Hamster Sperm Hyaluronidase

II. ITS RELEASE FROM SPERM IN VITRO IN RELATION TO THE DEGENERATIVE AND NORMAL ACROSOME REACTION

PRUDENCE TALBOT AND LUTHER E. FRANKLIN
University of Houston, Department of Biology, Houston, Texas 77004

ABSTRACT Hamster sperm incubated in vitro were used to determine: (1) when hyaluronidase is released from sperm undergoing acrosome detachment, i.e., either a degenerative or normal acrosome reaction, and (2) what ultrastructural changes occur in the acrosomes of living sperm during hyaluronidase release. Sperm were incubated up to 4 hours in normal saline, Ham’s F-10, or heat pretreated blood serum. At hourly intervals, the percentages of live (motile) and acrosome reacted sperm were counted, and the supernatant from each sperm sample was assayed for hyaluronidase activity. In normal saline, sperm died during the incubation period, underwent a degenerative acrosome reaction, and gradually released hyaluronidase. In Ham’s F-10, sperm lived throughout the incubation period, did not undergo acrosome detachment, and did not release detectable amounts of hyaluronidase. In heat pretreated blood serum, sperm death did not occur during the incubation period, a surge of hyaluronidase release was complete by 1 hour of incubation, and additional hyaluronidase activity was not detected in the incubation medium between 3 and 4 hours when the normal acrosome reaction was observed. The fine structure of the acrosome was unaltered immediately after the release of hyaluronidase in serum. It was concluded that more than 50% of the mechanically extractable hamster sperm hyaluronidase was released by a factor present in serum and that this release occurred prior to and independently of the normal acrosome reaction. The partial release of hyaluronidase in serum prior to the occurrence of a normal acrosome reaction may indicate that this enzyme has a bifunctional role in reproduction.

Mammalian sperm contain the enzyme hyaluronidase (Hechter and Hadidian, '47; Swyer, '47). Numerous investigators have shown that hyaluronidase is associated with the acrosome (Leuchtenberger and Schrader, '50; Austin, '60; Mancini et al., '64; Srivastava et al., '65; Stambaugh and Buckley, '69, '70).

The mechanism for the release of hyaluronidase from sperm is incompletely understood but is considered to be related to acrosome detachment. Acrosomes become detached from sperm either as a consequence of cell death (the degenerative acrosome reaction) or by the occurrence of a physiologically normal (true) acrosome reaction (Austin and Bishop, '58). The normal acrosome reaction consists of at least two phases—membrane vesiculation and subsequent detachment of the acrosome.

Direct evidence that acrosome reactions result in hyaluronidase release has come only from studies involving the degenerative acrosome reaction. When sperm death is artificially induced, e.g., by osmotic shock (Swyer, '47), freeze-thawing (Perlman et al., '48), or digitonin treatment (Austin, '60), hyaluronidase is released.

It has been inferred, although never directly demonstrated, that hyaluronidase release also occurs during the normal acrosome reaction (Barros et al., '67). Austin ('68) has suggested that the finely granular material observable within the acrosome at the ultrastructural level may be hyaluronidase or a precursor of this enzyme, and Yanagimachi and Teichman ('72) have shown cytochemically that this granular material in mammalian sperm acrosomes is probably a proteinase-hyaluronidase complex. During the normal
acrosome reaction, vesiculation occurs between the plasma membrane and outer acrosomal membrane, and most of the finely granular material is lost from the acrosome (Piko and Tyler, '64; Barros et al., '67; Bedford, '68, '70; Franklin et al., '70; Yanagimachi and Noda, '70). Barros et al. ('67) have suggested that the perforations formed in the acrosome through membrane vesiculation provide ports for the escape of enzymes, including hyaluronidase. Rogers and Morton ('73) have demonstrated that living hamster sperm release more hyaluronidase during incubation in media which support the occurrence of capacitation and the normal acrosome reaction than they release in Tyrode's solution. It is not clear from their data, however, whether or not hyaluronidase release and the normal acrosome reaction are simultaneous events.

Lewis and Ketchel ('73) concluded that the release of hyaluronidase from rabbit sperm incubated in vitro is "related to the completion of the process of capacitation." The definition of mammalian sperm capacitation varies but is widely accepted as a physiological change which precedes the normal acrosome reaction in time. Although Lewis and Ketchel ('73) did not monitor acrosome reactions in their study, their data imply that hyaluronidase is released from rabbit sperm before a normal acrosome reaction occurs.

