyses both benzoyl arginine ethyl ester (BAEE) and gelatin, and is inhibited by a variety of natural and synthetic proteinase inhibitors (Zaneveld, Polakoski & Williams, 1972; Zaneveld, Dragoje & Schumacher, 1972). In previous articles from this laboratory the terms acrosin and acrosomal proteinase have been used interchangeably. Justifiably, this enzyme has now officially been named acrosin by the International Commission on Enzyme Nomenclature (Enzyme No. 3.4.21.10) and we will adhere to this. A recent communication by Goldberg (1973) indicated that the injection of rabbits with purified sperm lactate dehydrogenase X resulted in a significant decrease in conception rate. Ejaculated spermatozoa also contain coating antigens (SCA) that originate from the seminal plasma (Weil, 1967). In man, one of these antigens appears to be identical to lactoferrin (Boettcher, 1969; Hekman & Rümke, 1969). The results of Ansbacher (1972) showed that a significant percentage of men produce sperm-agglutinating as well as -immobilizing antigens after vasectomy.

The purpose of the present study was to extend the knowledge on sperm-specific antigens. Those associated with the sperm envelope, including the acrosome, were of particular interest since it is reasonable to assume that such surface antigens are responsible for the induction of the immunological response. A preliminary report was presented earlier (Zaneveld & Schlumberger, 1972).

**MATERIALS AND METHODS**

**Fractionation of spermatozoa**

Fresh bull spermatozoa (5 x 10^9 to 1.0 x 10^10/ml) were washed three times by centrifugation at 900 g for 30 min with phosphate-buffered saline (PBS), pH 7.4, to remove the seminal plasma. The washed cells were resuspended in the original volume and the sperm heads and tails detached by sonication for 20 sec with an energy of 120 W at 10 kHz in a Branson Sonifier, model B12. Two ml of the sonicated spermatozoa were layered onto a discontinuous sucrose gradient consisting of 4 ml 30% (w/w) sucrose in PBS above 7 ml 60% (w/w) sucrose in PBS. Centrifugation was performed at 4°C in a Spinco rotor SW 40 for 45 min at 30,000 rev/min. Three bands were obtained, the upper consisting mostly of protein (Fraction P), the middle mostly of sperm tails although some contaminating heads were also present, and the bottom mostly of sperm heads with some sperm tails (Text-fig. 1). The bottom fraction was recovered by cutting the bottom of the tube whereas the other two fractions were removed from the top. Contaminating heads in the middle (sperm-tail) fraction were removed by re-centrifugation of this fraction at 40,000 rev/min for 30 min (4°C) in the discontinuous sucrose gradient described above. Contaminating tails were separated from the bottom (sperm-head) fraction by layering 1 ml of the fraction, diluted 1:1 in PBS, above 12 ml 60% (w/w) sucrose and centrifuging at 40,000 rev/min for 45 min. After centrifugation, the isolated sperm tails (Fraction T) and sperm heads (Fraction H) were recovered as described previously. Sucrose was removed by repeated washings in PBS at low centrifuge speeds and/or dialysis.
Detergent extracts were prepared by a modification (Polakoski, Zaneveld & Williams, 1971) of the detergent treatment of Hartree & Srivastava (1965). Frozen and thawed spermatozoa, or isolated sperm heads obtained after sucrose density gradient centrifugation, were washed in PBS and incubated with Hyamine 2389 (Rohm and Haas Co., Philadelphia) and Triton X-100 (Rohm and Haas Co., Philadelphia). The treated spermatozoa or sperm heads were removed by centrifugation, the soluble proteins precipitated with alcohol, dialysed against distilled water and freeze dried. Such treatment results in the dispersal of the acrosome from spermatozoa although the plasma membrane surrounding the mid-piece and tail is also frequently disrupted and removed (Churg, Zaneveld & Schumacher, 1974). Contamination from other parts of the spermatozoon is minimal, however. The detergent extracts of whole spermatozoa (Extract S) but not those from isolated sperm heads (Extract E) were used in the production of antisera. In one group of experiments, the detergent-treated spermatozoa were washed, sonicated and centrifuged using the sucrose density gradient technique described above.

