Effects of Female Reproductive Tract Secretions on Rabbit Sperm
II. Control of Sperm Hyaluronidase Release[1] (36859)

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Rabbit sperm incubated *in vitro* release hyaluronidase into solution at different rates in different media (1). Rabbit uterine fluid can directly influence this rate of release and degradation of sperm hyaluronidase depending on whether the fluid was collected before or after ovulation. The experiments described in this report were carried out to further explore this characteristic of uterine fluid and its relationship to sperm and sperm hyaluronidase.

Methods and Materials. The methods for sperm collection, incubation, and enzyme analysis have been described (1). Proteolytic activity was measured by the degraded hemoglobin method of Press, Porter, and Cebra (2). The enzyme–hemoglobin mixture was incubated at 37° at pH 7.4. Proteolytic activity was calculated from the change in extinction coefficient ($\Delta\epsilon$) resulting from hydrolysis of hemoglobin during a 30 min incubation. These values are expressed as the total activity contained in 1 ml of the original sperm incubation.

Uterine fluid was fractionated to determine if the hyaluronidase release effect could be correlated with a specific uterine macromolecule. Pooled uterine fluid collected as described previously (1), was concentrated by ultrafiltration in an Amicon cell using a UM-2 Diaflo membrane, and then dialyzed against a 0.15 *M* Tris/glycine/saline buffer, pH 8.3. The concentrated sample was applied to a Biogel P100 gel filtration column (2.5 × 100 cm) with a bed volume of approximately 495 ml. The flow rate was 10–11 ml/hr and the eluent was monitored at 280 nm using an LKB Uvicord II. The protein fractions from this column were dialyzed against Tyrode's solution and then concentrated by ultrafiltration before freezing.

Sperm were also incubated in postovulatory uterine fluid containing soybean trypsin inhibitor (Worthington Biochemicals) 10 μg/ml, or progesterone (Sigma) at various concentrations.

Results. Fluid from ovariectomized females. Two groups of three does each were first unilaterally ovariectomized and then both cornua were ligated (single cervical ligature on each horn) 45 days later. Seven to 10 days following uterine ligation each doe received an intravenous injection of human chorionic gonadotropin (HCG)[3] and fluid was aspirated from the intact (control) and ovariectomized horns 14–16 hr later. Sperm incubations in the control uterine fluid produced an increase in hyaluronidase activity after 4 hr incubation and this was followed by a decrease in hyaluronidase activity throughout the balance of the incubation (Fig. 1). The fluid from the ovariectomized horn of the same rabbits caused a continuous decrease in sperm hyaluronidase throughout the period of the incubation. Thus the fluid from the ovariectomized side of the female lacks the capacity to cause sperm hyaluronidase release.

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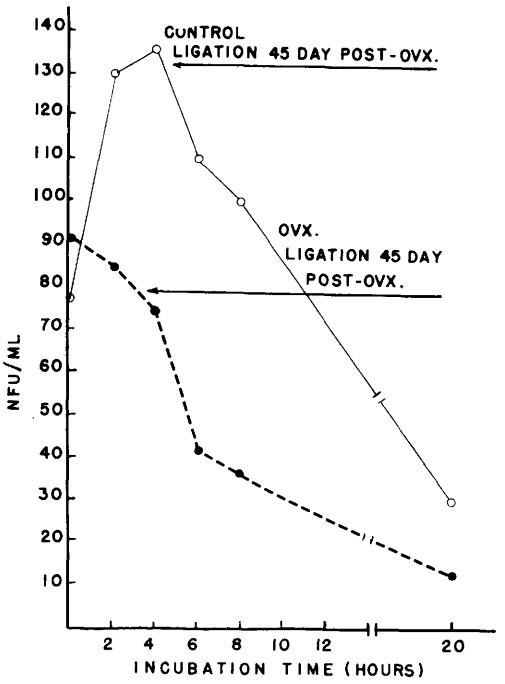

FIG. 1. Hyaluronidase release from sperm incubated in uterine fluid collected from unilaterally ovariectomized (ovx) rabbits. Control uterine fluid taken from the cornua on the intact side. Each point is the mean of two experiments using different pools of uterine fluid from two groups of does. Values for hyaluronidase activity are given in National Formulary Units (NFU) per milliliter of incubation medium.

