Effects of Female Reproductive Tract Secretions on Rabbit Sperm
I. Release of Hyaluronidase *in Vitro*[1] (36858)

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(Introduced by B. C. Wexler)

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It is well known that hyaluronidase is a component of mammalian sperm. Any hyaluronidase found in semen is the consequence of its loss or release from sperm (1, 2). Sperm will release hyaluronidase when incubated in either distilled water or salt solution (3, 4). The amount of hyaluronidase released into these media seems to depend on the concentration of the sperm. Swyer (4) demonstrated that the loss of hyaluronidase by rabbit sperm in Baker's solution could be reduced by the prior addition of bovine hyaluronidase to the solution. Based on his results, he postulated that the sperm in the uterus would lose small amounts of hyaluronidase and this, in turn, would prevent additional release of the remaining hyaluronidase. Such a mechanism would permit a slow and progressive loss of hyaluronidase as the sperm passed through the uterus into the oviduct. Consequently, the amount of hyaluronidase in solution when the sperm reached the tubal ampulla, would be adequate to disperse the cumulus cells surrounding the freshly ovulated ova.

In vitro hyaluronidase dissolves the matrix which binds the mass of cumulus cells surrounding mammalian ova. Consequently, it has been proposed that this is the *in vivo* function of the sperm hyaluronidase. Austin (5) has also proposed that the release of hyaluronidase occurs as a result of the process of capacitation. There is little doubt that the acrosomal contents are lost during or immediately following the vesiculation and fusion of the plasmalemmal and outer acrosomal membranes (6, 7). This "acrosome reaction" is believed to follow the process of capacitation. However, the events comprising capacitation are yet to be elucidated. Consequently, there are still questions concerning the timing and locus of hyaluronidase release within the female reproductive tract. Is hyaluronidase released following ejaculation or capacitation, and does it occur in the uterus or in the fallopian tube? This report is the first of a series of investigations concerning the dynamics of sperm hyaluronidase release, during *in vitro* incubation in various media, including female reproductive tract secretions.

Materials and Methods. In each experiment rabbit sperm were collected from 3 to 5 mixed breed rabbits of proven fertility, by means of an artificial vagina (8). The semen samples were pooled, divided into aliquots, and centrifuged at 600*g* for 10 min. The seminal plasma was decanted and the sperm pellet resuspended in the incubation medium to an estimated concentration of 25×10^6/ml.

Sperm suspensions were incubated under mineral oil at 37°. At intervals, usually every 2 hr, 0.5 ml aliquots were removed from the incubation, sperm motility was assessed by phase contrast microscopy and the aliquot was centrifuged at 55,000*g* for 30 min. The supernatant was decanted and assayed for enzyme activity.

To obtain uterine fluid, mature female rabbits were laparotomized while in estrus, and both uterine cornua were ligated near the

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TABLE I. Stability of Hyaluronidase in Tyrode's Solution and in Uterine Fluid.

Hyaluronidase dissolved in Tyrode's solution (0.5 mg/ml)							Hyaluronidase dissolved in uterine fluid (0.5 mg/ml)						
Temp (°)	N[a]	Length of incubation	NFU[b]/ml Start	NFU/ml finish	% Change	$t_{½}$	Temp (°)	N	Length of incubation	NFU/ml Start	NFU/ml finish	% Change	$t_{½}$
37	3	30 min	161	169	+5		37	2	2 hr	134	123	—8	
		3 hr	161	146	—9.3				6 hr	134	107	—20	
		20 hr	161	115	—28.7				25 hr	134	66	—51	
						47.1 hr							24.2 hr
60	3	15 min	161	137	—15		60	2	60 min	134	81	—39	
		60 min	161	114	—28				120 min	134	57	—58	
						2.18 hr							1.57 hr
24	2	1 hr	133	132.5	NC[c]		24	2	2 hr	134	127	—5	
		3 hr	133	132	NC				6 hr	134	121	—10	
		18 hr	133	134	NC				25 hr	134	80	—40	
													35.5 hr
—20	2	20 hr	182	179	NC								
		20 days	134	121	—10								

[a] N = number of different incubations.
[b] NFU = National Formulary units.
[c] NC = no change.

TABLE II. Stability of Rabbit Sperm Hyaluronidase in the Supernatant from Sperm Incubations.

