Properties of Acrosomal Hyaluronidase from Bull Spermatozoa

EVIDENCE FOR ITS SIMILARITY TO TESTICULAR HYALURONIDASE*

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SUMMARY

Sperm acrosomes contain hyaluronidase, the amount varying, respectively, from ram, rabbit, bull, human, boar, rat, and stallion sperm to rooster sperm that do not possess this enzyme.

Hyaluronidase was partially purified from bull sperm acrosomal extracts by DEAE-cellulose chromatography. Bull sperm acrosomal hyaluronidase has an optimum pH of 3.75 and is not affected by freezing and thawing or refrigeration, but it is unstable at a pH below 3.0 or temperatures above 50°. The enzyme requires salt for stability and activity and is inhibited by Fe++ and Fe+++ ions or heparin but not by Mn++, Mg++, and Ca++ ions or EDTA. Partially purified acrosomal hyaluronidase from rabbit spermatozoa possesses the same properties. The molecular weight of the hyaluronidase of bull sperm acrosomal extracts was estimated to be 110,000 by gel filtration using a Sephadex G-100 column. The acrosomal proteinase-proteinase inhibitor complex appeared as a separate fraction with a molecular weight of 68,000.

The activity of bull testicular hyaluronidase as well as bull sperm acrosomal hyaluronidase is inhibited by rabbit immunoantiserum prepared against bull testicular hyaluronidase preparations or bull sperm acrosomal extracts. Immuno-diffusion experiments showed that bull testicular hyaluronidase and bull sperm acrosomal hyaluronidase preparations have only one component in common. This component was demonstrated to be the hyaluronidase. The results suggest that sperm acrosomal hyaluronidase is identical with testicular hyaluronidase but is apparently different from lysosomal hyaluronidase, present in organs other than the testis.

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A large variety of organs and tissues possess hyaluronidase. In all of these, except the testis, hyaluronidase was shown to be of lysosomal origin (1-7). Lysosomal and testicular hyaluronidase differ significantly (7). The biochemical properties of lysosomal and testicular hyaluronidase have been studied extensively (5-8), although a highly purified preparation of testicular hyaluronidase has just recently been obtained (9).

McLean (10) and Hoffmann and Duran-Reynals (11) first indicated the presence of hyaluronidase in spermatozoa. Later, the enzyme was shown to be associated with the sperm head and in particular the acrosome or head cap (12-14). The hyaluronidase activity of a semen sample correlates with the number of sperm (15), and the hyaluronidase of seminal plasma was shown to originate from sperm and not from the accessory glands (16).

Hyaluronidase disperses the cumulus oophorus on addition to ova (17, 18), a process that can be inhibited using hyaluronidase inhibitors (14, 19, 20) or antisera (21, 22). It is generally assumed that sperm use this enzyme to lyse a path through the cumulus (23). Various authors reported an increase in the fertilizing capacity of sperm on addition of hyaluronidase (24, 25). Treating sperm with hyaluronidase inhibitors resulted in a decrease in fertility (20, 26). These data indicate that the inactivation of acrosomal hyaluronidase may be a feasible approach in the development of new contraceptive agents.

* Except for a brief statement about the pH optimum of the hyaluronidase of human spermatozoa (4), no data are available concerning the characteristics of sperm acrosomal hyaluronidase. This article describes the partial purification and some of the properties of bull sperm acrosomal hyaluronidase. Identity with testicular hyaluronidase was evaluated both biochemically and immunochemically. The hyaluronidase activities of sperm acrosomal extracts from various species and some of the properties of partially purified rabbit sperm acrosomal hyaluronidase were also determined. A preliminary report was presented previously (27).

