II in the cell. It has been suggested that alkaline ribonuclease II is involved in the degradation of messenger RNA and ribosomal RNA precursor in liver (*11*). If such is generally the case, it seems quite possible that the large increase in ribonuclease II activity noted here could well account for the increased muscle RNA and protein turnover in murine dystrophy (*3*).

Further study of this model disease holds promise for fresh insight into the elusive causes of muscular dystrophy and into the intriguing problem of the physiological function of the alkaline ribonuclease II inhibitor system.

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9. Control studies have shown that the values for free and latent ribonuclease in water and KCl extracts represent substantially all of the alkaline ribonuclease II activity of the muscle. No free or latent activity can be found in the residual particulate matter from normal muscle, and the small amounts of free activity remaining in the dystrophic pellet can be removed by repetition of the KCl extraction. Thus the data of Table 1 are not the result of mere differences in extractability of enzyme from dystrophic and normal muscle; they represent true differences in tissue content of enzyme activity.
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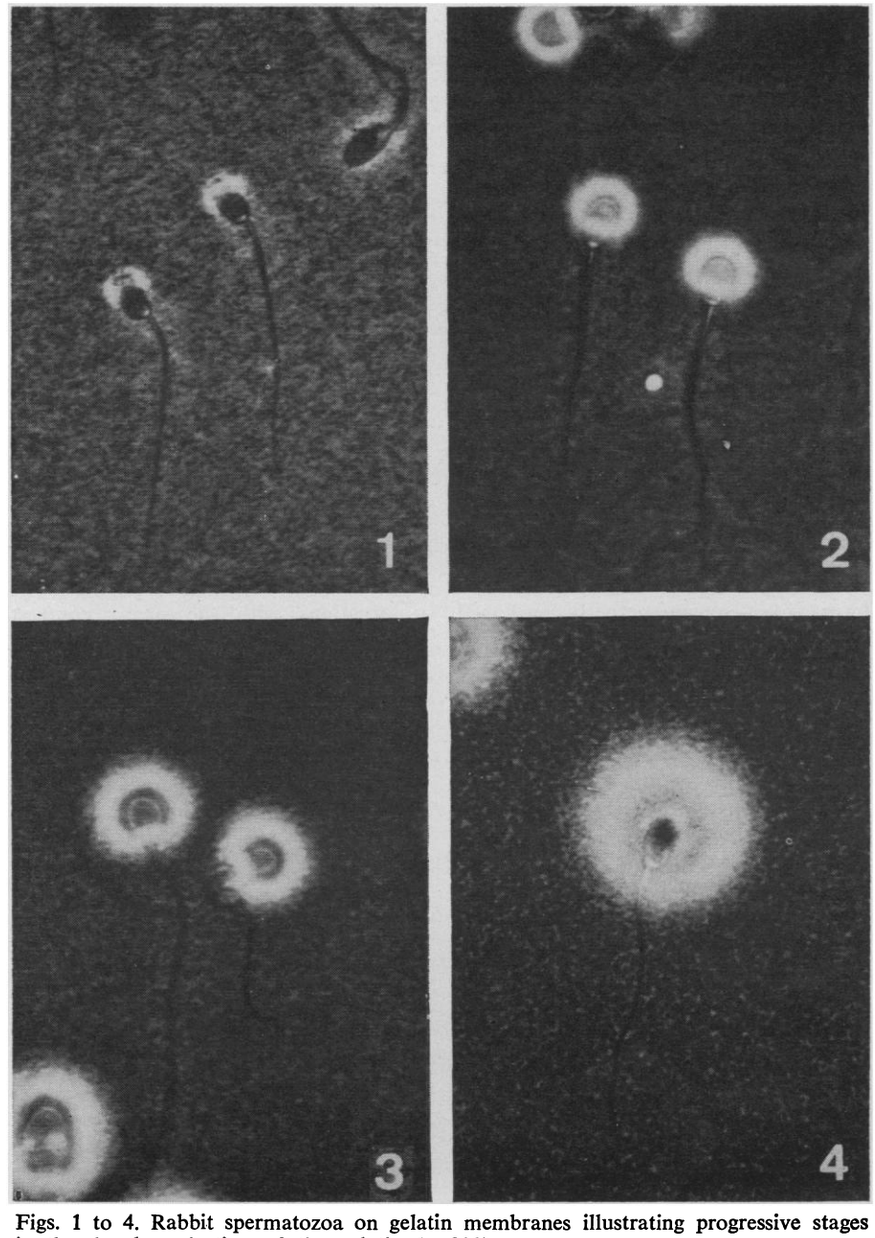
Proteolytic Reaction of Mammalian Spermatozoa on Gelatin Membranes

