munication between the sexes, could also produce the same effect. One further implication of our demonstration that the pheromone is volatile is that pathways other than renal excretion may be important—for example, excretion from the lungs.

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References and Notes

1. P. Karlson and A. Butenandt, *Ann. Rev. Entomol.* **4**, 39 (1959).
2. W. K. Whitten, *Advan. Reprod. Physiol.* **1**, 155 (1966).
3. F. H. Bronson and W. K. Whitten, *J. Reprod. Fertility* **15**, 131 (1968).
4. H. M. Marsden and F. H. Bronson, *Science* **144**, 1469 (1965).
5. W. K. Whitten, unpublished.
6. R. K. Chipman and K. A. Fox, *J. Reprod. Fertility* **12**, 233 (1966).
7. W. H. Bossert and E. D. Wilson, *J. Theoret. Biol.* **5**, 443 (1963).
8. Supported by PHS grants HD-00473 and HD-00767, and NSF grant GY 2550. The principles of laboratory animal care as promulgated by the National Society of Medical Research, the American Psychological Association, and the Council of the American Physiological Society are observed in our laboratory.

21 May 1968

Zona Pellucida Dissolution Enzymes of the Rabbit Sperm Head

Abstract. *Enzymes which dissolve the zona pellucida have been extracted from the rabbit sperm head and characterized as having the enzymic properties of hyaluronidase and trypsin. Their combined actions produce a rapid and complete dissolution of the zona pellucida, but the vitellus and its membrane are unaffected by prolonged exposure to this enzyme complex. Inhibition of the proteolytic component with lima bean or soybean inhibitors of trypsin prevents dissolution of the zona pellucida by the extract.*

Penetration of the zona pellucida of the ovum by a spermatozoon was observed as early as 1875 (*1*), but the mechanism of the process has remained obscure. The zona pellucida, a thick, transparent membrane, is best developed in the ova of placental mammals, but is also recognizable in those of marsupials, monotremes, and even of reptiles. The matrix of the zona pellucida appears essentially homogeneous, even when observed with electron microscopy, and consists of neutral or weakly acidic mucoprotein. Initially, the zona pellucida lies in close apposition to the vitellus, but it becomes separated by the fluid extruded from the vitellus when the first polar body is emitted. At the time of ovulation the zona pellucida is surrounded by the cumulus oophorus and corona radiata, but both of these cell masses are rapidly loosened and dispersed after ovulation (*2*). Thus, the principal barrier to fusion of a sperm with an ovum is the zona pellucida. Recently Srivastava *et al.* (*3*) have obtained occasional dissolution of the zona pellucida with extracts of rabbit sperm acrosomes. Obviously, the process of zona penetration is enzymic in nature, but no enzymes for zona dissolution have been characterized or identified in extracts of sperm.

We have examined extracts of washed rabbit epididymal spermatozoa disrupted by sonic oscillation for hyaluronidase and proteolytic activity, which might hydrolyze this mucoprotein layer. These extracts displayed high hyaluronidase activity against bovine vitreous humor hyaluronic acid, and high proteolytic activity with denatured bovine hemoglobin. The synthetic substrates *p*-toluenesulfonyl-L-arginine methyl ester (TAME) and benzoyl arginine ethyl ester (BAEE) were also hydrolyzed by the extracts. Elastin, L-leucinamide, and hippuryl-L-phenylalanine were not hydrolyzed.

Since the spermatozoon penetrates obliquely and head first through the zona pellucida, the enzymes for zona dissolution must be localized in the head of the spermatozoon, and probably within the acrosomal cap. Using a new procedure of sucrose density-gradient centrifugation (*4*) we have isolated from the disrupted rabbit epididymal sperm a homogeneous head fraction which contains most of the hyaluronidase and proteolytic activity. Prolonged sonic disruption of this purified head fraction results in solubilization of the hyaluronidase and proteolytic activities, which sediment as a single molecule with a molecular weight of 59,000, as determined by the procedure of Martin and Ames (*5*). This procedure does not visibly damage the dense nucleus of the sperm, but most of the fragile acrosomes are disrupted and dislodged by the sonic oscillation. It would appear, therefore, that this enzymic particle is being extracted from the acrosomal cap.