Addition of progesterone. Progesterone in a concentration of 5 ng/ml caused a slight delay in the timing of the maximum hyaluronidase release compared to the controls, while 20 ng/ml resulted in a stable level of hyaluronidase activity for the first 6 hr of the incubation (Fig. 2). Progesterone in a concentration of 100 ng/ml of incubation medium eliminated the sperm hyaluronidase release and hyaluronidase activity declined throughout the incubation. 17β-Estradiol (Sigma) in concentrations from 5 to 100 ng/ml had no effect on the sperm hyaluronidase release.

Addition of trypsin inhibitor. Although the addition of progesterone delayed or eliminated the sperm hyaluronidase release in the incubations discussed above, it had no effect on the apparent proteolytic activity which develops when sperm are incubated in uterine fluid. The rapid disappearance of hyaluronidase suggests the possible release of a proteolytic enzyme by the sperm. The effect of soybean trypsin inhibitor was tested by adding 10 μg/ml of soybean trypsin inhibitor to an aliquot of uterine fluid just prior to the addition of sperm. The inhibitor did not prevent the rapid rise in hyaluronidase concentration, but did slow the rate of disappearance from the supernatant for a period of 4–6 hr (Fig. 3). This finding suggests that the disappearance of hyaluronidase is due to a trypsin-like enzyme. Since the rate of proteolysis of hyaluronidase in uterine fluid alone is considerably slower (1), it is possible that this proteolytic enzyme is released by the sperm.

Heated uterine fluid. Postovulatory uterine fluid was heated at 56° for 30 min. After allowing the fluid to cool to 37° the sperm were added. Hyaluronidase activity level in heated uterine fluid is constant for the first 6 hr of incubation, however, a rapid decrease followed (Fig. 4A). The supernatant proteolytic activity in heated uterine fluid declines throughout the period of the incubation (Fig. 4B). Thus, the heated uterine fluid appears to lose the ability to cause any increase in sperm hyaluronidase release and also shows a concomitant loss in proteolytic ability. A cessation of sperm motility after 6 to 8 hr of incubation was noted.

Uterine fluid fractionation. Fractionation of uterine fluid on a Biogel P100 gel filtration column resulted in the separation of four fractions which contained the majority of the uterine fluid protein (Fig. 5). Sperm incubation in Tyrode's solution containing the individual fractions indicated that in fraction III the pattern of sperm hyaluronidase release is similar to that found with postovulatory uterine fluid. Fractions I and IV did not result in hyaluronidase release from the sperm but did produce proteolytic effects similar to those found with preovulatory uterine fluid. However, neither of these fractions was able to sustain sperm motility beyond 6 to 8 hr. Hyaluronidase release by sperm incubated in fraction II was very similar to the results obtained with heated uterine fluid

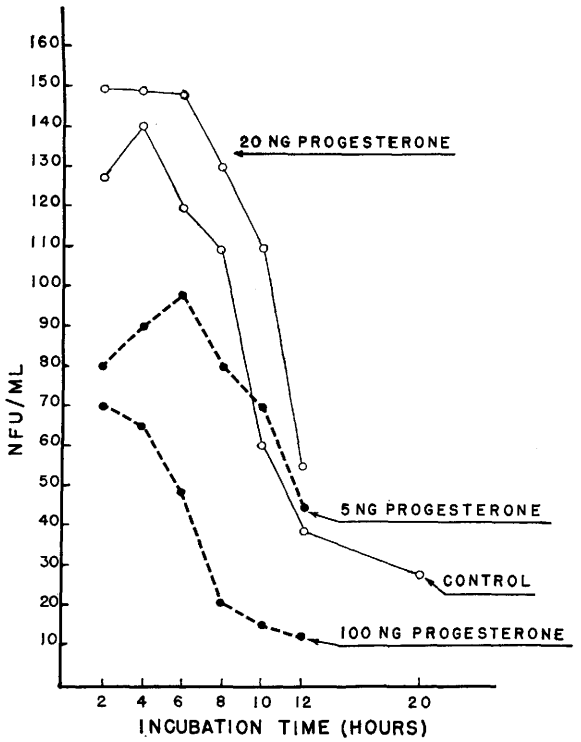

FIG. 2. Hyaluronidase release from sperm incubated in uterine fluid containing additional progesterone. Each point is the mean from replicate experiments using two different pools of uterine fluid. Values for hyaluronidase activity are given in National Formulary Units (NFU) per milliliter of incubation medium.

(Fig. 4A). The hyaluronidase activity was maintained at a constant value for about 4 hr and then decreased rapidly. Fraction III was found in the eluent from the gel filtration column only when postovulatory uterine fluid was the major component in the sample applied to the column.

