OBSERVATIONS ON THE MAMMALIAN ACROSOME: EXPERIMENTAL REMOVAL OF ACROSOMES FROM RAM AND BULL SPERMATOZOA

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Summary. Various procedures have been used to extract or detach the acrosomes from ram and bull spermatozoa. Spermatozoa from pooled ram ejaculates, or individual bull ejaculates, were first washed free of the seminal plasma, suspended in Ringer's solution, and then incubated at 37° C with solutions of sodium hydroxide, sodium carbonate, or cetyltrimethylammonium bromide (ctab). An alternate procedure consisted of shaking the Ringer's suspension of spermatozoa with small glass beads. Staining of spermatozoa by the Giemsa or Pas methods showed that the acrosomes were apparently dissolved by 0-0125 N-NaOH, and that they were detached as such by 1-5 mM ctab or by shaking with glass beads. Nitrogen and sugar determinations on extracts from spermatozoa confirmed the removal of materials from the cells. Because most of the extractable sugar was released in 0-0125 N-NaOH, it is suggested that a structural element, such as the acrosome, was being detached. Release of hyaluronidase from the treated spermatozoa was also observed. Ram sperm extracts contained four antigens, all of which were similar to antigens in ram seminal plasma, as revealed in double-diffusion agar plates. Antisera also had sperm agglutination titres up to 81.

INTRODUCTION

The acrosome, a membranous cap-like structure covering the anterior part of the sperm head, contains material which gives a positive reaction with the periodic acid–Schiff (Pas) reagent (Wislocki, 1949; Schrader & Leuchtenberger, 1951; Clermont & Leblond, 1955; Hancock, 1957). This material was extracted by Clermont, Glegg & Leblond (1955) from guinea-pig spermatozoa by means of 0-1 N-NaOH and, after hydrolysis, was found to contain galactose, mannose, fucose and hexosamine. Madera-Orsini (1956) and Miller & Mayer (1960) extracted washed bull spermatozoa with 0-01 N-NaOH and reported that the product contained protein, phospholipid, cholesterol, glucosamine and glucuronic acid. As an introduction to a study of the chemical composition

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of acrosomes, we describe, in the present paper, various procedures designed to extract quantitatively, or to detach, the acrosome from mammalian spermatozoa. The endeavour has been to accomplish this end under the mildest possible conditions in order to minimize denaturation of the extracted substances. This paper also includes the results of some experiments aimed at detecting spermatozoal antigens in the extracts.

MATERIALS AND METHODS

Semen was collected from sixteen rams by means of an artificial vagina. Thirty to 40 ml of pooled semen were allowed to cool slowly to 20° C, and small samples were removed for cytological and chemical analysis. In order to separate the spermatozoa from the seminal plasma, one volume of semen was mixed with three volumes of sperm–Ringer solution (Mann, 1946), and centrifuged for 15 min at 900 g in an angle-head centrifuge. The supernatant was discarded, and was replaced by an equal volume of Ringer’s solution in which the spermatozoa were resuspended and recentrifuged. The second supernatant was discarded, and the cells were resuspended in Ringer’s solution to twice the volume of the original sample of semen. To effect dissolution or detachment of acrosomes this suspension of spermatozoa was mixed with an equal volume of sodium hydroxide, sodium carbonate or cetyltrimethylammonium bromide (CTAB), made up to different concentrations in 0.9% saline solution, and incubated at 37° C for 45 min. In some experiments mechanical agitation was substituted for chemical extraction. The suspension of spermatozoa was mixed with one volume each of Ringer’s solution and glass beads (Ballotini Glass Beads for Mickle Disintegrator, Grade 12, 150 µ diameter). The mixture was placed in an Erlenmeyer flask and shaken in a water bath at 37° C for 45 min (120 strokes per min; amplitude 3 cm). The size of the flask was selected so that the contents covered the bottom to a depth of about 1 cm. The Mickle disintegrator was used on a few occasions, but since only mild agitation was required, the first method was preferred.