EXPERIMENTAL PROCEDURE

Enzyme Assays—Hyaluronidase assays were performed by a modification of the methods of Bollet et al. (1) and Aronson and Davidson (6). In a tube, 0.3 ml of hyaluronic acid (Worthington) at a concentration of 0.8 mg per ml in 0.1 M acetate buffer, pH 3.8, containing 0.15 M NaCl was mixed with 0.2 ml of the enzyme solution and incubated at 37° for 1 hour. The mixture
was adjusted to pH 8.9 by addition of 0.1 ml of 0.8 M potassium tetraborate buffer, pH 9.1, and 0.25 ml of 1 M NaOH and tested for N-acetylatedoxaminine released according to the method of Rieioig et al. (28). Controls were made by either omitting the enzyme or the substrate. N-Acetylglycosaminine (Sigma) was used as standard. One unit of hyaluronidase activity was defined as that amount of enzyme that causes the release of 1 μmole of N-acetylglycosaminine in 1 hour at 37°. Specific activity was recorded as the number of units per mg of protein assuming that 1 mg of protein per ml has an optical density of 1.0 at 280 nm using a 1-cm light path. Bovine testicular hyaluronidase was obtained from Worthington and β-glucuronidase from Warner Chilcott Laboratory (General Diagnostics Division). Acrosomal proteinase determinations were made using benzoyl-arginine ethyl ester (BAEE, Sigma, 10 mg per 50 ml) (20) in 0.1 M borate buffer, pH 8.0, containing 0.05 M CaCl₂ as substrate (30). The presence of a proteinase-proteinase inhibitor complex was indicated by an increase in activity after incubation of the solutions at pH 3.0 (31).

Preparation of Acrosomal Extracts—Ram, rabbit, boar, stallion, rooster, and human ejaculated sperm were collected and treated immediately, whereas the bull sperm was obtained frozen in pools of 100 ejaculates from Eastern Artificial Insemination Company, Ithaca, New York and Curtiss Breeding Service, Cary, Illinois. Rabbit and rooster sperm were treated in pools of 40 ejaculates and human sperm in pools of 15 ejaculates. The ejaculate of the other species were treated individually. Epididymides were either obtained fresh from the School of Veterinary Medicine, University of Georgia, or were purchased from Pel-Freeze Biologicals and shipped on ice. The epididymides were flushed according to Polakoski et al. (32), and the sperm were removed by centrifugation at 700 x g for 15 min.

Acrosomal enzymes were precipitated from the supernatant solution by addition of ethanol (80%), and the precipitate was washed with 0.5 M NaCl and 0.5 M NaCl for 24 hours. Chromatography was performed using a stepwise NaCl gradient. A flow rate of 1 drop per 5 s was maintained.

Preparation of Antisera—Antibodies against rabbit and bull sperm acrosomal extracts were prepared by mixing the extracts (25 mg) with 2.3 ml of Freund's adjuvant using a tissue microhomogenizer and injecting the mixture subcutaneously into the inguinal and axillary regions. Rabbits were used for bull acrosomal extracts and guinea pigs were used for rabbit acrosomal extracts. After 3 weeks a booster injection of 5 mg of acrosomal extract was administered intraperitoneally every 2 days for 12 days. The animals were bled 10 days later. Bull testicular hyaluronidase antisera was a generous gift from Dr. Trueschel, Research Laboratories, Farbenfabriken, Bayer AG (Wuppertal-Elberfeld, Germany).

Immunochromatographic Techniques—Bull testicular and bull acrosomal hyaluronidase preparations were incubated with antisera or normal rabbit sera as control at room temperature for 15 min before testing for activity using the colorimetric test. The solutions were centrifuged before measuring the absorbance since a
cloudy precipitate was frequently present. To determine the specificity of inhibition, rabbit sperm acrosomal hyaluronidase was incubated with bull acrosomal extract antisera. The inhibitory effect of antisera on hyaluronidase activity was also evaluated using the immunoenzyme diffusion test (see below).

Regular immunodiffusion experiments were performed according to the method of Ouchterlony (35) using 1% agarose gel buffer with 0.1 M acetate buffer, pH 6.5. A new method was devised that allowed for the observation of both the immunoprecipitin bands and the enzyme activity. Agarose (1%) was mixed with 0.1% hyaluronic acid in 0.1 M acetate buffer, pH 6.5, containing 0.05 M NaCl, and immunodiffusion slides and Wilson plates (36) were prepared. After the precipitin bands had formed, the plates were submerged in 1% cetylpyridinium chloride dissolved in the same buffer. This resulted in a white precipitation of the hyaluronic acid, whereas the areas of hyaluronidase diffusion remained transparent.