Abstract. *The acrosomes of spermatozoa of several mammalian species show proteolytic activity when applied to fixed gelatin membranes. The technique permits continuous observation of the enzymatic reaction of an individual spermatozoon. Release of the enzyme occurs solely in the region of the acrosome, in a manner which is species-specific.*

The mechanism by which spermatozoa penetrate the cumulus oophorus and zona pellucida of the mammalian egg to effect fertilization is not fully understood. Extracts prepared from the acrosomes of ram, bull, or rabbit spermatozoa have proteolytic and hyaluronidase activities and can bring about removal of the cumulus oophorus, corona radiata and sometimes the zona pellucida of rabbit eggs (*1*). In addition, Stambaugh and Buckley have isolated hyaluronidase and a "trypsin-like" enzyme from acrosomal extracts of rabbit



Figs. 1 to 4. Rabbit spermatozoa on gelatin membranes illustrating progressive stages in the depolymerization of the gelatin (× 800).

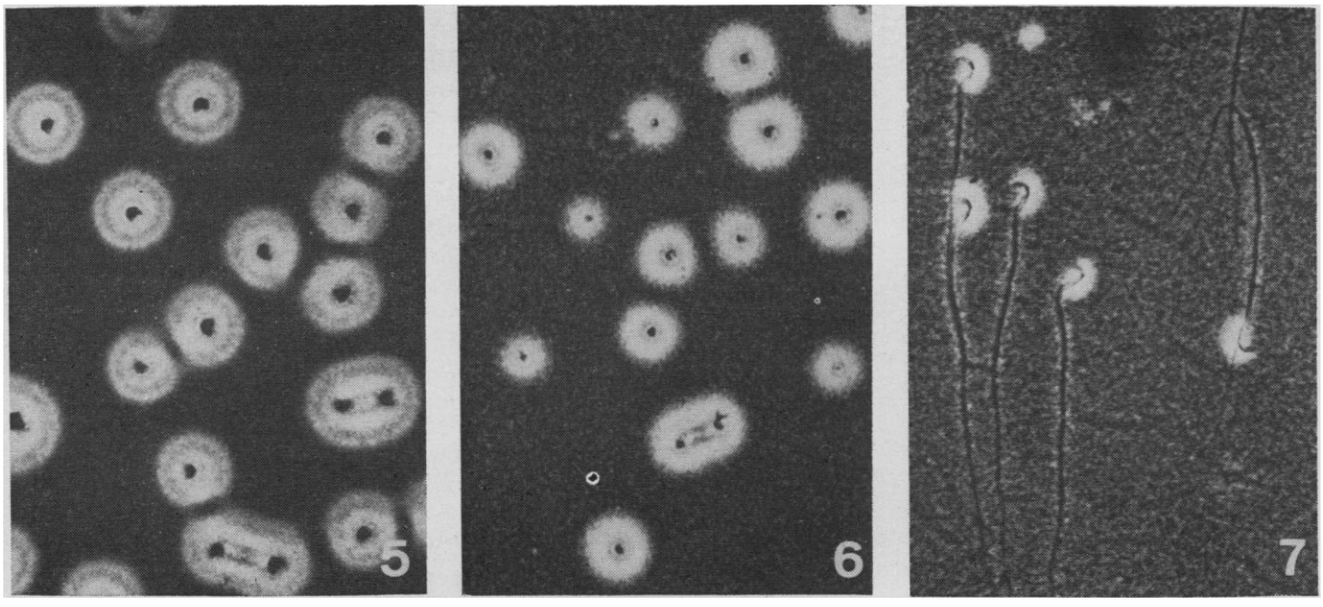


Fig. 5. Rabbit spermatozoa on a gelatin membrane showing the intense and uniform reaction. In Figs. 5 and 6 the sperm flagella are obscured (× 320). Fig. 6. Human spermatozoa on a gelatin membrane showing similar reaction (× 320). Fig. 7. Rat spermatozoa on a gelatin membrane. The sperm heads are hook-shaped and the reaction occurs mainly on their convex aspects (× 320).

spermatozoa, whose combined action produces rapid and complete dissolution of the zona pellucida of rabbit eggs. The trypsin-like enzyme is apparently the more important, since the dissolution process can be completely stopped by inhibitors specific for trypsin (*2*). The same trypsin inhibitors will inhibit sperm penetration of rabbit ova in vitro as well (*3*).