**Preparation of antisera and immunoglobulins**
Incomplete Freund’s adjuvant (0-5 ml) was mixed with 0-5 ml of either Fraction H (containing $10^8$ sperm heads), Fraction T (containing $10^8$ sperm tails), or Fraction P (containing 0-5 mg protein) and injected intramuscularly.
Sperm fraction antibodies

into rabbits together with 50,000 units penicillin and 50 mg streptomycin. Ten weeks after the first injection, booster injections with each particular antigen preparation were administered subcutaneously as follows: Day 1 (0-25 ml each), 5 x 10^7 heads (Fraction H), 5 x 10^7 tails (Fraction T) and 0-25 mg Fraction P; Day 2 (0-5 ml each), 10^8 heads, 10^8 tails and 0-5 mg Fraction P and Day 3 (1 ml each), 2 x 10^8 heads, 2 x 10^8 tails and 1 mg Fraction P. The animals were bled 9 days after the last injection.

Antisera against the detergent extracts of whole spermatozoa (Extract S) were prepared by mixing 10 mg of the freeze-dried extracts with 2-0 ml complete Freund’s adjuvant (Hyland Labs.) using a tissue microhomogenizer and injecting the mixture subcutaneously into the inguinal and axillary region of rabbits. Three weeks later, 15 mg freeze-dried detergent extract was administered in a series of six booster injections beginning with small amounts every 2 days for 12 days. The material was dissolved in saline, centrifuged and the supernatant solution injected intravenously during the first five injections in increasing portions. The combined sediments were administered intraperitoneally at the same time as the final intravenous injection. The animals were bled 9 days later.

After clotting, the sera were centrifuged at 900 g for 20 min (‘untreated’ antisera). Seminal plasma (0-1 ml) was added to 1 ml of the antisera and incubated at 5°C for 2 days to remove seminal plasma-specific antibodies. The mixture was centrifuged at 900 g for 30 min and the supernatant solution collected (‘absorbed’ antisera).

Immunoglobulins were precipitated from ‘untreated’ antisera by addition of one part saturated (NH₄)₂SO₄ to two parts antiserum (Campbell, Garvey, Cremer & Sussdorf, 1970). After 2 to 3 hr at room temperature, the mixture was centrifuged at 1400 g for 30 min and the precipitated material resuspended to its original volume with 0-15 M-NaCl (saline). This procedure was repeated twice. The final precipitate was resuspended in a small volume of 0-15 M-saline and dialysed against three changes of saline. The resultant antibody solution was approximately five times more concentrated than the original antiserum. All further dilutions were made with saline. This preparation was used throughout the proteinase inhibition tests since whole sera contain proteinase inhibitors (Heimburger, Haupt & Schwick, 1971) that would interfere with the results (Schumacher, 1971; Schumacher & Zaneveld, 1972).

Sperm agglutination, sperm immobilization and immunodiffusion tests

An equal volume of ‘untreated’ or ‘absorbed’ antiserum was mixed with 25 μl of a sperm solution containing 10⁶ spermatozoa. The spermatozoa were either in the presence of seminal plasma (‘unwashed’ spermatozoa) or had been washed three times with PBS (‘washed’ spermatozoa). After incubation at room temperature for 45 min, the spermatozoa were observed for agglutination. The extent of agglutination was recorded on a decreasing scale from 5+ (all the spermatozoa agglutinated) to 0 (no agglutination).

To determine the effect of antiserum on sperm motility, ‘washed’ and ‘unwashed’ spermatozoa (4 x 10⁷/ml) were mixed with equal volumes of undiluted ‘untreated’ or ‘absorbed’ antisera and were examined under the microscope.
after various periods of time at room temperature. Motility was evaluated by randomly counting motile and immotile spermatozoa in ten different high-power fields and determining the percentage of motile spermatozoa. No differentiation was made between vigorously and sluggishly moving spermatozoa, although they were usually either very motile or dead. Three different rabbit sera obtained from healthy, untreated rabbits were used as controls in the agglutination and immobilization experiments. In certain experiments, normal guinea-pig serum was used as source for complement.