Medium	N[a]	Temp (°)	Length of incubation (hr)	NFU[b]/ml Start	NFU[b]/ml Finish	% Change
Uterine fluid	1	24	2	111[c]	85	—23
	2	24	6	245	163	—33
	2	4	16	119	109	—8
	2	—20	20	103	49	—52
Tyrode's solution	2	4	16	214	202	—5
	2	—20	24	208	164	—21

[a] N = number of different sperm incubations.
[b] NFU = National Formulary units.
[c] NFU values are averages from replicate determinations in each incubation.

cervical junction. After 7–10 days the accumulated uterine fluid was removed with a syringe. Fluid showing gross contamination with blood was discarded. In certain of the experiments 75 IU of human chorionic gonadotropin (HGG)[3] was administered intravenously to induce ovulation. Fluid was then collected either before or after ovulation as required by the experimental design.

The uterine fluid collected for each group of 3–5 does was pooled, filtered through a sterile 0.8 μm membrane filter and frozen at —20° until use. Protein concentration of each pool of uterine fluid was determined by a microbiuret method (9). Before use, the fluid was warmed at 37° and equilibrated with 5% CO_2 in air to pH 7.6 to 7.8. Just prior to the addition of the sperm 500 IU penicillin, 500 μg streptomycin (GIBCO) and 5.4 mg glucose were added/ml of medium.

Hyaluronidase was measured by the fluorometric assay of Guilbault, Kramer and Hackley (10). Bovine testicular hyaluronidase (Sigma), 370 national formulary units (NFU)/mg, was used as the standard.

Where applicable, data was subjected to statistical analysis by the method of the analysis of covariance (11).

Results. Hyaluronidase stability in solution. In order to determine whether or not the rate of hyaluronidase release from sperm could be measured by successive determinations in the supernatant, without being affected by natural inactivation, bovine testicular hyaluronidase was incubated (0.5 mg/ml) in either Tyrode's solution or uterine fluid at specific temperatures. The results (Table I) indicate that at 37° the half-life of hyaluronidase in uterine fluid is about 50% of that in Tyrode's solution (24.2 hr vs 47.1). At 24° the hyaluronidase in Tyrode's solution shows no change in activity after 18 hr, while in uterine fluid there is a 40% loss after 25 hr. This indicates that there is a certain amount of natural proteolysis in uterine fluid which is not present in Tyrode's solution. The uterine fluid *per se* shows no hyaluronidase activity.

Supernatants from sperm incubations, which contained hyaluronidase released by the incubated sperm, were held at room temperature or below (Table II). At 24°, the hyaluronidase activity in uterine fluid supernatant declined 33% in 6 hr; a rate of inactivation at least 3 times that found in uterine fluid without sperm. The hyaluronidase activity in the supernatant from Tyrode's solution also declined, though not as rapidly as in the uterine fluid supernatant. Although uterine fluid itself will inactivate hyaluronidase, the prior incubation of sperm in the uterine fluid results in a considerable increase in the rate of enzymatic inactivation.

Sperm incubations. When sperm are suspended in distilled water, there is an almost immediate cessation of motile activity. Incubation of sperm in distilled water, therefore, reflects the release of hyaluronidase by dead or dying sperm. Hyaluronidase activity in

[3] HCG (APL) was kindly provided by Dr. John B. Jewell, Ayerst Laboratories, New York.

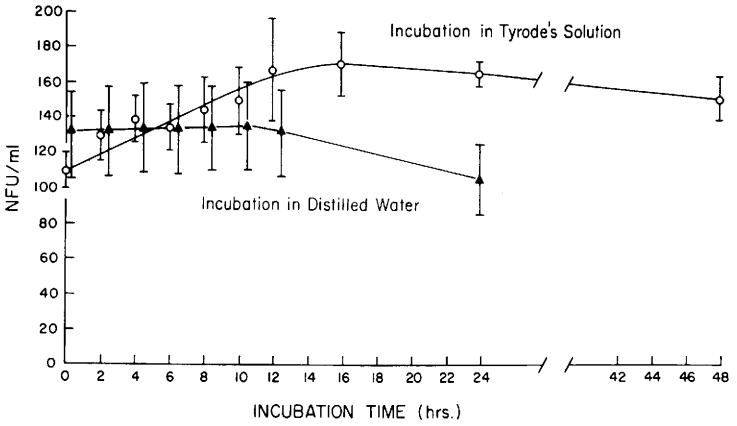

FIG. 1. Hyaluronidase released from sperm incubated in distilled water or in Tyrode's solution. Hyaluronidase activity is expressed in terms of national formulary units (NFU) per milliliter of incubation medium. Each point is the mean of 6 to 8 experiments, ± the standard error.

distilled water is relatively constant for the first 10–12 hr of the incubation, after which time there is a gradual decline (Fig. 1). Since there is a natural degradation of hyaluronidase in solution, the stable level of activity during the first 10–12 hr indicates that, after the initial release of hyaluronidase—which occurs as the sperm are resuspended—there is a gradual release of hyaluronidase which approximates its rate of degradation. This is followed by a slower rate of release for the next 12 hr (ratio of release to degradation <1) concomitant with a loss of activity.