The time relationship between hyaluronidase release and the normal acrosome reaction is not clear from the literature and has never been directly investigated. In this study, hamster sperm incubated in vitro in three cell culture media were used to determine: (1) when hyaluronidase is released from sperm undergoing acrosome detachment, i.e., either a degenerative or normal acrosome reaction, and (2) what ultrastructural changes occur in the acrosomes of living sperm during hyaluronidase release. Hamster sperm were chosen because they will undergo a normal acrosome reaction in vitro in heat pretreated blood serum (Barros and Garavagno, '70; Yanagimachi, '70) and detachment of the acrosome is readily observed with the phase contrast microscope.

MATERIALS AND METHODS

Release of hyaluronidase. Hamster sperm from lacerated cauda epididymidis were suspended and carefully washed in normal saline. Washed sperm were added to 0.5 ml droplets (10^7 sperm per droplet) of normal saline, F-10 (Ham, '63), or heat pretreated (60°C for 60 minutes) serum (primate or calf) which had previously been tested for its ability to support the occurrence of capacitation and a normal acrosome reaction. Sperm in these media were incubated in Falcon tubes (Catalogue number 2095) at 37°C in air. At time zero (no incubation) and at hourly intervals thereafter up to 4 hours, incubations were terminated and the percentages of live and acrosome reacted sperm were counted. In evaluating these percentages, a random field of 100 sperm was examined and any motile sperm were scored as live. In a separate field, 50 motile sperm were examined, and any without acrosomes were scored as having undergone a normal acrosome reaction. Sperm were separated from the incubation media by centrifugation, and the supernatants were assayed for hyaluronidase activity using the bioassay based on cumulus dispersion rate which was described in the previous report (Talbot and Franklin, '74). This assay procedure was used because it is sensitive to low levels of hyaluronidase activity and is compatible with complex incubation media.

Electron microscopy. At time zero and after 75 minutes of incubation, sperm in baboon serum and in Ham's F-10 were fixed in 1/2 strength glutaraldehyde-parafomaldehyde (Karnovsky, '65), postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in a graded series of acetone, embedded in low viscosity plastic (Spurr, '69), sectioned, stained with uranium and lead salts, and examined with an AEI EM6B electron microscope.

To distinguish between sperm which were living and sperm which were dead at the time of fixation, the following procedures were used. Sperm incubated in Ham's F-10 were picked up on the zona pellucida surface of eggs prior to processing for electron microscopy. Living sperm...
were thus selected for the incubation droplet, and this was verified with the phase contrast microscope prior to fixation. Sperm maintained in serum did not readily adhere to the zona surface of eggs added at time zero and time 75 minutes and were fixed for electron microscopy directly in the incubation droplet; dead sperm were thus included in this fixation procedure. Examination of the serum incubated sperm with the electron microscope revealed three sperm groups based on the condition of the acrosome. Sperm either (1) had unswollen, intact acrosomes (figs. 6–9); (2) had swollen, semi-detached acrosomes (fig. 10); or (3) lacked acrosomes (fig. 10). Sperm in the latter two groups correspond to hamster sperm undergoing degenerative changes as described by Yanagimachi and Noda ('70) and by Franklin et al. ('70). In this study, these sperm were readily distinguishable as dead (immotile) with phase contrast microscopy prior to fixation. Sperm in group 1 did not have any characteristics which could be associated with degeneration. With the electron microscope, 100 sperm from the 75 minute incubation were examined; 68% of these could be categorized as belonging to group 1. This percentage is approximately equal to the percentage of living sperm (70%) determined with phase contrast microscopy for this sample during incubation and consequently supports the assumption that group 1 sperm were considered to have been alive and group 2 and 3 were considered to have been dead at the time of fixation.

RESULTS

Hyaluronidase release by sperm in normal saline (fig. 1a). At the concentration used in this study, sperm in normal saline died during the course of the incubation period and underwent a degenerative acrosome reaction. All sperm were dead by the end of the third hour of incubation, although not all of the sperm acrosomes were detached. Degenerative acrosome reactions had occurred in most sperm by 4 hours. Approximately a 4-fold increase in hyaluronidase activity was observed in the supernatant by the end of 4 hours. No normal acrosome reactions occurred.

Hyaluronidase release by sperm in Ham’s F-10 (fig. 1b). In the defined medium F-10, which does not support the occurrence of the normal acrosome reaction, a significant increase over the background level of hyaluronidase was not detected during 4 hours of incubation. Sperm survived throughout the incubation period, although by 4 hours their motility had become sluggish.