Bull semen was collected and treated in the same way as ram semen except that individual, rather than pooled, samples were processed. For removal of the seminal plasma, the semen was diluted with one volume of Ringer’s solution before centrifuging.

After incubation or agitation, the suspensions were centrifuged for 10 min at 8000 g, and the supernatants were removed for analysis. Total nitrogen in supernatants was determined by Nesslerization (King & Wootton, 1956) after digestion in 72% perchloric acid (Long & Staples, 1961). Sugars were estimated as glucose equivalents with an orcinol reagent (Vasseur, 1948); citric acid was determined by the method of Speck, Moulder & Evans (1946), as adapted for semen by Mann (1951). Hyaluronidase activity was measured turbidimetrically by a modification of the mucin-clot prevention method, in which results are expressed in ‘turbidity reducing units’ (Tolksdorf, 1954).

In preparation for staining, samples taken from the sperm suspensions were diluted 200 times either in 0.9% saline solution containing 0.2% formaldehyde or in Ringer’s solution without a fixative. Five drops of the sus-
removal of ram and bull acrosomes

pensions were placed on a microscope slide, and the excess fluid was removed. When the slides were dry, they were washed and placed in the staining solution. Giemsa stain was freshly mixed according to the directions given by Hancock (1952). The PAS technique (H Hancock, 1957; Pearse, 1961) was used as an alternative method of staining. Dimensions of stained sperm heads were determined with an ocular micrometer.

Antisera to extracts of ram spermatozoa were produced in two female rabbits of mixed breed, by means of the series of injections given in Table 1. The rabbits were bled regularly after 8 weeks, and the blood serum was dialysed against 0-9% saline solution. The sera were tested for sperm agglutination, and for precipitating antibodies in double-diffusion agar plates prepared on microscope slides (Crowle, 1961). The titre is expressed as the reciprocal of the highest dilution of antiserum that agglutinates or immobilizes spermatozoa.

**Table 1**

INJECTIONS OF EXTRACTS OF RAM SPERMATOZOA INTO RABBITS FOR THE PRODUCTION OF ANTISERUM

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Route</th>
<th>Volume (ml)</th>
<th>Injection mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Subcutaneous</td>
<td>2</td>
<td>Two parts adjuvant* to one part sperm extract</td>
</tr>
<tr>
<td>10</td>
<td>Subcutaneous</td>
<td>3</td>
<td>Two parts adjuvant to one part sperm extract</td>
</tr>
<tr>
<td>16</td>
<td>Subcutaneous</td>
<td>1</td>
<td>Two parts adjuvant to one part sperm extract</td>
</tr>
<tr>
<td>20</td>
<td>Intravenous</td>
<td>0.5</td>
<td>Sperm extract</td>
</tr>
<tr>
<td>22</td>
<td>Intravenous</td>
<td>0.5</td>
<td>Sperm extract</td>
</tr>
<tr>
<td>23</td>
<td>Subcutaneous</td>
<td>3</td>
<td>Two parts adjuvant to one part sperm extract</td>
</tr>
</tbody>
</table>

*Freund's complete adjuvant (Difco).

Spermatozoa were extracted with a final concentration of 0.0125 N-NaOH. Solutions were adjusted to pH 7.4 before use.

RESULTS

SEPARATION OF SPERMATOZOA FROM SEMINAL PLASMA

In order to assess the extent to which seminal plasma was still present in suspensions of washed spermatozoa, we determined the concentrations of citric acid, since this substance is known to be derived exclusively from the seminal plasma (Mann, 1954). In a typical experiment, the citric acid concentration was 0.47 m-moles/100 ml of ejaculated semen, as compared to 0.03 m-moles/100 ml in the suspension of spermatozoa prepared for extraction. Thus about 94% of the seminal plasma was removed by the washing procedure. It was possible to remove all of the citric acid by centrifuging at higher velocities, but this caused a decline in sperm motility and a release of nitrogen-containing materials from the cells. High speed centrifugation was therefore avoided.