Sperm were also incubated in fraction III at two different protein concentrations. At a protein concentration of 1.5 mg/ml, the maximum hyaluronidase release was attained at 2 hr incubation, while at a protein concentration of 0.5 mg/ml, maximum hyaluronidase release was not reached until after 4 hr incubation.

Discussion. These results define certain aspects of the mechanism by which uterine fluid interacts with sperm. We have shown that there is some uterine fluid component which causes the release of hyaluronidase from sperm incubated in postovulatory uterine fluid. This factor is apparently proteinaceous, is heat sensitive, and its effect on sperm is directly related to its concentration in the medium. The source of this factor (or factors) is not yet known, nor is the means by which it causes the sperm hyaluronidase release understood.

In addition, a proteolytic factor is involved in the inactivation of hyaluronidase as was demonstrated by the effect of the soybean trypsin inhibitor. The lack of proteolytic activity in the warmed uterine fluid indicates that the proteolysis is not a residual effect caused by incomplete removal of seminal plasma proteolytic enzymes. Additionally, proteolysis was not measurable in sperm incubated in whole seminal plasma (unpublished results).

Although this proteolytic enzyme may be the acrosomal proteinase of Stambaugh and Buckley (6, 7) it was not measurable using benzoyl arginine ethyl ester as the substrate (8). This would indicate that either it is not

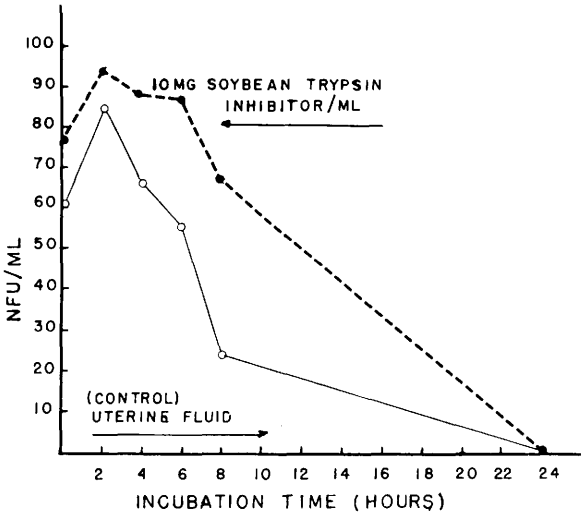

FIG. 3. Hyaluronidase release from sperm incubated in uterine fluid containing additional soybean trypsin inhibitor. Each point is the mean from replicate experiments using two different pools of uterine fluid. Values for hyaluronidase activity are given in National Formulary Units (NFU) per milliliter of incubation medium.

the acrosomal proteinase (as described by Stambaugh and Buckley) or that the concentration of this proteolytic enzyme is very low in these sperm incubations.

The interpretation of these results must be considered in the context of data obtained by others with regard to the process of capacitation. First, we have demonstrated that preovulatory uterine fluid and postovulatory uterine fluid are quite different in their effects on hyaluronidase release in that the former prevents the release and the latter induces the release of hyaluronidase (1). Secondly, ovariectomy also alters the ef-

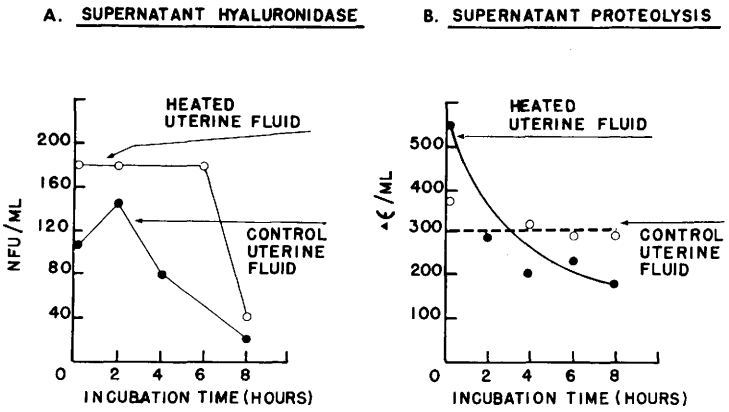

FIG. 4A. Hyaluronidase release from sperm incubated in uterine fluid warmed to 56° for 30 min prior to adding the sperm. Each point is the mean from replicate experiments using two different pools of uterine fluid. Hyaluronidase values are given in National Formulary Units (NFU) per milliliter of incubation medium. (B) Proteolytic activity in uterine fluid sperm incubations. Uterine fluid was either heated to 56° prior to sperm addition or was untreated. Each point is the mean from replicate experiments using two different pools of uterine fluid. Values for proteolytic activity are given as the change occurring in the extinction coefficient of hemoglobin and calculated per milliliter of incubation medium.