Hyaluronidase release by sperm undergoing a normal acrosome reaction in serum (fig. 1c). In heat pretreated serum, there are no detectable cell deaths during the incubation period. The normal acrosome reaction (detachment) occurred between 3 and 4 hours of incubation. A surge of hyaluronidase release was observed between time zero and 1 hour of incubation in calf, baboon, Rhesus monkey, and human serum. Typically, a 20 to 100-fold increase in hyaluronidase activity was detected in the supernatant at the end of 1 hour, after which time the activity remained constant. In no experiment was a significant increase in hyaluronidase activity detected concomitant with the occurrence of the normal acrosome reaction.

Hyaluronidase release by sperm which did not undergo a normal acrosome reaction in serum (fig. 1d). Several serum samples which did not support the occurrence of the normal acrosome reaction were tested to determine whether or not sperm incubated in these sera would release hyaluronidase. A surge of hyaluronidase release was observed and was completed by 1 hour of incubation. Cell deaths were not detectable during incubation, and only 2% of the sperm had undergone an acrosome reaction by 4 hours.

Hyaluronidase release from sperm in serum following repetitive washing. To determine if additional amounts of hyaluronidase could be released from living sperm after the initial surge, sperm were incubated in serum for 1 hour, washed with normal saline, incubated a second hour in fresh serum, washed in normal saline and incubated a third hour in fresh serum. Hyaluronidase activity was measured in the supernatant of each serum sample. Results (table 1) indicate that of the total amount of hyaluronidase recoverable in the incubation media, 95% was released during the first hour of incubation. Subsequent washing and incubation in fresh serum did not elicit significant additional hyaluronidase release.
Fig. 1 Hyaluronidase release, sperm viability, and the normal acrosome reaction in different incubation media: (a) normal saline; (b) Ham's F-10; (c) heat pretreated sera supporting the occurrence of a normal acrosome reaction; (d) heat pretreated sera not supporting the occurrence of a normal acrosome reaction. ▲, percentage of sperm showing a normal acrosome reaction (detachment); ○, percentage of live (motile) sperm; □, hyaluronidase activity (TRU/ml) measured in supernatants. For normal saline and Ham's F-10, each point is the mean ± SE of 4 to 5 trials. For both serum plots, each point is the mean ± SE of 5 to 6 trials. To evaluate the percentage of sperm showing a normal acrosome reaction, 50 sperm were counted in each trial. To evaluate the percentage of live sperm, 100 sperm were counted in each trial.

**Percentage of total extractable hyaluronidase released by sperm in serum.** To determine how much, if any, residual hyaluronidase is present in sperm after serum incubation, the following experiments were performed. Washed epididymal sperm were added to tubes of serum. At time zero, one sample was either sonicated for 1 minute or frozen and thawed 5 times, and one sample received no treatment. Additional tubes of serum containing living sperm were incubated at 37°C for 1 hour to elicit hyaluronidase release. At the end of the incubation period, serum was re-
HYALURONIDASE RELEASE

TABLE 1

Release of hyaluronidase by living sperm in serum after repetitive washings

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Time</th>
<th>% Live sperm</th>
<th>% Normal Acrosome Reaction</th>
<th>TRU/ml (^1,2) in Supernatant</th>
<th>TRU/ml Released per incubation</th>
<th>% of total activity released in supernatants released per incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial hour of serum</td>
<td>zero</td>
<td>59</td>
<td>0</td>
<td>0.128</td>
<td>4.650</td>
<td>95.3</td>
</tr>
<tr>
<td>incubation (^3)</td>
<td>1 hour</td>
<td>68</td>
<td>0</td>
<td>4.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second hour of serum</td>
<td>zero</td>
<td>63</td>
<td>0</td>
<td>0.176</td>
<td>0.208</td>
<td>4.3</td>
</tr>
<tr>
<td>incubation (^3)</td>
<td>1 hour</td>
<td>70</td>
<td>0</td>
<td>0.384</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third hour of serum</td>
<td>zero</td>
<td>63</td>
<td>0</td>
<td>0.092</td>
<td>0.024</td>
<td>0.5</td>
</tr>
<tr>
<td>incubation (^3)</td>
<td>1 hour</td>
<td>71</td>
<td>0</td>
<td>0.053</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total activity recovered in supernatants: 4.882

\(^1\) Mean of duplicate trials.
\(^2\) For comparisons between serum samples, all values are ± a factor of 1.85.
\(^3\) Sperm samples were washed in normal saline prior to initiating serum incubation.

moved by centrifugation. The sperm were washed in normal saline, resuspended in fresh serum, and either sonicated, frozen and thawed, or left untreated. Supernatants were assayed for hyaluronidase activity. Results (table 2) from both extraction procedures were comparable and indicate that during 1 hour of serum incubation, about 55–60% of the hyaluronidase which is extractable by sonication or freeze-thawing is released.