Immunodiffusion tests were made using 0.5% agar gel with PBS according to the method of Ouchterlony (1962). The slides were observed after 48 hr at 5°C, washed in PBS and subsequently in water, dried and stained with amido black (Crowle, 1961).

**Enzyme tests**

Hyaluronidase assays were performed as described by Zaneveld, Polakoski & Schumacher (1973). The esterolytic activity of acrosin was evaluated by addition to benzoylarginine ethyl ester (BAEE, 0.2 mg/ml) in 0.05 M-tris-HCl, pH 8.0, containing 0.05 M-CaCl₂ (Schwert & Takenake, 1955). To 3.0 ml of BAEE, 0.1 ml of the test solution was added. One milliunit (mU) was arbitrarily defined as that amount of enzyme causing a rate of change in optical density of 0.001/min at a wavelength of 253 nm. The proteolytic activity of the enzyme was tested by the gelatin-plate technique using exposed and developed Kodak projector slides (Anderer & Hoernle, 1968; Schumacher, 1971; Zaneveld, Schumacher & Travis, 1973). For this purpose, 30 μl of the solution to be assayed was placed as a drop on the surface of the gelatin layer and incubated for 3 hr at 37°C in a humid chamber.

The presence of a proteinase–proteinase inhibitor complex was evaluated by decreasing or increasing the pH of the solutions with 0.1 M-HCl or 0.1 M-NaOH before addition to BAEE at pH 8.0 (Polakoski et al., 1971; Zaneveld, Polakoski & Williams, 1973). Since proteinase–proteinase inhibitor complexes dissociate at acidic pH and associate at basic pH (Laskowski, 1970), an increase and decrease, respectively, in proteinase activity will occur if such a complex is indeed present.

The inhibitory activity of the immunoglobulins concentrated from Extract S antisera was evaluated at neutral pH by incubating 0.1 ml of the immunoglobulin solution, either undiluted or diluted in physiological saline, with 0.1 ml of the following proteinase solutions that each possessed almost identical activities (approximately 300 mU/ml) at pH 8.0:

1. lyophilized detergent extract of unsonicated bull spermatozoa (Extract S), dialysed extensively against 0.001 M-HCl to remove as much inhibitor as possible (25 mg/ml in borate buffer, pH 8.0);
2. Fraction P (2 mg/ml in PBS, pH 7.4);

After 15 min at room temperature, 0.1 ml of the mixture was added to 3.0 ml of the BAEE–borate buffer solution, and 30 μl to a photographic plate. As control, enzyme was incubated with 0.15 M-NaCl. Protein was estimated by
absorption readings at 280 nm, assuming that 1 mg of protein/ml has an optical density of 1.0 using a 1-cm light path.

RESULTS

**Preparation and partial characterization of sperm fractions**

Three fractions were obtained after the first sucrose density gradient centrifugation of sonicated bull spermatozoa (Text-fig. 1). Fraction P was the least dense and consisted mostly of protein based on a spectrophotometric evaluation at wavelengths of 260 and 280 nm (Dawson, Elliott, Elliott & Jones, 1969). The middle fraction contained mostly sperm tails and the bottom fraction mostly sperm heads. At this point, both fractions were still slightly contaminated with each other; 10% of the sperm-head fraction being made up of sperm tails and 10% of the tail fraction being heads. A pure preparation of sperm heads (Fraction H) and tails (Fraction T) was obtained after the second sucrose density gradient centrifugation. Light microscope studies showed that the heads and tails had separated at the junction of the midpiece and the head.

<table>
<thead>
<tr>
<th>Table 1. Hyaluronidase and acrosin activity of various sperm fractions</th>
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<tr>
<td><strong>Sperm fraction</strong></td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Fraction P</td>
</tr>
<tr>
<td>Detergent extract of:</td>
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<tr>
<td>Whole spermatozoa (Extract S)</td>
</tr>
<tr>
<td>Sperm heads (Extract E)</td>
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</tbody>
</table>

See 'Materials and Methods' for the fractionation procedures, assay techniques and measurements.