COMPOSITION AND PROPERTIES OF MATERIAL EXTRACTED FROM SPERMATOZOA

Samples of pooled whole ram semen contained an average of 110 m-equiv. of nitrogen per 100 ml, with a range of 92 to 120, and an average of 4.6 m-moles
of orcinol-reactive sugar per 100 ml, with a range of 3·2 to 5·7. The latter includes bound carbohydrate as well as any free fructose originating in the seminal plasma. Suspensions of washed spermatozoa contained an average of 52 m-equiv. nitrogen (range 48 to 58) and 1·4 m-moles of orcinol-reactive sugar (range 1·1 to 1·8). In each case the range is based upon at least six determinations. Values for washed spermatozoa are expressed as the amounts present in spermatozoa derived from 100 ml semen.

We found that about 31 % of the sugar in ram spermatozoa can be extracted by sodium hydroxide in concentrations varying from 0·0125 to 0·25 N (Text-fig. 1). Much less sugar is extracted at lower concentrations but the curve rises progressively at concentrations above 0·25 N-NaOH. The curve for nitrogen content of extracts is similar to that for sugar except that relatively more nitrogen-containing material is released at the higher concentrations of sodium hydroxide. When sodium carbonate was used in place of sodium hydroxide the extracts obtained at concentrations of 0·05 M and 0·5 M contained, respectively, 11·9 % and 21·4 % of the nitrogen and 28·2 % and 33·0 % of the sugar in spermatozoa. Similarly, 0·75 mM, 1·5 mM and 3·0 mM CTAB extracted, respectively, 9·2, 13·3 and 14·4 % of the nitrogen and 19·0, 19·3 and 19·0 % of the sugar in ram spermatozoa.

An extract of ram spermatozoa prepared with 1·5 mM CTAB contained 5250 turbidity reducing units of hyaluronidase per ml while a control extract prepared with saline solution contained 767 units per ml.

**Text-fig. 1.** Percentage of nitrogen (open circles) and sugar (solid circles) extracted from washed ram spermatozoa by various concentrations of sodium hydroxide. On the percentage scale 100 would represent the material in unextracted spermatozoa.

In washed intact ram spermatozoa stained by the Giemsa method the acrosome was a deep red, the equatorial segment was pale red, the post-nuclear region was pale blue and the midpiece and tail were deep red. A microphotograph of normal ram spermatozoa stained by the Giemsa method is reproduced in
Magnification of spermatozoa in all figures: ×2400. All spermatozoa were stained by the Giemsa method.

Fig. 1. Control ram spermatozoa.

Figs. 2 and 3. Ram spermatozoa after treatment with 0.0125 N-NaOH.

Fig. 4. Ram spermatozoa after treatment with 0.1 N-NaOH.

Fig. 5. Ram spermatozoa after treatment with 0.25 N-NaOH.

Fig. 6. Double-diffusion agar plate. Centre well contained antiserum to extracts of ram spermatozoa. Outer wells, beginning with the left-hand well as number 1 and progressing in a clockwise direction, contained ram seminal plasma in Wells 1, 3 and 5, and 0.0125 N-NaOH extract of ram spermatozoa in Wells 2, 4 and 6.