Since hydrolysis of BAEE, TAME, and denatured hemoglobin is characteristic of the trypsin type of specificity, the sperm head extract was further compared with purified bovine pancreatic trypsin. The *p*H optimum for

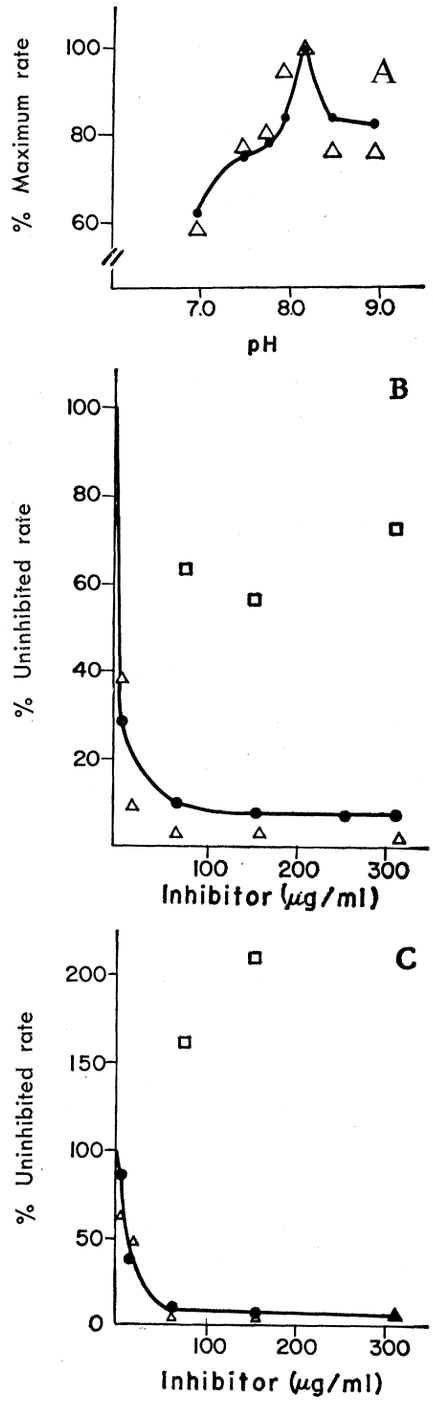

Fig. 1. (A) Effect of *p*H on BAEE hydrolysis by sperm head extract (●) and by crystalline bovine trypsin (△). (B) Inhibition of BAEE hydrolysis with soybean trypsin inhibitor of sperm head extract (●), crystalline bovine trypsin (△), and crystalline papain (□). (C) Inhibition of BAEE hydrolysis with lima bean trypsin inhibitor of sperm head extract (●), crystalline bovine trypsin (△), and crystalline papain (□).

the extract was 8.2 with BAEE as substrate, identical to the *p*H optimum for crystalline bovine trypsin (Fig. 1A).

Among the proteolytic enzymes found in nature, trypsin is unique in that it is sensitive to a number of naturally occurring inhibitors. Accordingly, the sperm head extract was tested for sensitivity to two of these inhibitors and compared with crystalline bovine trypsin and crystalline papaya papain, a plant enzyme also active on this substrate. Soybean trypsin inhibitor (10 μg/ml) depressed the enzymic activity of both trypsin and sperm head extract to 10 to 20 percent of the maximum rate when no inhibitor was present (Fig. 1B). Papain was inhibited only slightly by this concentration of inhibitor. Lima bean trpsin inhibitor (Fig. 1C) (50 μg/ml) also depressed the activity of both trypsin and sperm extract to 10 percent of the maximum rate. However, the activity of crystalline papain was increased in the presence of lima bean trypsin inhibitor.

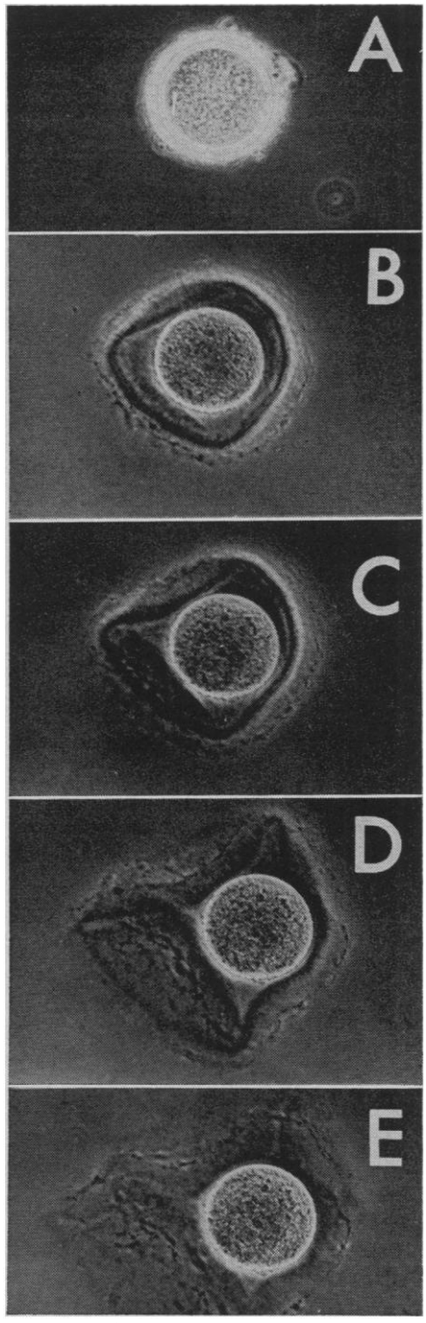

Fig. 2. Dissolution of the zona pellucida by sperm head extract at 37.5°C; (A) 0 time; (B) 20 minutes; (C) 40 minutes; (D) 60 minutes; (E) 90 minutes.

