THE RELEASE OF HYALURONIDASE FROM CAPACITATING HAMSTER SPERMATOZOA

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Summary. The release of acrosomal hyaluronidase from hamster spermatozoa capacitating in test-tubes was measured. In order to do this, a colorimetric hyaluronidase assay of increased sensitivity was developed, based upon the finding that an albumin–substrate complex was hydrolysed much more rapidly than hyaluronic acid alone. To estimate the degree of morbidity of the sperm samples, cytoplasmic lactic dehydrogenase loss into the media was monitored. This loss did not correlate with the percentage of motile spermatozoa, but was almost identical to the amount of hyaluronidase appearing in the medium when the spermatozoa were incubated in non-capacitating Tyrode's solution. Sperm hyaluronidase release, but not lactic dehydrogenase loss, was elevated by the addition of serum albumin in the presence or absence of human serum dialysate. This hyaluronidase release was magnified when heat-detoxified human serum was used to capacitate the spermatozoa. The relationship of these findings to capacitation-associated acrosome vesiculation and detachment is discussed.

INTRODUCTION

The cumulus oophorus, an outer barrier surrounding most mammalian ova, can be dispersed by hyaluronidase (McClean & Rowlands, 1942). This enzyme is present within the acrosomes of spermatozoa (Austin, 1960). It is released in a soluble form by conditions causing the detachment of the acrosome from the spermatozoon (Swyer, 1947). Although acrosome loss and hyaluronidase release are most commonly correlated with the loss of sperm motility (Blom, 1945; Kooner & Ludwick, 1971), acrosome-free, motile spermatozoa have been observed after sperm capacitation (Barros & Austin, 1967). Capacitated rabbit spermatozoa require the presence of ova for acrosome loss to occur (Bedford, 1970), but hamster spermatozoa do not (Yanagimachi, 1969). Because the acrosome is perforated by vesiculation sometime before it becomes detached (Barros, Bedford, Franklin & Austin, 1967), it is of interest to know whether the time of hyaluronidase release coincides with that of acrosome vesiculation or detachment.

A serum-based *in-vitro* system has recently been developed that capacitates

sufficient numbers of hamster spermatozoa to investigate biochemically (Morton, Rogers & Chang, 1973). Here, we report the results in our measurements of hyaluronidase release by hamster spermatozoa incubated under *in-vitro* conditions causing capacitation.

MATERIALS AND METHODS

Sperm incubation conditions

Our methods for sperm capacitation and *in-vitro* fertilization in the golden hamster are described in detail elsewhere (Morton *et al.*, 1973). Hamster spermatozoa from the cauda epididymidis were suspended in Tyrode's solution at a concentration determined with a haemocytometer to be 2 to 4×10^7 /ml. Of this suspension, 0.5 ml was added to 0.5 ml of the following five media: (1) Tyrode's solution; (2) Tyrode's solution with 10 mg crystalline human serum albumin (Nutritional Biochemical Corporation); (3) heat-detoxified human serum; (4) the dialysate of heat-detoxified human serum; or (5) this dialysate with 10 mg of the above albumin. These suspensions were incubated in stoppered 15-ml Corex tubes for 4 hr at 37° C. Media 3 and 5 capacitate the spermatozoa within this time period.

Heat-detoxified serum, derived from blood drawn without anticoagulants, was prepared by heating human serum at 56° C for 30 min, passing it through a 0.3- μ m millipore filter and adjusting the pH to 7.5 with 0.2 N-HCl. The serum dialysate was prepared by dialysis of this serum against an equal volume of Tyrode's solution for 18 to 20 hr at 4° C.

Assay for sperm capacitation

To determine whether the spermatozoa were capacitated, aliquots of sperm suspensions incubated for 4 hr were combined with hamster ova in Tyrode's solution under mineral oil in small watch-glasses. The ova were examined 1 to 2 hr later under phase contrast at a magnification of $\times 400$. If capacitation had previously occurred, spermatozoa with swollen heads were found within the ova.

Assay of sperm motility

Samples (approximately 25 μ l) from the sperm incubation tubes were placed at room temperature on a microscope slide without a cover-slip. The samples were immediately evaluated under phase contrast at a magnification of $\times 100$ both for percentage motile spermatozoa and rate of movement on an arbitrary scale of 0 to 10.