Important questions arise from these studies concerning the relation of these data to the known morphological characteristics of spermatozoa. How is the trypsin-like enzyme actually packaged in the sperm cell and in what manner is it released during penetration of the egg membranes? We now report a technique which provides a direct, visual demonstration of the release of a proteolytic enzyme from individual sperm heads of several mammalian species.

Membranes of fixed gelatin were prepared on microscope slides according to the technique devised by Owers for the detection of proteases released in vitro (*4*). Simply stated, these membranes are thin layers of gelatin impregnated with India ink and fixed in glutaraldehyde. Spermatozoa were obtained from rabbits and other laboratory species by making small cuts in the caudae epididymides of freshly killed animals and allowing the sperm to exude into Hanks solution. A few drops of the concentrated sperm suspension were mixed with a few drops of a barbital acetate buffer solution of specific *p*H. The buffers used were from *p*H 2.6 to 9.5, with increments of approximately 1.0 between each. One drop of the sperm-buffer mixture was placed on a gelatin membrane and drawn across with the edge of a cover slip or microscope slide, in a manner similar to the preparation of a blood smear. A square cover slip was applied, and the preparation was sealed with molten vaseline to prevent evaporation. Preparations were placed in individual petri dishes and kept at 37°C both during and between observations with the phase contrast microscope.

When rabbit spermatozoa are placed on the membranes, the first sign of reaction between a spermatozoon and the gelatin is a lightening of the dark background in the areas immediately adjacent to the lateral edges of the acrosome (Fig. 1). This effect is due to depolymerization of the gelatin and consequent dispersal of the India ink particles. Such a reaction may be seen within a few minutes of making the preparation. At this point the light areas are entirely confined to the region immediately adjacent to the acrosome and do not occur near the postnuclear cap or any part of sperm flagellum.

As depolymerization of the surrounding gelatin continues, the anterior two-thirds of the sperm head become enveloped in a white, spherical area (Fig. 2). In a later stage of the reaction the sperm appears to have a dark cap over the anterior part of the head (Fig. 3). Finally, the zone of depolymerization forms an area large enough to include the entire sperm head and even a short segment of the flagellum. A dark "halo" of concentrated India ink particles appears around the anterior two-thirds of the head (Fig. 4). In highly reacted spermatozoa, a second halo may be seen toward the periphery of the reaction site (Fig. 5).

When buffers from *p*H 7.5 to 9.5 are added to the sperm suspension just prior to smearing, most sperm in the preparation show a strong reaction within 1 hour. The reaction intensifies slowly over the next hour. At low *p*H levels (2.6, 3.6, 4.66) the reaction is significantly slower and does not reach the same intensity. We have found that sperm in Hanks solution alone (*p*H 7.2), with no buffer added, will show a rapid and strong reaction. When soybean trypsin inhibitor (*5*) dissolved in Hanks solution is mixed with the sperm suspension in a concentration of 1.0 mg/ml, the reaction is suppressed drastically but not completely. Ten percent formalin added to the sperm suspension just before smearing destroys the reaction entirely.

The manner in which the sperm are applied to the surface of the gelatin membrane is important in the rapidity and extent of depolymerization. Spermatozoa near the beginning and through

the middle of the smear often show a more rapid and intense reaction than those at the end and sides. We believe that the speed and intensity of reaction depend upon physical phenomena related to the proximity of the sperm to the substrate.

In addition to rabbit spermatozoa, experiments have been carried out on ejaculated spermatozoa from humans and epididymal sperm from rats, mice, guinea pigs, and hamsters. There are considerable differences between species in the capacity of sperm acrosomes to react on the gelatin membranes. So far it appears that human sperm are the only ones comparable to rabbit sperm in the rate of reaction (Fig. 6). The human spermatozoon is much smaller than that of the rabbit [4.6 μm as opposed to 8.4 μm in head length (*6*)], but its "reaction circle" is proportional in size. The reaction is somewhat more rapid and intense at the higher *p*H's (7.5 to 9.5) than at lower ones, but this difference is not so clear as in the case of rabbit sperm. In their favorable *p*H range, human spermatozoa show a maximum reaction in less than 1 hour. Washing them twice in calcium-free Ringer solution and resuspending in Hanks solution did not significantly alter their capacity to depolymerize the membranes. Human seminal plasma from which the sperm had been removed showed a definite capacity to digest the gelatin.