Electron microscopy. With the phase contrast microscope, no changes in the sperm acrosome were observed during the first hour of serum incubation when hyaluronidase release occurred. To determine if vesiculation between the periacrosomal plasma membrane and outer acrosomal membrane might be taking place, sperm from F-10 and serum incubations were examined with the electron microscope at time zero and after 75 minutes of incubation.

Comparison of acrosomes from sperm judged to have been alive at the time of fixation (MATERIALS AND METHODS) from both F-10 (figs. 2–5) incubations and both serum (figs. 6–9) incubations revealed no difference in the conditions of the acrosome, including the equatorial segment. Swelling and leaching were not evident in any region of the acrosome. The outer acrosomal membrane was always observed to be intact and showed no evidence of vesiculation or breaking after 75 minutes of incubation in either serum or F-10. In all incubations, the periacrosomal plasma membrane was observed to be either intact, elevated and intact, or elevated and broken in several places.

DISCUSSION

Investigations directly relating hyaluronidase release to acrosome detachment have been limited to the degenerative acrosome reaction associated with sperm death. The experiments presented in this report were designed to examine the relationship be-
tween the acrosome reaction, both as a normal event and a postmortem change, and the release of hyaluronidase from sperm.

Hamster sperm incubated in a medium (normal saline) which did not sustain sperm viability underwent a degenerative acrosome reaction and released hyaluronidase during the incubation period. Both hyaluronidase release and acrosome detachment continued to occur after all sperm movement ceased (3 hours). These data indicate that hyaluronidase release occurs at the time of and/or subsequent to a degenerative acrosome reaction and therefore support results reported by several other investigators (Austin, '60; Hathaway and Hartree, '63; Srivastava et al., '65). The possibility that a small amount of hyaluronidase is released before motility ceases or acrosome detachment occurs cannot be precluded.

In Ham's F-10, hamster sperm remained alive during the incubation period, i.e., the percentage of motile sperm did not decrease, but no acrosome reactions occurred, nor was there any detectable release of hyaluronidase.

In contrast to the situation in Ham's F-10, hamster sperm incubated in serum released hyaluronidase whether or not a normal acrosome reaction occurred. This suggests that serum contains a factor which promotes the release of hyaluronidase from structurally intact acrosomes. This factor in serum may be albumin which Rogers and Morton ('73) have shown enhances hyaluronidase release from hamster sperm incubated in Tyrode's solution. Lewis and Ketchel ('72b) have reported a hyaluronidase releasing factor in post-ovulatory rabbit uterine fluid, but unlike the factor in serum, it is heat labile.

The surge of hyaluronidase release from sperm was complete in all sera by 1 hour of incubation. A normal acrosome reaction (detachment) occurred in some, but not all, sera between 3 and 4 hours of incubation. The fine structure of the acrosome was not changed (vesiculated) during or immediately after hyaluronidase release. This establishes that (1) when the normal acrosome reaction occurs in serum, it follows and is well separated in time from the surge of hyaluronidase release and (2) hyaluronidase release occurs in serum whether or not normal acrosome detachment is observed.

If it is accepted that capacitation precedes the normal acrosome reaction in time, these findings are in good agreement with data obtained from studies of rabbit sperm. Lewis and Ketchel ('73) have shown that hyaluronidase release is related to the completion of the process of capacitation, and Bedford ('64, '70) has demonstrated that no ultrastructural changes occur in rabbit sperm during capacitation. These data imply that the release of hyaluronidase may in some way be related to the capacitation process of both hamster and rabbit sperm.

Rogers and Morton ('73) have shown a surge of hyaluronidase release at 1 hour with very little increase at later times for hamster sperm incubated in heat pre-treated blood serum. Their results regarding hyaluronidase release by hamster sperm in serum are in good agreement with the data in our study. However, the assumed relationship between the normal acrosome reaction and release of hyaluronidase from sperm (Barros et al., '67; Rogers and Morton, '73) is not supported by our current findings.

After 1 hour of serum incubation, 40–45% of the total complement of mechanically extractable hyaluronidase is still present in the sperm. It is not clear when or if this residuum of hyaluronidase is released, since it was not detected in the supernatant following the normal acrosome reaction. Three possibilities exist: (1) the residual hyaluronidase may be tightly bound to the sperm head (e.g., to the inner acrosomal membrane) and carried to the egg at fertilization, (2) it may be represented by the finely granular contents of the acrosomal vesicle, or (3) it may be retained in the detached acrosomal ghost. In any case, it was not released in active form (except by sonication or freeze-thawing) under the conditions of these experiments.