RESULTS

Hyaluronidase Activity of Acrosomal Extracts—Acrosomal extracts in the absence of hyaluronic acid or hyaluronic acid in the absence of enzyme did not result in N-acetylhexosamine formation. Dott and Dingle (37) reported that β-glucuronidase may be present in the acrosomal extracts. This does not interfere with the assay since 2500 units of β-glucuronidase did not cause the release of N-acetylhexosamine when incubated with hyaluronic acid under the same conditions. Large differences exist among the hyaluronidase activities of the extracts of the species tested, decreasing in order from the ram, rabbit, bull, human, boar, rat, and stallion to rooster acrosomal extracts that did not possess any hyaluronidase activity (Table I). No significant differences in hyaluronidase activity could be found between the acrosomal extracts of epididymal and ejaculated sperm of the same species.

Purification of Rabbit and Bull Sperm Hyaluronidase—Both the bull and rabbit acrosomal hyaluronidase appeared in the first protein fraction after DEAE-cellulose column chromatography (Fig. 1). Respectively, a 27- and 25-fold increase in specific activity was obtained (Table II). Individual tubes possessed specific activities as high as 90 units per mg. The partially purified hyaluronidase preparations were used throughout the following experiments.

Optimum pH—The optimum pH of both bull and rabbit acrosomal hyaluronidase is 3.75 (Fig. 2). Activity ranged approximately from pH 3.0 to pH 7.0.

Stability—Neither bull nor rabbit acrosomal hyaluronidase activity was altered significantly by either freezing and thawing or refrigeration. Rabbit acrosomal hyaluronidase lost its ac-

TABLE I

Hyaluronidase activity of sperm acrosomal extracts from various species

The acrosomal extracts were prepared by treating washed sperm in Krebs-Ringer phosphate buffer, pH 7.0, with equal volumes of a 0.15% solution of Hyamine 2389 and Triton X-100 for 1½ hours at 37°. The sperm were removed by centrifugation at 700 × g for 15 min, and the supernatant solution was treated with 80% alcohol. The precipitated material was dialyzed against distilled water and lyophilized.

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>Specific activity (range)</th>
<th>Specific activity (average)</th>
<th>Number of pools or ejaculates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ram</td>
<td>Ejaculate</td>
<td>0.840–2.940</td>
<td>1.54</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Epididymide</td>
<td>0.610–1.840</td>
<td>1.22</td>
<td>3</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Ejaculate</td>
<td>0.470–0.652</td>
<td>0.561</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Epididymide</td>
<td>0.600</td>
<td>0.600</td>
<td>1</td>
</tr>
<tr>
<td>Bull</td>
<td>Ejaculate</td>
<td>0.141–0.300</td>
<td>0.221</td>
<td>2</td>
</tr>
<tr>
<td>Human</td>
<td>Ejaculate</td>
<td>0.045–0.089</td>
<td>0.067</td>
<td>2</td>
</tr>
<tr>
<td>Boar</td>
<td>Ejaculate</td>
<td>0.016–0.086</td>
<td>0.045</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Epididymide</td>
<td>0.009–0.032</td>
<td>0.029</td>
<td>8</td>
</tr>
<tr>
<td>Rat</td>
<td>Epididymide</td>
<td>0.014–0.018</td>
<td>0.016</td>
<td>3</td>
</tr>
<tr>
<td>Stallion</td>
<td>Ejaculate</td>
<td>0.000–0.030</td>
<td>0.004</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Epididymide</td>
<td>0.000–0.006</td>
<td>0.003</td>
<td>2</td>
</tr>
<tr>
<td>Rooster</td>
<td>Ejaculate</td>
<td>0.000</td>
<td>0</td>
<td>2</td>
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</tbody>
</table>

* Pooled before preparation of acrosomal extract (see text).

TABLE II

Purification of rabbit and bull acrosomal hyaluronidase

<table>
<thead>
<tr>
<th>Species</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm extracts</td>
<td>100</td>
<td>14.1</td>
<td>0.141</td>
<td>1</td>
</tr>
<tr>
<td>DEAE chromatography</td>
<td>5.04</td>
<td>14.1</td>
<td>3.54</td>
<td>25</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm extracts</td>
<td>20.3</td>
<td>19.1</td>
<td>0.652</td>
<td>1</td>
</tr>
<tr>
<td>DEAE chromatography</td>
<td>0.33</td>
<td>5.7</td>
<td>17.2</td>
<td>27</td>
</tr>
</tbody>
</table>

FIG. 1. DEAE column chromatography of acrosomal extracts from ejaculated bull sperm. ■—■ absorbance at 280 nm; ○—○, hyaluronidase activity. Lyophilized bull sperm acrosomal extracts (100 mg) in 3 ml of 0.05 M phosphate buffer, pH 7.0, were applied to the column and eluted with the same buffer using a stepwise salt gradient. A similar elution profile was obtained with rabbit sperm acrosomal extracts.