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Figs. 7 and 8. Ram spermatozoa after treatment with 1.5 mM CTAB.
Fig. 9. Ram spermatozoa after shaking with glass beads.
Fig. 10. Control bull spermatozoa.
Fig. 11. Bull spermatozoa with loosened acrosome, and a large mass of material removed from the acrosomal region of bull spermatozoa by shaking with glass beads.
Fig. 12. Masses of membranous material detached from bull spermatozoa, and heads of bull spermatozoa without acrosomes, resulting from shaking with glass beads.
Pl. 1, Fig. 1. After extraction with 0.0125 n-NaOH, the acrosome region was stained unevenly and appeared either rough or torn (Pl. 1, Fig. 2); or else it stained the same blue colour as the post-nuclear region, although the equatorial segment was still visible (Pl. 1, Fig. 3). Ram spermatozoa treated with more concentrated NaOH stained a uniform deep red, and it was no longer possible to distinguish the acrosome region except in outline (Pl. 1, Figs. 4 and 5). Tails were missing below the midpieces in cells extracted with 0.1 n-NaOH, and midpieces were separated from the heads by 0.25 n-NaOH. The dimensions of ram sperm heads before and after extraction with NaOH are given in Table 2. Cells extracted with 0.0125 n-NaOH are smaller than unextracted cells by an amount that probably represents the loss of materials from the acrosome region. Higher concentrations of sodium hydroxide caused a further decrease in the size of heads and also a slight change in shape inasmuch as the equatorial segment was now distinctly wider than other parts of the head. Since ram spermatozoa treated with 0.5 mM-Na₂CO₃ stained uniformly red by the Giemsa method it was impossible to detect changes in the acrosome region. Lower concentrations of sodium carbonate had no apparent effect.

A membranous structure was partly or completely dislodged from the acrosome region of ram spermatozoa treated with 1.5 mM CTAB. This structure, which was stained red by the Giemsa technique, appeared to peel off the head from the equatorial segment toward the anterior; it was often seen lying free of the cells (Pl. 2, Figs. 7 and 8). The material in the acrosome region which stains red by the Giemsa method was removed from ram spermatozoa by agitation with glass beads. The detached material had a tendency to clump and it was readily seen after staining (Pl. 2, Fig. 9). The details of the acrosome region were less obvious in spermatozoa stained by the PAS technique; however, the material that was PAS-positive appeared to be the same as that coloured red after Giemsa staining. Spermatozoa which appeared by the Giemsa method

**Table 2**

<table>
<thead>
<tr>
<th>Spermatozoa</th>
<th>Length (μ)</th>
<th>Width at the widest place (μ)</th>
</tr>
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<tbody>
<tr>
<td>Unextracted</td>
<td>8.47 ±0.27*</td>
<td>4.99 ±0.21*</td>
</tr>
<tr>
<td>Extracted with 0.0125 n-NaOH (no red stain remaining in acrosome region)</td>
<td>8.04 ±0.20*</td>
<td>4.67 ±0.20*</td>
</tr>
<tr>
<td>Extracted with 0.1 n-NaOH</td>
<td>6.4</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>(6.3 to 6.5)†</td>
<td>(3.5 to 3.9)†</td>
</tr>
<tr>
<td>Extracted with 0.25 n-NaOH</td>
<td>5.8</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>(5.6 to 6.1)†</td>
<td>(3.1 to 3.4)†</td>
</tr>
</tbody>
</table>

*Standard deviation; N = 60, Student’s t = 10 for differences in both length and width. Differences of the means are significant beyond the 1% level.
†Range of measurements on ten spermatozoa.
Measurements were made on preparations stained by the Giemsa method.
to have lost the membranous material from the acrosome region were not stained by the PAS reagent.

Bull spermatozoa were affected by mechanical agitation and by 1.5 mM CTAB in much the same way as ram spermatozoa, although the cells had a tendency to agglutinate in the CTAB. Periodic acid–Schiff and Giemsa stains were used to observe that material from the acrosome region had been removed by these treatments. The material removed by the glass-bead method was seen as agglutinated masses of red-staining material (Pl. 2, Figs. 10, 11 and 12).

**ANTIGENS IN SPERM EXTRACTS**

Antisera to extracts of washed ram spermatozoa usually had an agglutination titre of 81, and an immobilization titre of 3, toward ram spermatozoa. Control sera, as would be expected (see Chang, 1947), had agglutination and immobilization titres of 3.