Assay for lactic dehydrogenase

The 1-ml sperm suspensions were centrifuged at 10,000 *g* for 5 min at 2° C to remove the spermatozoa. The supernatant fraction (0.3 ml) was assayed for lactic dehydrogenase activity (Morton & Albagli, 1973). The Calbiochem LDH-L kit employing the spectrophotometric assay of Wacker, Ulmer & Vallee (1956) which follows the appearance of NADH at 340 nm was used. To obtain a value for the total lactic dehydrogenase content of the spermatozoa, the

sperm suspension was treated with Triton X-100 at a final concentration of 1% before the centrifugation step to release the enzyme.

Assay for hyaluronidase

Sperm supernatant fractions (0.2 ml), prepared as for the lactic dehydrogenase assay and also containing 2 mg crystalline human serum albumin, were added to 0.3 ml hyaluronic acid reagent. This contained 0.36 mg hyaluronic acid (Sigma, grade III-P, potassium salt from human umbilical cord) in 0.15 M-NaCl and 0.1 M-acetate buffer, pH 3.5, unless otherwise indicated. After incubation for 1 hr at 37° C, the hydrolysis was stopped by the addition of 0.1 ml of 0.8 M-potassium tetraborate, pH 9.1. Total sperm hyaluronidase content was determined by adding Triton X-100 to a final concentration of 1% before centrifugation. In some cases, 0.2 ml commercial bovine testicular hyaluronidase (Nutritional Biochemical Corporation, 460 National Formulary Units/mg) was substituted for the sperm supernatant fraction in the amounts indicated.

The extent of substrate hydrolysis by hyaluronidase was determined colorimetrically by measuring the appearance of *N*-acetylglucosamine end-groups, using the method described by Reissig, Strominger & Leloir (1955). Colour formation was linear up to 150 nmol *N*-acetylglucosamine standard.

RESULTS

Evaluation of hyaluronidase assay

Under our hyaluronidase assay conditions, which were quite similar to those of Males & Turkington (1970), *N*-acetylglucosamine end-group colour formation was proportional to the amount of bovine testicular hyaluronidase added over the 3- to 100- μ g range tested. Since serum albumin is required for the capacitation of spermatozoa *in vitro*, the effect of crystalline human serum albumin upon the assay was investigated. A fivefold stimulation (Text-fig. 1) of hyaluronic acid hydrolysis occurred as the concentration of albumin in the incubation medium was increased beyond 0.3 mg/ml. This was paralleled by the appearance of a white turbidity which disappeared when the pH was raised by the addition of the tetraborate 'stopping reagent'.

The effect of albumin upon hydrolysis was further investigated by varying the hyaluronic acid concentration in the presence and absence of 2 mg albumin/ml. The results shown in Text-fig. 2 indicate that in the absence of albumin, the amount of *N*-acetylglucosamine colour formation was fairly constant above 0.1 mg hyaluronic acid/ml. In the presence of albumin, however, colour formation was highly dependent upon substrate concentration.

Since, theoretically, albumin could stimulate hyaluronic acid hydrolysis by modification of either the enzyme or its substrate, we performed experiments to determine which was the independent variable. In Table 1, the effects of the molecular ratios of albumin (mol. wt 69,000; Tanford, Kawahara & Lapanje, 1967) to hyaluronidase (mol. wt 61,000; Borders & Raftery, 1968), and of albumin to hyaluronic acid (mol. wt about 400,000; Tolksdorf, 1954) upon the stimulation of hydrolysis are compared. It may be seen that whether or not a given albumin-hyaluronidase ratio was present when the stimulation occurred

depended on whether the albumin-hyaluronic acid ratio was above 8. From these data, we concluded that albumin acts not upon hyaluronidase but upon hyaluronic acid to cause the observed three- to tenfold stimulation in the rate of hydrolysis. We found this stimulus in rate to be immediate and linear over the range of the assay.

To take advantage of the increased sensitivity that the presence of albumin gave to the hyaluronidase assay, the albumin-hyaluronic acid molecular ratio in the assay was maintained at about 60, unless indicated otherwise. This was often accomplished automatically, due to the albumin content of the sperm capacitation media.