For the first 12–14 hr of sperm incubation in Tyrode's solution there is a very gradual increase in hyaluronidase activity, followed by a very gradual decline (Fig. 1). Sperm motility in Tyrode's solution remains at high levels, often for as long as 24 hr. Thus, the maximum hyaluronidase activity is measurable when the majority of the sperm appear most active.

In sperm incubations in uterine fluid collected after ovulation had occurred, the hyaluronidase activity shows an increase from the initial value to a definite maximum (Fig. 2). This is followed by a rapid decline in measurable activity. The length of time that the sperm must be incubated for the hyaluronidase activity to reach a maximum value is related both to the length of time the uterus was ligated and to the time which elapsed between the HCG injection and the collection of the uterine fluid. Reduction of the length of the ligation period or shortening the interval between the time of HCG injection and fluid collection, or both results in shifting the timing of the attainment of maximum hyaluronidase activity closer to the beginning of the incubation period.

When sperm were incubated in uterine fluid collected prior to ovulation the hyaluronidase activity follows a much different pattern than that seen in the incubations in postovulatory uterine fluid (Fig. 3). The maximum hyaluronidase activity was measured in the solution at the start of the incubation. From that point on there was a very rapid decrease in hyaluronidase activity. This rate of enzymatic inactivation results in a half-life of about 3 hr. There was no difference between preovulatory fluid from HCG-primed does and fluid from nonprimed does. Consequently, the data from both groups was combined and is collectively referred to as preovulatory uterine fluid.

Sperm motility. Observation of sperm incubated in postovulatory uterine fluid showed that motility remains high (60–80%) for 3–4 hr following the maximum hyaluronidase activity. There is a rapid decrease in motility leading to a total cessation of activity after 16–20 hr of incubation. In addition, head to head agglutination of sperm was observed with many aggregates containing 50 or more sperm as well as aggregates with only a few

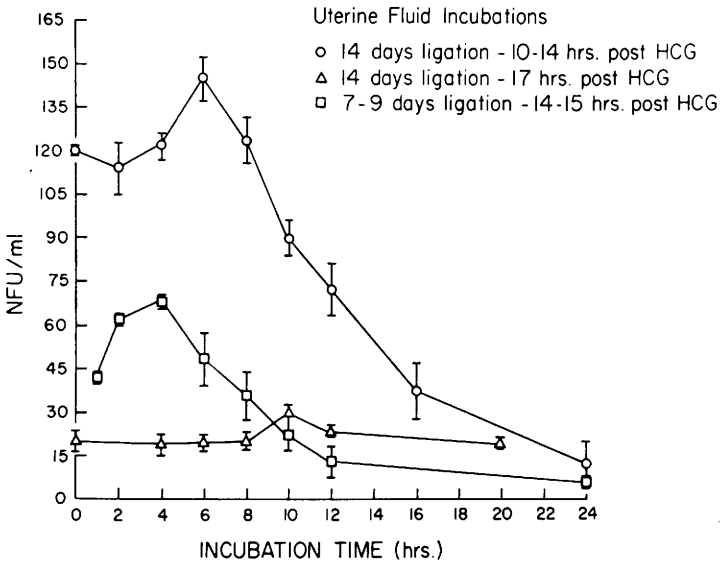

FIG. 2. Hyaluronidase released from sperm incubated in postovulatory uterine fluid. Hyaluronidase activity is expressed in terms of national formulary units (NFU) per milliliter of incubation medium. Each point is the mean of 4 to 6 experiments, ± the standard error.

sperm. Relatively few (10–20%) of the active sperm in uterine fluid exhibit individual and strong linear motion. It is these highly active sperm which can no longer be found following the appearance of the maximum hyaluronidase activity in the supernatant.

Discussion. In considering the results of these *in vitro* incubations there are several factors which make comparison of the absolute values of hyaluronidase from one experiment to another, difficult, if not impossible. The hyaluronidase content of seminal plasma has been shown to be proportional to the number of sperm present in the ejaculate. As the sperm content of each ejaculate pool was quite variable we attempted to standardize the number of sperm in each incubation. However, the single washing did not permit complete removal of the seminal plasma, thus, the initial values for hyaluronidase in each incubation reflect the incomplete removal of seminal plasma as well as the variable sperm concentration in the original ejaculate. This source of error, coupled with the inherent error of counting sperm by the hemocytometric method, makes it impossible to standardize the initial hyaluronidase values in each incubation. Consequently, it is the relative changes in hyaluronidase activity which reflect the effectiveness of uterine fluid in releasing sperm hyaluronidase.