FIG. 2. The pH optimum of bull and rabbit sperm acrosomal hyaluronidase. ■—■, bull sperm acrosomal hyaluronidase (30 μg); ○—○, rabbit sperm acrosomal hyaluronidase (10 μg).
tivity after 1 hour at pH 3.0. At this same pH, bull testicular and bull acrosomal hyaluronidase lost most of their activity in 1 hour and all activity in 12 hours.

Dialysis against H2O caused a significant decrease in bull sperm acrosomal hyaluronidase activity, the amount of decrease depending on the extent of dialysis. No activity was lost if bull sperm hyaluronidase was dialyzed against a solution of 0.15 M NaCl. The preparations dialyzed against H2O showed identical activities using substrate with or without NaCl, indicating that dialysis in the absence of salt permanently destroys the activity of the enzyme. Salt is also necessary for the hydrolysis of substrate since the absence of NaCl in the substrate solution resulted in a significant decrease in activity of preparations dialyzed in the presence of NaCl. Similar results were obtained with the rabbit acrosomal hyaluronidase preparations. Apparently salt is a requirement for both enzyme activity and enzyme stability.

Effect of Temperature—Bull and rabbit acrosomal hyaluronidase, as well as bull testicular hyaluronidase, lost most of their activity at 50° and all of their activity at temperatures above 60° (Fig. 3).

Effect of Ions, EDTA, and Heparin—Mg++ and Ca++ ions had no apparent effect on the activity of bull and rabbit acrosomal hyaluronidase, whereas Mn++ caused a slight decrease in activity at a concentration of 0.1 M. Fe+++ and Fe++ ions effectively decreased enzyme activity at 0.1 M and 0.01 M concentrations. Fe++ inhibited both bull and rabbit acrosomal hyaluronidase to a greater extent than Fe+++. Care had to be taken to correct for the values obtained with Fe++, Fe+++, and Mn++ ions since these ions affected the color formation by N-acetylglucosamine although never more than 20%. Bull acrosomal hyaluronidase was generally more effectively inhibited by metal ions than rabbit acrosomal hyaluronidase.

Even at concentrations of 0.5 M EDTA had no effect on the activity of either bull or rabbit acrosomal hyaluronidase. Heparin (100 units) completely inhibited bull (0.090 unit) and rabbit (0.070 unit) acrosomal hyaluronidase. EDTA and heparin had no effect on color formation by N-acetylglucosamine.

Molecular Weight Estimation—Judging from the elution pattern after Sephadex G-100 gel filtration, bull acrosomal hyaluronidase has a molecular weight of 110,000 (Fig. 4). This is approximately the same as the molecular weight of 126,000 reported for the dimeric form of bull testicular hyaluronidase (9). Similar to the proteinase-proteinase inhibitor complex present in boar sperm acrosomal extracts (31), data that will be published later show that such a complex is also present in bull sperm acrosomal extracts. This complex was eluted separately from the acrosomal hyaluronidase and possessed a molecular weight of 68,000.

Immunochemical Tests—Bull acrosomal extract antisera inhibited the activity of bull testicular and bull acrosomal hyaluronidase but not that of rabbit acrosomal hyaluronidase (Table III). Similarly, antisera against bull testicular hyaluronidase inhibited bull acrosomal and bull testicular hyaluronidase. Normal rabbit sera did not affect the hyaluronidase activity. The same results were obtained using the immunoenzyme diffusion test (Fig. 5a). Ouchterlony plate tests showed that only one precipitin band is formed between bull testicular hyaluronidase preparations and bull acrosomal extract antisera (Fig. 5b). This precipitin band showed a pattern of identity with one of the precipitin bands formed with crude or partially purified bull acrosomal hyaluronidase preparations. Using the immunoenzyme diffusion technique (Fig. 6) this precipitin band was identified as the complex formation between hyaluronidase and its antibody since the line of enzyme inhibition is identical with this band. No immunoprecipitin bands were formed between species.