In the double-diffusion agar plates, up to four lines of precipitation formed between ram sperm extracts and the antisera. These four lines appeared to be continuous with some of the six lines that formed between ram seminal plasma and the antiserum. Some, but not all, of these relationships can be seen in Pl. 1, Fig. 6. If the antiserum was preabsorbed with ram seminal plasma, precipitation lines failed to appear between sperm extracts and antiserum. These results indicate that all of the precipitating antigens in the sperm extract were also present in the seminal plasma. A maximum of two lines formed between antisera and the suspending medium of washed, but unextracted, spermatozoa. These lines probably represent the small amount of seminal plasma remaining with the washed spermatozoa.

**DISCUSSION**

Spontaneously detached acrosomes are frequently observed in a small proportion of ejaculated spermatozoa in several species. Blom (1945) photographed bull and stallion spermatozoa with detached galeae (acrosomes), and was able to relate the proportion of such cells in bull semen to the fertility and reproductive behaviour of the animals. In the present experiments the removal of acrosomes was effected both by chemical methods and by agitation. Evidence for this is based upon identification of the acrosome by Giemsa staining and by the PAS method. Because of its apparent thickness (Table 2) and its carbohydrate content the red-staining material lost from the anterior end of the head would appear to be the acrosome itself rather than the plasma membrane.

The 0.0125 N-NaOH seemed to dissolve the acrosome inasmuch as detached material was never seen after treatment. However, partially disrupted acrosomes remained on some cells, especially if the incubation at 37° C was omitted or shortened. The 1.5 mM-CTAB yielded cell suspensions in which detached acrosomes persisted in an insoluble form; a condition which is reflected in the lower concentrations of nitrogen and sugar in the soluble extract obtained with CTAB. According to Koefoed-Johnsen & Mann (1954), a marked disruption of head membranes and tail ends can be detected by
electron microscopy in ram spermatozoa which have been treated with 2·5 mm CTAB. Agitation with glass beads appears to be the most effective means of removing acrosomes although some separation of heads from midpieces also takes place. Most of the carbohydrate in the spermatozoa, and much of the nitrogen-containing material, that were soluble in 0·1 n-NaOH were also dissolved by 0·0125 n-NaOH (Text-fig. 1). This observation supports the idea that a structural element, the acrosome, is extracted by the weaker alkaline solution. Other material may well be extracted by dilute sodium hydroxide but this is not obvious from the stained preparations.

Our finding that disruption of the acrosome is accompanied by a release of hyaluronidase suggests that at least part of the enzyme is present in the acrosome. Austin (1960) observed that hyaluronidase was released from spermatozoa treated with digitonin, which removed the acrosome. However, the enzyme was not liberated from spermatozoa which had been treated with formalin which preserves the attachment of the acrosome to the spermatozoon. These observations are consistent with the work of Masaki & Hartree (1962) who found that most of the cellular enzyme of bull spermatozoa is present in the heads.

The similarity between antigens in extracts of ram spermatozoa and in ram seminal plasma might be due either to the small proportion of seminal plasma remaining in the sperm suspensions from which the extracts were prepared or to seminal plasma antigens that were absorbed to the spermatozoa. Weil and his associates (Weil, Kotsevalov & Wilson, 1956; Weil & Finkler, 1958; Weil & Rodenburg, 1960) were unable to find differences between antigens in seminal (ejaculated) spermatozoa and seminal plasma, and found that rabbit epididymal spermatozoa and human testicular (spermatocele) spermatozoa lacked the antigens shared by the seminal plasma and seminal spermatozoa. They concluded accordingly that antigen is taken up from the seminal plasma by the spermatozoa. Since after various attempts at extraction seminal spermatozoa released only trace amounts of antigen, a strong bond must exist between seminal plasma antigens and spermatozoa (Weil, 1961).

Earlier studies with guinea-pig spermatozoa have shown that sperm extracts contain several antigens (Pernot, 1956; Katsh & Katsh, 1961). One of these antigens is probably a complex polysaccharide (Katsh & Katsh, 1961), and as such it could be acrosomal material.

ACKNOWLEDGMENTS

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