The rate of inactivation of hyaluronidase in Tyrode's solution and in uterine fluid follows a logarithmic pattern of decay with time. The calculated half-lives are sufficiently different from the results derived from sperm incubations that we do not consider natural inactivation of hyaluronidase activity to be a factor in the uterine fluid incubations. A logarithmic decay pattern was observed only in the preovulatory uterine fluid with a half-life considerably shorter than that found in the hyaluronidase incubations indicative of active proteolysis.

The results of these experiments demonstrate that freshly ejaculated rabbit sperm incubated in preovulatory uterine fluid do not release or secrete hyaluronidase. Further, the data indicates that the release of hyaluronidase in postovulatory uterine fluid and in Tyrode's solution is not simply a result of a dead or dying sperm population (3, 12). Instead, the maximum hyaluronidase activity was measured at times when the sperm populations were exhibiting very high levels of activity.

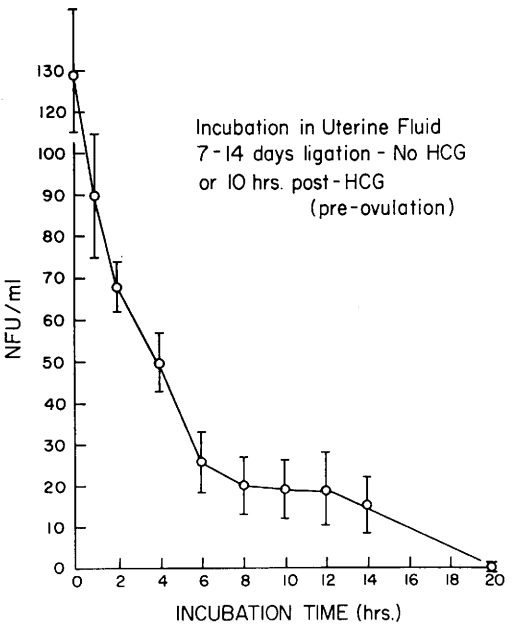

FIG. 3. Hyaluronidase released from sperm incubated in uterine fluid categorized as preovulatory. Hyaluronidase activity is expressed in terms of national formulary units (NFU) per milliliter of incubation medium. Each point is the mean of six experiments, ± the standard error.

The evidence demonstrating that the sperm incubated in preovulatory uterine fluid retain acrosomal hyaluronidase is not consistent with the proposal by Swyer (4) that hyaluronidase is retained in rabbit sperm as a result of a "balancing effect" with hyaluronidase in the medium. Rather, it indicates that preovulatory uterine fluid lacks a factor, or factors, found in postovulatory uterine fluid, which can cause active release of hyaluronidase by the sperm.

Although these measurements were made in uterine fluid there is no evidence to indicate that a similar pattern may not occur in fluid in the oviduct. However, there is a considerable body of evidence which does suggest that uterine fluid and oviduct fluid in the rabbit are quite similar, both biochemically and physiologically, even following periods of tract ligation (13–17).

The retention of hyaluronidase by rabbit sperm until ovulation occurs is certainly consistent with the *in vivo* time sequence of events leading to fertilization in this induced ovulator. Considering that a 10 to 11 hr interval passes between coitus and ovulation, the release of hyaluronidase prior to ovulation would probably result in its complete dilution and inactivation before the sperm came into contact with the ova. The retention of hyaluronidase in preovulatory uterine fluid, and its release from sperm in postovulatory uterine fluid, or possibly in fallopian tube fluid in the proximity of the ova, would seem to support the proposal that the function of hyaluronidase is related to penetration of the ovum or its cellular investments.

Summary. The release of hyaluronidase by rabbit sperm was measured during *in vitro* incubation, in: (a) distilled water, (b) Tyrode's solution, and (c) rabbit uterine fluid. There was no apparent release of hyaluronidase in fluid obtained from the uterus of an estrus rabbit and collected prior to HCG-induced ovulation. In fluid obtained after HCG-induced ovulation, increases in hyaluronidase activity occurred, indicating hyaluronidase release by the sperm. The peak of maximum hyaluronidase activity was related to the timing of the fluid collection. In contrast, no discernible peak of hyaluronidase activity occurred when sperm were incubated in Tyrode's solution or in distilled water. Postovulatory uterine fluid appears to actively promote the release of hyaluronidase from sperm.

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