![Fig. 3](http://www.jbc.org/) The effect of temperature on bull and rabbit sperm acrosomal hyaluronidase and bull testicular hyaluronidase. •—• bull sperm acrosomal hyaluronidase; —— bull testicular hyaluronidase. Bull (30 µg) and rabbit (5 µg) acrosomal hyaluronidase and bull testicular hyaluronidase (100 µg) in 0.1 M acetate buffer, pH 3.5, containing 0.15 M NaCl were maintained in a water bath for 10 min at the indicated temperatures before testing for activity (see text).

![Fig. 4](http://www.jbc.org/) Molecular weight estimation of bull sperm acrosomal hyaluronidase and proteinase-proteinase inhibitor complex by Sephadex G-100 gel filtration. See text for detailed description.

**Table III**

Effect of normal rabbit sera and antisera on acrosomal and testicular hyaluronidase activity

Tests were performed as described in Table V. See text for further experimental details.

<table>
<thead>
<tr>
<th>Source and amount of hyaluronidase</th>
<th>Control serum</th>
<th>Bull acrosomal extract antisera</th>
<th>Bull testicular hyaluronidase antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull acrosomes (35 µg)</td>
<td>0.120/100</td>
<td>0.019/16</td>
<td>0/0</td>
</tr>
<tr>
<td>Bull testicles (60 µg)</td>
<td>0.110/100</td>
<td>0.017/16</td>
<td>0/0</td>
</tr>
<tr>
<td>Rabbit acrosomes (7 µg)</td>
<td>0.115/100</td>
<td>0.120/100</td>
<td></td>
</tr>
</tbody>
</table>
FIG. 5. a, effect of rabbit anti-bull acrosomal extract serum and normal rabbit serum on various hyaluronidase preparations in the immunoenzyme diffusion test. Hyaluronic acid (0.1%) was mixed with agarose (1%) in acetate buffer and immunodiffusion slides were prepared as described. After 16 hours of diffusion at 23°, submerging of the slides in 1% cetylpyridinium chloride dissolved in the same buffer resulted in a white precipitation of the unhydrolyzed hyaluronic acid, whereas the radial diffusion zones of the hyaluronidase appeared transparent. The area of complex formation between hyaluronidase and its antibody can be seen as a distinct zone of enzyme inhibition, whereas the diffusion of rabbit acrosomal hyaluronidase proceeds without inhibition. b, agar gel immunodiffusion test of bull testicular hyaluronidase preparations and bull acrosomal extracts versus rabbit anti-bull acrosomal extract serum. Precipitin band p is identical between the preparations. The other bands derive only from the acrosomal extracts. Due to the large excess and higher diffusion rate, one of these bands almost completely surrounds the central well. Similarly, an identical precipitin band was present using bull sperm acrosomal hyaluronidase partially purified by DEAE column chromatography as antigen instead of bull acrosomal extracts. Such a band was also formed using anti-bull testicular hyaluronidase serum instead of anti-bull acrosomal extract serum. No precipitin bands were formed between rabbit acrosomal hyaluronidase preparations and bull testicular or bull acrosomal extract antisera. BAE, bull acrosomal extracts (20 mg per ml); BTH, bull testicular hyaluronidase (10 mg per ml); BTH2, bull testicular hyaluronidase (20 mg per ml); RAE, rabbit acrosomal extracts (20 mg per ml); A, anti-bull acrosomal extract serum; N, normal rabbit serum.

FIG. 6. Immunoenzyme diffusion test of bull testicular and bull acrosomal hyaluronidase preparations versus rabbit anti-bull acrosomal extract serum. BTH, bull testicular hyaluronidase (10 mg per ml); BAE, bull acrosomal extracts (20 mg per ml); A, anti-bull acrosomal extract serum. Precipitin band p corresponds to the band of Fig. 5 (see Fig. 5 for description of test). A disadvantage of cetylpyridinium chloride is that the immunoprecipitin lines disappear as the unhydrolyzed hyaluronic acid is precipitated. 1, 5 min; 2, 15 min; 3, 30 min after addition of cetylpyridinium chloride; and 4, diagram combining results of 1, 2, and 3.

lyosomal hyaluronidase does not require salt for activity (7). Acrosomal hyaluronidase therefore differs from lysosomal hyaluronidase. Although the acrosome is considered to be a lysosome (38), we now know that at least two of the acrosomal enzymes, hyaluronidase and the acrosomal proteinase (30), have characteristics that are different from similar enzymes usually found in lysosomes, emphasizing the uniqueness of the sperm as a cell.