TEXT-FIG. 1. The effect of serum albumin upon the rate of hyaluronidase activity. The conditions were as described in the Materials and Methods section. The testicular hyaluronidase concentration was 50 μ g/tube. Crystalline human serum albumin was used. The incubation time was 30 min.

Since we, and others, use 56° C-treated sera for sperm capacitation *in vitro*, it was necessary to determine whether the high molecular weight hyaluronidase-inhibitors reported to be present in sera (Mathews & Dorfman, 1955) would interfere with the assay. The 56° C-treated serum was found to be as effective as albumin in stimulating hyaluronic acid hydrolysis by testicular hyaluronidase, provided the albumin-hyaluronic acid ratio was above 6. Thus, it seems likely that the high molecular weight inhibitors were denatured by the heat treatment of the sera.

TEXT-FIG. 2. The effect of albumin upon hyaluronidase activity in the presence of increasing concentrations of hyaluronic acid. The conditions were as described in the Materials and Methods section. The testicular hyaluronidase concentration was 35 μ g/tube. ○, 2 mg/ml crystalline human albumin present. △, no albumin present. The incubation time was 60 min.

Table 1. The effect of the molecular ratio of albumin to hyaluronidase and to hyaluronic acid upon rates of hydrolysis

	Comparison 1		Comparison 2					Comparison 3		
Alb./hyal.*	1	1	1	3	10	30	100	10	30	100
Alb./h.a.	5	10	0.8	2.5	8.4	25	84	1.7	5.0	17
Stimulation†	—	+	—	—	+	+	+	—	—	+

* Alb = albumin; hyal = hyaluronidase; h.a. = hyaluronic acid. In Comparison 1, 30 μ g hyal. 180 μ g h.a. and 0.3 mg alb. were used. For Comparison 2, see Text-fig. 2. In Comparison 3, 10 μ g hyal., 360 μ g h.a. and 0.1, 0.3 and 1.0 mg alb. were used. For other conditions see Materials and Methods section.

† Stimulation was defined as an increase in the rate of hydrolysis by hyaluronidase greater than 100%.

Application of assay to sperm capacitation in vitro

Our goal was to measure the release of hyaluronidase from the spermatozoa into the media over the 4-hr period of the capacitation incubation. To give the results meaning, the total hyaluronidase content of the capacitating sperm samples was needed. It has been shown that 0.02% Triton X-100 caused the

complete release of soluble lactic dehydrogenase from hamster spermatozoa (Morton & Albagli, 1972). At 0.04% Triton, no acrosomes were visible under the light microscope. Gibbons & Gibbons (1972) found that 0.25% Triton removed all membranes from sea-urchin spermatozoa. Here, 1% Triton X-100 was used to dissolve the acrosome and release hyaluronidase. This concentration solubilizes the mitochondria as well but does not inhibit hyaluronidase activity in the albumin-containing assay. When we added 1% Triton X-100 to epididymal hamster spermatozoa, centrifuged them and assayed the supernatant in the presence of albumin for hyaluronidase activity, we found 3600 ± 960 nmol *N*-acetylglucosamine end-groups were formed/hr/ 10^8 cells. This value was the average of fourteen experiments. In each of the subsequent experiments, the Triton-releasable sperm lactic dehydrogenase was determined, assigned as 100% of the sperm hyaluronidase content and the hyaluronidase released from spermatozoa not extracted with Triton was plotted relative to this.

TEXT-FIG. 3. Appearance of extracellular hamster sperm lactic dehydrogenase under capacitating and non-capacitating conditions (see Materials and Methods section for conditions). Numbers next to data points refer to the percentage of motile spermatozoa and rate of movement (0 to 10). Δ, Spermatozoa incubated in Tyrode's solution (average of seven experiments). O, Spermatozoa incubated in serum dialysate and albumin (average of five experiments).

To determine the extent of cell death occurring during the capacitation period, the appearance of cytoplasmic lactic dehydrogenase outside the spermatozoa was measured. We had previously found that 0.02 to 3% Triton X-100 did not inhibit this enzyme but released a constant amount of lactic dehydrogenase from hamster spermatozoa into the media (Morton & Albagli, 1972).