The dissolution of the zona pellucida by the extract concentrated by lyophilization is shown in Fig. 2. Complete dissolution of the zona occurred within 1.5 hours at 37.5°C, but the vitellus was not visibly affected by a 25-hour incubation in the concentrated extract. Addition of soybean trypsin inhibitor (300 μg/ml) or lima bean trypsin inhibitor (300 μg/ml) to this extract completely inhibited dissolution. Thus, the trypsin component of this extract is the more important enzyme in the dissolution process. This is supported by the fact that crystalline hyaluronidase (375 unit/ml) causes no visible dissolution of the zona pellucida even after a 24-hour incubation at 37.5°C, but crystalline trypsin (5500 unit/ml) completely dissolves the zona in 10 minutes at this temperature.

These data indicate that penetration of the zona pellucida by the spermatozoon is an enzymic process involving mainly an acrosomal enzyme similar to trypsin. The hyaluronidase might supplement this dissolution process, or its physiologic function may be to thin the cervical mucus or disperse the hyaluronic acid matrix of the cumulus oophorus of undenuded ova.

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References and Notes

1. E. Van Benenden, *Bull. Acad. Roy. Belg. Classe Sci.* **40**, 686 (1875).
2. R. Stambaugh, C. Noriega, L. Mastroianni, Jr., *J. Reprod. Fertil.*, in press; C. R. Austin, *The Mammalian Egg* (Blackwell Scientific Publication, Oxford, 1961), pp. 86–102; L. Zamboni, C. Hongsanand, L. Mastroianni, Jr., *Fertil. Steril.* **16**, 177 (1965).
3. P. N. Srivastava, C. E. Adams, E. F. Hartree, *Nature* **205**, 498 (1965); *J. Reprod. Fertil.* **10**, 61 (1965).
4. R. Stambaugh and J. Buckley, in preparation.
5. R. G. Martin and B. N. Ames, *J. Biol. Chem.* **236**, 1372 (1961)
6. This work was supported by the Ford Foundation grant 65-58A and PHS grant 3 RO1 HD-01810-03.

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Bioluminescence: pH Activity Profiles of Related Luciferase Fractions

Abstract. *The 27,000g supernatant from crude extracts of the dinoflagellate* Gonyaulax *can be fractionated into two components having luciferase activity that differ both in molecular weight (35,000 and 150,000) and in* p*H activity profile. The smaller component has activity over a broad range from* p*H 5 to 9, while the larger one is active only in the acid region. This clarifies the previous ambiguity in the literature regarding the optimum* p*H for the assay of luciferase.*

Gonyaulax polyedra is one of the bioluminescent marine dinoflagellates. These are unicellular algae in which light emission commonly occurs as a brief (0.2-second) bright (10^8- to 10^{10}-photon) flash of light evoked by mechanical stimulation (*1*). Biochemical studies with *Gonyaulax* have shown that bioluminescence may be obtained in vitro from two distinctly different systems—one being soluble (*2*) and the other particulate (*3*).

The particulate system involves a large particle (molecular weight, $> 10^9$), termed the scintillon, which has the potential for bioluminescence when isolated and kept at *p*H 8.2. Activity occurs specifically when the *p*H is lowered to about 5.7, as a brief flash with a duration of about 0.2 second. The only additional factor required is oxygen.

The soluble system was first characterized (*2*) as the supernatant from a cell homogenate that had been centrifuged at 36,000*g* for 10 minutes. The activity was shown to involve a dialyzable, heat-stable component (*Gonyaulax* luciferin), a heat-labile protein (*Gonyaulax* luciferase), oxygen, and a high concentration of salt. The luminescence in this system is relatively long-lived, having a half-life of about 10 minutes at concentrations normally used in partially purified preparations (*4, 5*).

Using Sephadex gel filtration, we have now resolved the luciferase of the soluble system into two components that have molecular weights of approximately 150,000 and 35,000. The component with the higher molecular weight has a narrow *p*H activity profile similar to that described by Hastings and Sweeney (*2*), while the one with the lower molecular weight is active over a wider range from *p*H 6.0 to *p*H