The biochemical properties of acrosomal hyaluronidase described in this communication correspond to those reported for testicular hyaluronidase (7, 8, 30, 40) with one exception. Meyer and Rapport (39) showed that Fe+++ is a more effective inhibitor for testicular hyaluronidase than Fe++. Although this is in contrast to the relative effect of these ions on acrosomal hyaluronidase, it is probably due to the different assay method.
Bull testicular hyaluronidase antisera and bull acrosomal extract antisera show cross inhibitory activity in the colorimetric enzyme assay experiment. In the immunodiffusion test, one precipitin line between acrosomal extract and its antisera shows a pattern of identity with the only precipitin band between testicular hyaluronidase and this antisera. In the combined immunoenzyme diffusion test, these precipitin lines delineate clearly the zone of hyaluronidase inhibition. Based on these observations, there seems to be little doubt that testicular and sperm acrosomal hyaluronidases display the same immunological properties. Rabbit sperm acrosomal hyaluronidase is neither precipitated nor inhibited by antisera against bull sperm acrosomal extract produced in rabbits, indicating significant differences in antigenicity of the heterologous bull acrosomal hyaluronidase and the homologous rabbit acrosomal hyaluronidase.

Lysosomal and testicular (acrosomal) hyaluronidases differ biochemically (1, 2, 7); however, differences in immunological characteristics remain to be determined. In case testicular (acrosomal) hyaluronidase could be identified as specific for spermatozoa, it is indeed conceivable that the isoimmunization of females with testicular hyaluronidase results in impaired fertility without affecting other cells, provided the antibodies enter into the Fallopian tube fluid.

The pH optimum of rabbit and bull acrosomal hyaluronidase is similar to that of human sperm hyaluronidase (4) although a much wider range of optimal activity was obtained with the human enzyme. This is most likely due to the impurity of the human sperm preparation tested since pH experiments with unpurified rabbit acrosomal extracts produced a similar wide pH optimum. The relative hyaluronidase activities of the acrosomal extracts of the species tested correlate with those reported for whole sperm (41). Srivastava et al. (14) reported a somewhat higher hyaluronidase activity in bull than in rabbit acrosomal extracts. This difference can be explained since in the present study the bull semen was obtained frozen and had to be thawed causing hyaluronidase release into surrounding plasma and therefore a decrease in the specific hyaluronidase activity of the acrosomal extracts.

Stambaugh and Buekly (42) reported that the zona penetrating enzymes of rabbit spermatozoa occur as a single complex of hyaluronidase and a proteinase with a combined molecular weight of 39,000. These authors based their conclusion on a sucrose density gradient centrifugation experiment involving crude rabbit extracts that had been obtained by sonication of whole sperm. The present results show that bull sperm acrosomal hyaluronidase and proteinase appear in separate fractions after Sephadex G-100 column chromatography, indicating that at least in this species the enzymes are most likely not in complex.

Sperm acrosomes possess β-glucuronidase as well as N-acetylglucosaminidase (37, 38). The function of these enzymes in the fertilization process is still unknown. Similar enzymes are present in lysosomes. Since hyaluronidase produces only tetra- and disaccharides on hydrolysis of hyaluronic acid (8), it is generally assumed that in the case of lysosomes β-glucuronidase breaks down oligosaccharides further down to disaccharides which in turn are hydrolyzed to monosaccharides by N-acetylglycosaminidase (5). It seems likely that a similar process occurs when sperm hydrolize the hyaluronic acid moiety of the cumulus and that it may be a combined action of all three enzymes that aids sperm in the penetration through the cumulus oophorus.

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Addendum—Since the submittal and acceptance of this article, data were published by Metz et al. (43, 44) showing that hetero- and iso-antibodies produced against epidual and ejaculated rabbit semen prevented the dispersion of the cumulus oophorus or hydrolysis of hyaluronic acid by epidual, ejaculated, and capacitated rabbit semen or rabbit sperm extracts. Similar results were obtained with human material. Reactions were completely species specific. Castro and Metz (45) also demonstrated differences in antibody inhibition between human sperm hyaluronidase and human serum (lysozyme) hyaluronidase, emphasizing the dissimilarity of these enzymes.

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