Text-figure 3 shows that the amount of lactic dehydrogenase activity appearing outside spermatozoa incubated in Tyrode's solution increased about 28% over the 4-hr incubation period. When serum dialysate and albumin were also present, about 16% of the Triton-extractable lactic dehydrogenase was lost from the cells. This loss of lactic dehydrogenase was less than the loss in the percentage of motile spermatozoa over the 4-hr period. The latter was 60% in Tyrode's solution and 50% in serum dialysate-albumin. As reported elsewhere, these capacitation conditions did not greatly increase sperm survival but did maintain an extremely high rate of motility (Morton & Chang, 1973).

Although it is conceivable that the sperm acrosome could remain intact in the presence of a disintegrating plasma membrane, the data of Text-fig. 4(a), lower curve, suggest this is not the case. There, the 22% of hyaluronidase lost from spermatozoa incubated in Tyrode's solution is seen to be qualitatively similar to the loss of lactic dehydrogenase (Text-fig. 3). In addition, the extracellular levels of both enzymes at the beginning of the incubation were essentially identical. If albumin was omitted from the hyaluronidase assay, the apparent activity of the sperm enzyme was reduced three- to tenfold, in agreement with our observation on purified testicular hyaluronidase. This was expected because the testicular enzyme appears to originate from the spermatozoa (Males & Turkington, 1970).

TEXT-FIG. 4. The effect of albumin and serum dialysate upon the release of acrosomal hyaluronidase from hamster spermatozoa (see Materials and Methods section for conditions). (a) Spermatozoa in Tyrode's solution: ●, plus 10 mg albumin/ml; Δ, no albumin. (b) Spermatozoa in serum dialysate: ○, plus 10 mg albumin/ml; ▲, no albumin.

Completely independent of this, when hamster spermatozoa were incubated in Tyrode's solution containing 10 mg/ml crystalline human serum albumin, significantly more hyaluronidase was released from the cells (Text-fig. 4, upper curve). This is consistent with another experiment where a hamster sperm suspension incubated for 4 hr in Tyrode's solution and albumin was able to disperse the cumulus masses surrounding hamster ova, while a similar suspension incubated only in Tyrode's solution was not.

When serum dialysate is added to the albumin-containing Tyrode's solution, the spermatozoa not only remove the cumulus masses around the ova but fertilize them as well (Yanagimachi, 1970; Morton *et al.*, 1973). Yet the presence of serum dialysate in the absence of albumin (Text-fig. 4b, lower curve) did not increase the release of hyaluronidase by spermatozoa and only slightly increased hyaluronidase release in the presence of albumin (upper curve) above that of spermatozoa incubated in Tyrode's solution and albumin (Text-fig. 4a).

This contrasts with the effect of heat-detoxified human serum, shown in Text-fig. 5, upon hyaluronidase release. Approximately one fourth more hyaluronidase appeared in the medium from hamster spermatozoa incubated in these capacitating conditions than from those incubated in Tyrode's solution.

This is consistent with our earlier report that after a 4-hr incubation in this medium, about 50% of the spermatozoa were motile and more than half of these were without acrosomes (Morton *et al.*, 1973).

TEXT-FIG. 5. The effect of heat-detoxified serum upon the release of acrosomal hyaluronidase from hamster spermatozoa (see Materials and Methods section for conditions). Δ , Spermatozoa in Tyrode's solution; O, spermatozoa in heat-treated serum.

DISCUSSION

The ability of serum albumin to form an insoluble complex with hyaluronic acid has long been known and forms the basis of at least four different hyaluronidase assays (Tolksdorf, 1954). Our finding that testicular hyaluronidase hydrolysed this complex up to ten times more rapidly than hyaluronic acid alone is apparently new.

Without the increase in the sensitivity made possible by this phenomenon, it would have been very difficult to have measured hyaluronidase levels in a sperm-capacitating system. The non-specific protein stimulus to hyaluronidase activity, mentioned by Meyer & Rapport (1952) for their assay, appears to be different from the albumin effect reported here because it could be produced by gelatin, an agent which has no effect upon hydrolytic rates in this assay. The fact that the average molecular weight of umbilical cord hyaluronic acid is six times that of albumin, and that six times as much albumin as hyaluronic acid was required before hydrolysis stimulation occurred, while interesting, may only be coincidental.