* Tested after adjusting the pH of the solutions to 3.0.

Many of the purified sperm heads possessed a small hole in the anterior, abaxial portion of the acrosome. Fraction P was absent after sonication and sucrose density gradient centrifugation of spermatozoa from which the acrosome had been removed by detergent treatment.

Fraction P contained the highest amount of hyaluronidase and acrosin activity (BAEE as substrate) relative to the protein concentration (Table 1). This fraction, as well as the detergent extract preparation obtained from whole spermatozoa (Extract S) also possessed gelatin-digesting activity. The BAEE hydrolysing activity of Fraction P increased two- to four-fold if the pH of the solutions was lowered to 3.0 and decreased if the pH was raised to 8.0, indicating the presence of an acrosin–proteinase inhibitor complex similar to the one present in detergent extracts (Polakoski *et al.*, 1971; Zaneveld, Dragoje & Schumacher, 1972; Zaneveld, Polakoski & Williams, 1973). Apparently, at least half of the acrosin present in Fraction P is in complex with inhibitor. The acrosin activity of the fractions was therefore recorded at pH 3.0 (Table 1) since this reflected the true activity without interference from the proteinase.
inhibitor. Hyaluronidase activity was absent from detergent extracts of isolated sperm heads (Extract E) (Table 1) which also possessed only small amounts of proteinase activity. Some of the Extract E acrosin was bound to a proteinase inhibitor like that in Fraction P and Extract S, although to a much smaller extent since only a 1.3 times increase in activity was noted on lowering the pH to 3.0 or less.

Immunodiffusion experiments
With double immunodiffusion techniques, Extract S antisera formed four precipitin bands with bull seminal plasma and Fraction H antisera two to three bands, whereas antisera of Fractions T and P did not cross-react with seminal plasma. No precipitin bands were observed after 'absorption' of the antisera with bull seminal plasma. None of the antisera cross-reacted with detergent extracts from human or rabbit spermatozoa or with human or rabbit seminal plasma. Similarly, rabbit and human detergent extract antisera did not cross-react with bull seminal plasma or bull sperm detergent extracts, showing the species specificity of the sperm antigens obtained by detergent treatment. At least four bands were formed between Extract S antisera and Extract S itself. One of these bands was identified as hyaluronidase and is identical to bull testicular hyaluronidase (Zaneveld, Polakoski & Schumacher, 1973). Fractions H, T and P antisera formed respectively three, two and two precipitin bands with Extract S.

Sperm agglutination tests
The antisera against Extract S consistently gave rise to the highest degree of agglutination, followed in decreasing order by the antisera against Fractions H, T and P (Table 2). Agglutination was frequently more pronounced with 'washed' than with 'unwashed' spermatozoa. Antisera that had been 'absorbed' with seminal plasma still caused the agglutination of the spermatozoa, although to a much smaller extent. The control sera did not agglutinate spermatozoa under the conditions of the assay.

<table>
<thead>
<tr>
<th>Sperm fraction</th>
<th>Antisera 'absorbed' (+) or 'untreated' (−) with seminal plasma</th>
<th>Agglutination</th>
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<tbody>
<tr>
<td>Detergent extracts (Extract S)</td>
<td>−</td>
<td>5+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5+</td>
</tr>
<tr>
<td>Sperm heads (Fraction H)</td>
<td>−</td>
<td>5+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5+</td>
</tr>
<tr>
<td>Sperm tails (Fraction T)</td>
<td>−</td>
<td>4+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3+</td>
</tr>
<tr>
<td>Fraction P</td>
<td>−</td>
<td>3+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2+</td>
</tr>
<tr>
<td>Control serum 1</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>Control serum 2</td>
<td>−</td>
<td>0</td>
</tr>
</tbody>
</table>

See ‘Materials and Methods’ for details concerning the preparation and test procedure.
The antisera caused various agglutination patterns, although head to tail agglutinations and agglutination of those parts of the spermatozoon against which the antisera had been prepared, were most common (Pl. 1, Fig. 1). The Extract S antisera produced almost exclusively head-to-head agglutinations after being stored for 6 months at $-20^\circ$C and repeatedly being thawed and frozen.