When rat spermatozoa react on the membranes, the zone of activity is invariably confined to the convex surface of the head (Fig. 7). The reaction rarely occurs except at *p*H 8.55 to 9.5. It does not begin until at least 1 hour after the preparation is made and seldom achieves maximum intensity until about 24 hours. Mouse spermatozoa show a similar reaction (beginning on the convex aspect of the head), but the reaction is more rapid and occurs over a wider *p*H range compared to the reaction of rat sperm. In the hamster and the guinea pig strong reactions may occur also in 4 to 6 hours from *p*H 7.5 to 9.5 and only slight or moderate reactions below *p*H 7.5. The guinea pig spermatozoon displays a large reaction circle similar to that of the rabbit.

To our knowledge this is the first demonstration of a proteolytic enzyme in the individual mammalian spermatozoon. Reaction between the spermatozoon and gelatin membrane begins in the region of the acrosome in a manner which is species-specific.

The features of the enzyme noted so far (its proteolytic activity, high *p*H optimum, inhibition by a trypsin inhibitor, and localization in the acrosome) suggest that it may be the "trypsin-like" enzyme identified by others as the primary agent effecting dissolution of the zona pellucida (*2, 7*). If this is true, we have a useful method for studying the potential of different populations of spermatozoa to effect penetration of the zona.

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Alcohol Dehydrogenase in Maize: Genetic Control of Enzyme Activity

Abstract. *A gene* (Adh_r) *which controls the activity of alcohol dehydrogenase in the scutellum of maize has been found. This gene is not allelic to the* Adh_1 *locus, which specifies the charge of the enzyme molecule and hence its migration rate. The two genes are linked and located about 17 crossover units apart. The* Adh_r^N *allele specifies equal activities of both the* Adh_1^S *and* Adh_1^F *products. The* Adh_r^L *allele gives lower activities of the* Adh_1^S *products only and operates in both the* cis *and the* trans *configurations.*

Variation in enzyme activities between different inbred lines of maize has been demonstrated recently for a number of enzymes (*1–3*). Hybrids between lines with different activities usually showed intermediate activities (*1*). Unfortunately, these studies showed only an average activity of the products of the two alleles contributed by the two parental lines. This is probably the reason for the absence of information concerning the mode of inheritance of differences in enzyme activities. Allelic isozymes having different electrophoretic mobilities were used in the present study to investigate this problem.

Maize is polymorphic for the alcohol dehydrogenase (E.C.1.1.1.1) gene Adh_1. Four alleles have been described thus far (*4*): Adh_1^S, Adh_1^F, and $Adh_1^{C(t)}$, which specify enzymes that differ in charge; and $Adh_1^{C(m)}$, which produces an enzyme having the same electrophoretic mobility specified by $Adh_1^{C(t)}$ but is much less active. The enzyme is a dimer and three isozymes are formed in heterozygotes (*4*).

Two homozygous lines of maize were used: $N,Adh_1^F/N,Adh_1^F$, a line with normal activity of alcohol dehydrogenase (ADH) in the scutellum of the mature kernel (N) and with a fast migrating isozyme form (Adh_1^F); and $L,Adh_1^S/L,Adh_1^S$, a rare line with lower ADH activity (L) and slow migrating isozyme form (Adh_1^S). The difference in activity between the two lines is easily detected visually (Fig. 1A) after starch-gel electrophoresis and specific staining for ADH (*4*). In quantitative tests (*5*) the intensities of the ADH bands that were developed after 1 hour of staining were measured in 18 different gels from each line by a densitometer (*6*) fitted with a filter of 545 nm (Fig. 2A). Mean curve areas of 98.8 ± 13.6 mm^2 (100 percent) and 62.2 ± 14.7 mm^2 (63 percent) were found for the $N,Adh_1^F/N,Adh_1^F$ and the $L,Adh_1^S/L,Adh_1^S$ lines, respectively. The difference in activity as measured by the curve area between the two lines is significant ($\alpha = .001$). When the enzymes from the two lines were extracted from mature dry kernels and assayed spectrophotometrically as described (*7*), similar results were found. The $L,Adh_1^S/L,Adh_1^S$ line had 60.7 percent of the activity shown by the $N,Adh_1^F/N,Adh_1^F$ line.

As judged by electrophoretic mobility, three isozyme bands were found in Adh_1^F/Adh_1^S heterozygotes since the