The events leading up to and including a normal acrosome reaction are undoubtedly complex. Nevertheless, from the limited data available, a sequence for some of these events is known. Upon initiation of incubation in a suitable medium, hamster sperm release 55–60% of their hyaluronidase over an interval of time, $T_h$. 


Provided the medium is suitable, some process occurs over a time interval $T_v$ at the end of which the acrosome is observed to be vesiculated with the electron microscope and becomes detached.

If this sequence of events occurs in vivo, the size of the time intervals might change; however, for the hamster, it is known that the time required for a normal acrosome reaction to occur in vivo is about 3 to 4 hours (Yanagimachi, '66). If $T_h$ is approximately the same in vitro and in vivo, then the hyaluronidase released in vivo during $T_h$ would, for the most part, not be present in the vicinity of the cumulus. The hyaluronidase liberated during this time interval may assist in sperm passage through the cervical mucus, may stimulate an environmental change in the uterus, and/or may alter the sperm surface so as to allow membrane changes (the acrosome reaction) to take place. Austin ('48) has suggested that hyaluronidase released from individual sperm allows their passage through the follicle cell layers of the egg. This hyaluronidase would most likely correspond to the hyaluronidase which is not released during $T_h$.

While clarification of these postulations is required for the in vivo situation, the early partial release of hyaluronidase from hamster sperm in vitro may indicate that this enzyme has a bifunctional role in reproduction.

In summary, the relationship between hyaluronidase release and acrosome detachment has been studied in vitro for hamster sperm. In normal saline, the only unambiguous sequence of events is cell death followed by a degenerative acrosome reaction; some hyaluronidase release follows this acrosome reaction. In F-10, sperm do not release hyaluronidase, undergo acrosome detachment, or die during 4 hours of incubation. In serum, hyaluronidase release occurs well before and is independent of the normal acrosome reaction. A factor in serum apparently promotes the release of 55–60% of the sperm hyaluronidase extractable by sonication or freeze-thawing. It is not known when or if the remainder of the hyaluronidase is released. The sequence of some of the events leading up to and including the normal acrosome reaction was discussed for hamster sperm incubated in vitro, and the possible significance of these findings for the in vivo situation was considered.

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LITERATURE CITED


Lewis, B. K., and M. M. Ketchel 1972a Effects of female reproductive tract secretions on rab-


PLATE 1

EXPLANATION OF FIGURES

2–3 Sections through hamster sperm acrosomes at time zero from an F-10 incubation. The acrosome (a) contains finely granular material of moderate electron density. The plasma membrane is elevated (arrows) in places and occasionally broken; in the region of the equatorial segment (e), the elevation of the plasma membrane is quite pronounced. The outer acrosomal membrane, subjacent to the plasma membrane, is intact. n, nucleus, p, perforatorium. × 34,000.

4–5 Sections through hamster sperm acrosomes 75 minutes after incubation in F-10. The appearance of the acrosome proper (a) and equatorial segment (e) is the same as in the time zero incubation. The plasma membrane (arrows) is slightly elevated and breakage in the membrane in the equatorial region is evident in this micrograph. n, nucleus; p, perforatorium. × 34,000.

6–7 Sections through group 1 hamster sperm acrosomes at time zero from an incubation in heat pretreated baboon serum. The acrosome (a) appears identical to acrosomes from incubations done in F-10. The plasma membrane (arrows) is sometimes elevated and broken, particularly around the equatorial segment (e). n, nucleus; p, perforatorium. × 34,000.

8–9 Sections through group 1 hamster sperm acrosomes 75 minutes after incubation in heat pretreated baboon serum. The acrosome (a) appears identical to acrosomes from the three other incubation conditions. The finely granular material is still present in the acrosome. Leaching and swelling of the acrosome is not evident in either the acrosome proper (a) or the equatorial segment (e). The plasma membrane (arrows) is occasionally elevated and broken, especially in the equatorial region. n, nucleus; p, perforatorium. × 34,000.

10 Sections of hamster sperm (groups 2 and 3) judged to be dead at the time of fixation from an incubation done in heat pretreated baboon serum. The acrosome has become detached from one sperm resulting in exposure of the perforatorium (p). In the second sperm, the acrosome (a) is swollen and semidetached; most of the acrosomal contents have been lost. n, nucleus. × 9,000.