**Text-Fig. 2.** Effect of normal sera and antisera against bull sperm fractions on the motility of bull spermatozoa (see text for experimental details). (a) Normal sera obtained from three different animals; (b) 'untreated' antisera; (c) antisera absorbed with seminal plasma. ●, Detergent extracts (Extract S); □, Fraction P; ■, sperm heads (Fraction H); ○, sperm tails (Fraction T).

After mixing 'unwashed' spermatozoa with Extract S or Fraction H antisera, precipitating particles similar to those found by Metz (1972) appeared in the suspending medium and were frequently attached to the spermatozoa (Pl. 1, Fig. 2). These particles did not appear if 'washed' spermatozoa were mixed
with these antisera, or if 'unwashed' spermatozoa were treated with either 'absorbed' antisera or Fraction T and P antisera. The particles apparently represent precipitates of seminal plasma antigen–antibody complexes.

**Sperm immobilization tests**

Approximately 60% of the 'unwashed' spermatozoa were motile at the time the tests were performed, whereas only 20 to 30% of the 'washed' spermatozoa were motile. The antisera against Fractions H, T and P completely immobilized 'unwashed' spermatozoa within 30 to 40 min, whereas the Extract S antiserum immobilized the spermatozoa within 10 min (Text-fig. 2). The control experiments with normal rabbit sera showed only 30 to 50% immobilization after 1 hr. Incubation of spermatozoa with antisera 'absorbed' with seminal plasma resulted in complete immobilization within 10 to 50 min (Text-fig. 2), times that were not significantly different from those obtained with 'untreated' antisera. Essentially identical results were obtained with 'washed' spermatozoa, except that the immobilization times were somewhat less, probably because of the lower percentage of motile spermatozoa in the starting solution.

After 6 months storage at -20°C and repeated thawing and freezing, the Extract S antiserum had lost most of their sperm-immobilizing activity, although the sperm-agglutinating activity remained the same. Addition of fresh guinea-pig serum to the antiserum resulted in an immediate restoration of the immobilizing activity. Guinea-pig serum by itself affected the motility of the bull spermatozoa only insignificantly.

**Enzyme inhibition tests**

Immunoglobulins prepared from Extract S antisera inhibited the acrosin of Extract S and Fraction P using gelatin as substrate (Text-fig. 3). Even 1:8 dilutions effectively inhibited enzyme activity. Bovine pancreatic trypsin was
Fig. 1. 'Washed' bull spermatozoa treated with detergent extract 'Extract S' antisera. See text for further details.

Fig. 2. 'Unwashed' bull spermatozoa treated with detergent extract 'Extract S' antisera. A much higher degree of agglutination was obtained throughout the sample than shown in the picture. This view was selected, however, because it clearly illustrates the particulate matter that adheres to the spermatozoa. See text for further details.
not inhibited by these immunglobulin preparations except at high antibody concentrations, probably because of residual serum proteinase inhibitors. Using BAEE as substrate, however, neither bovine pancreatic trypsin nor bull acrosin was inhibited by their homologous antibodies.

DISCUSSION

A very short exposure of washed, ejaculated bull spermatozoa to ultrasound results in the separation of heads and tails, and in the partial disruption of the plasma membranes and acrosomes. The latter is followed by the release of considerable amounts of acrosomal enzymes, and also of some sperm surface constituents that were either part of the plasma membrane or were constituents of seminal plasma absorbed by the sperm surface, or both. The various sperm components can be effectively separated by sucrose density gradient centrifugation into three fractions containing heads, tails and a soluble protein material. Recent ultrastructural investigations on rabbit and human spermatozoa (Churg et al., 1974) indicate that most of the plasma membrane and most of the acrosomal materials are removed by detergent treatment. The soluble protein fraction (Fraction P) was missing when detergent-treated bull spermatozoa were sonicated and subjected to sucrose density gradient centrifugation. This observation, together with the fact that Fraction P was high in acrosomal enzyme activity, strongly indicates that this fraction mostly consists of acrosomal material. The acrosomal membranes themselves apparently remain attached to the head during the sonication process since these membranes were mostly intact on isolated sperm heads with the exception of a small hole at the anterior region. It is possible that the acrosomal material escaped through this hole.