The hyaluronidase content we report here for hamster spermatozoa is difficult to compare to that of spermatozoa from other species. This is because the turbidimetric methods used in the past describe hyaluronidase activity more in physical than chemical terms when compared to the present colorimetric assay.

Measuring the extracellular appearance of the cytosol marker enzyme, lactic dehydrogenase, appears to be a more quantitative and less artifact-prone method for evaluating the integrity of the sperm plasma membrane than vital stain methods. The great adsorptive affinity of albumin for vital stains precluded their use here. Ultrasonic treatment released a greater quantity of lactic dehydrogenase than detergent treatment did (B. J. Rogers and B. Morton, unpublished observations), suggesting that there is a bound lactic dehydrogenase isozyme (Harrison & White, 1972) among the six reported for this enzyme (Goldberg, 1963), which is not released by detergent.

It is clear from Text-fig. 3 that loss of motility need not be correlated with cell lysis. Thus, although over 50% of the spermatozoa in these experiments were immotile after incubation for 4 hr, only half of these were sufficiently moribund to lyse, assuming the loss of cytosol into the media occurs on an all-or-none basis. How soon sperm lysis follows loss of motility is at present unknown; however, Garbers, Lust, First & Lardy (1971) activated the motility of quiescent bull spermatozoa with caffeine, indicating that lysis is not always immediate.

It is interesting that spermatozoa incubated in Tyrode's solution lost hyaluronidase from their acrosomes as soon as, and to the same extent as, the plasma membrane lost its ability to retain lactic dehydrogenase. Even more interesting was the finding that capacitating conditions which were less harmful to plasma membrane integrity, as judged by extracellular lactic dehydrogenase appearance, substantially increased the appearance of hyaluronidase in the medium. This is biochemical evidence that vesiculation of the sperm acrosome (Barros *et al.*, 1967) occurs during capacitation in such a way as to release soluble acrosomal contents without loss of sperm cytosol and consequently of motility.

Detailed studies of the relationship of acrosome vesiculation, hyaluronidase loss and acrosome detachment await future investigation, but the following conclusions are suggested by the work presented here. The elevated release of sperm hyaluronidase in the presence of albumin occurs without a corresponding release of cytosol and therefore is not related to increased sperm senescence. The additional presence of the small molecules of serum increases the release of hyaluronidase by albumin, slightly in the case of serum dialysate and substantially with the more concentrated heat-treated serum.

We had previously found that up to 60% of motile spermatozoa incubated for 4 hr in heat-treated serum were without acrosomes compared to up to 6% in serum dialysate albumin and none in Tyrode's solution (Morton *et al.*, 1973). Yanagimachi (1969) reported that albumin in Tyrode's solution also caused a small percentage of motile spermatozoa to lose their acrosomes. Thus, in the case of the spermatozoa incubated in serum, the amount of hyaluronidase loss and acrosome detachment at 4 hr was in good agreement. At 4 hr, however, acrosome detachment lagged behind hyaluronidase release for spermatozoa incubated with albumin in Tyrode's solution or serum dialysate. In the latter case, the percentage of acrosome-free, motile spermatozoa was substantially increased by an additional 3-hr incubation period (Morton *et al.*, 1973). It therefore appears that the detachment of the acrosome occurs a variable length of time after hyaluronidase is released. This gives meaning to our observation

that although spermatozoa capacitated in the serum dialysate-albumin medium show much less acrosome loss, they are as effective in fertilizing as are spermatozoa capacitated in heat-treated serum (Morton *et al.*, 1973). It also suggests that the percentage of acrosome-free, motile spermatozoa may not be an accurate criterion for estimating the efficiency of the capacitation of hamster spermatozoa *in vitro* because some acrosome-bearing spermatozoa can be capacitated.

The results presented here differ from those of Kooner & Ludwick (1971), who reported that hyaluronidase release from bull and rabbit spermatozoa in the presence of uterine fluid or β -amylase was correlated with cell morbidity. Although this may be due to species differences, we believe it to be safer to investigate phenomena related to capacitation in systems that can be monitored for capacitative success.

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