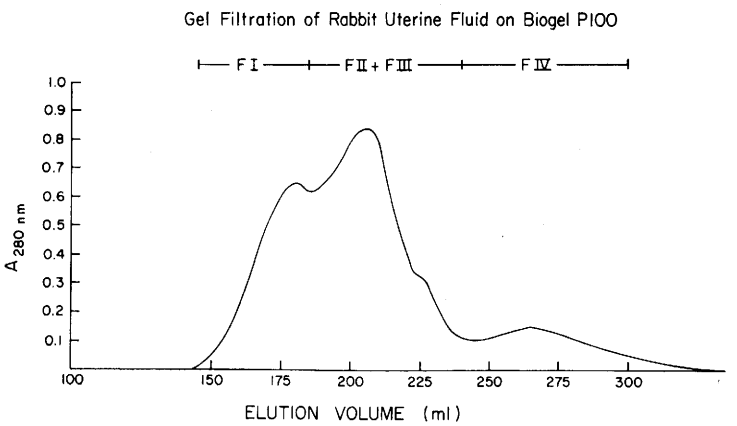

FIG. 5. Representation of the ultraviolet absorbance (280 nm) pattern of the eluent from gel filtration of rabbit uterine fluid. The four major protein containing fractions are identified by the roman numerals.

fect of postovulatory fluid, converting it (in effect) to preovulatory fluid. This variability in the effect of uterine fluid may be related to results obtained by Bedford (9). He has shown that the uterus, when separated from the oviduct, has a limited capability for capacitating sperm, and that certain elements from the oviduct can increase uterine capacitating ability. Although the ability of rabbit follicular fluid to capacitate sperm has not been tested, studies have shown that hamster sperm can be capacitated by hamster follicular fluid (3, 4). Thus, the effect of postovulatory uterine fluid may be due to the action of tubal or follicular elements. Although there is no direct evidence that fluid from the oviduct has any interchange with uterine fluid following mating, uterine fluid does capacitate sperm. A third consideration is that sperm recovered from the uterus 14 to 16 hr after mating exhibit maximum fertility in capacitation tests, while 6 to 8 hr later they become infertile (9, 10). Thus, under *in vivo* conditions rabbit sperm in the uterus seem to have a postcapacitation stage where they are motile, morphologically intact, and yet are incapable of fertilization. It should be kept in mind that this occurs in the uterus several hours after the time the ova would have been fertilized in the oviduct. These data agree with the proposal that capacitation shortens the life-span of mammalian sperm (11). These reports can be correlated with our results in these experiments where we have used uterine fluid collected 14 to 16 hr after the induction of ovulation. In addition, we observed that the sperm in these postovulatory uterine fluid incubations became immotile 6 to 8 hr after the maximum hyaluronidase release was measured. A final observation bearing on the interpretation of our results concerns the suppressing effect of progesterone on the release of sperm hyaluronidase. It has been shown that capacitation is suppressed or prevented in the progesterone dominated uterus (12, 13). Although *in vitro* results cannot be directly related to *in vivo* effects, it would be interesting to determine whether progesterone is one component of rabbit uterine fluid.

Finally, these observations have led us to hypothesize that the hyaluronidase release from sperm, which we have measured in postovulatory uterine fluid, and in one specific protein fraction of uterine fluid, may be directly related to the process of capacitation. It is quite probable that the sperm hyaluronidase release occurs following the completion of the capacitation process. It is suggested that uterine fluid components interact with the sperm during capacitation to bring about the release of hyaluronidase. Whether the acrosome reaction is involved in this hyaluronidase release is still not clear. The apparent shortening in the time required for capacitation to occur *in vitro*, may be the

result of a higher concentration of the appropriate factor(s).

Summary. The release of hyaluronidase by rabbit sperm was measured, *in vitro*: (a) in uterine fluid from unilaterally ovariectomized rabbits, (b) in uterine fluid heated to 56°, (c) in postovulatory uterine fluid with added progesterone, and (d) in protein containing fractions of uterine fluid. Ovariectomy suppresses sperm hyaluronidase release as does heating of the uterine fluid or the increased availability of progesterone. The hyaluronidase releasing activity of uterine fluid is confined to one macromolecular fraction following gel filtration. The possible relationship of these results to the process of capacitation is discussed.

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