The acrosin present in Fraction P was mostly in complex with an inhibitor. Fraction P was obtained in the absence of detergents, demonstrating that the previously reported acrosin–inhibitor complex (Polakoski et al., 1971; Zaneveld, Polakoski & Williams, 1973) was not the result of non-specific detergent–acrosin binding. Only small amounts of inhibitor appear to be present in detergent extracts of isolated sperm heads (Extract E). This may well explain why Stambaugh & Buckley (1970) have not been able to locate an inhibitor in their detergent extracts, since these authors obtain their material from sonicated rabbit sperm heads purified through a sucrose density gradient and not from whole spermatozoa.

The rapid release of hyaluronidase from spermatozoa probably accounts for the absence of hyaluronidase in the detergent extracts obtained from isolated sperm heads (Extract E) and for the high activity of this enzyme in Fraction P. The specific activity of hyaluronidase in Fraction P is higher than that in Extract S since the latter also possesses the acrosomal and plasma membranes that are released during detergent treatment. Acrosin concentration is similarly highest in Fraction P although this enzyme is apparently more tenaciously bound to the sperm head than is hyaluronidase.

Immunization of rabbits with detergent extracts obtained from washed bull spermatozoa (Extract S) results in the production of antibodies that pre-
represents pellucida to weight the guinea-pig ment mobilizing glutinating ing resulted are the gradient tails.

occasionally caused sperm serum subsequent observation. (Zaneveld, & Protzman, 1967), Antibodies similar results of our observations indicate that at least some of the sperm antigens in detergent extracts (Extract S) either originate from, or are shared with, seminal plasma constituents. This is not surprising since various seminal plasma components adhere to the spermatozoon during ejaculation. Removal of the seminal plasma-specific antibodies by absorption resulted in significant decreases of the agglutinating properties of all antisera although complete absence of agglutination was never obtained. Such absorption did not affect the immobilizing activity of the antisera. The immobilizing antibodies appear to be sperm specific, therefore, in contrast to the agglutinating antibodies that are probably induced by a mixture of sperm and seminal plasma antigens. This is especially interesting because sperm immobilizing iso-antibodies seem to be the ones associated with infertility (Menge & Protzman, 1967; Isojima, 1969). Another observation was that the addition of guinea-pig serum to frozen–thawed antisera restored their immobilizing ability. A similar effect is obtained if the antisera are first heated. Apparently, complement is essential for antisera-induced sperm immobilization.

Antibodies against detergent extracts not only cause sperm agglutination and immobilization, but they also effectively inhibit the acrosomal hyaluronidase (Zaneveld, Polakoski & Schumacher, 1973) and acrosin, enzymes essential for the fertilization process. The inhibition of acrosin by immunoglobulins using a high molecular weight substrate and the lack of such using a low molecular weight substrate indicates that the antibody-binding sites are not identical with the active site of the enzyme since the active site is apparently accessible to the low molecular weight substrate even in the presence of antibody. The antibody does prevent the enzyme from digesting a gelatin membrane which represents an insoluble, high molecular weight substrate similar to the zona pellucida of the ovum. The specificity of the reaction was shown by the absence
of inhibition of pancreatic trypsin, an enzyme very similar to acrosin. Similar results were obtained with human acrosin and its antibody (Zaneveld, Schumacher & Travis, 1973). The antigens obtained from spermatozoa by detergent treatment are, therefore, of particular interest for further developments in the immunological approach to fertility control.

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