Biochemical Changes in the Zona Pellucida of Rabbit Ova Induced by Fertilization and Sperm Enzymes (35180)

KENNETH GOULD, L. J. D. ZANEVELD, P. N. SRIVASTAVA, AND WILLIAM L. WILLIAMS
Department of Biochemistry, University of Georgia, Athens, Georgia 30601

The presence of protein in the zona pellucida of the mouse and rabbit ovum has been established by removal of the zona with Pronase (1) and trypsin (2, 3). The zona pellucida of the mouse (4–6), rat and rabbit was shown to be more resistant to digestion by trypsin after fertilization than before (2).

Sialic acid has been demonstrated in the zona pellucida of the rabbit ovum (7) and treatment of rabbit ova with bacterial neuraminidase reduced the number of penetrating sperm (8). The vitelline coat of invertebrate ova can be dissolved with cysteine or mercaptoethanol (9, 10). The zona pellucida is the analogous layer in mammalian ova and is also soluble in dilute mercaptoethanol solutions.

The sperm acrosome contains various enzymes, such as a trypsin-like enzyme (TLE) (11, 12), a neuraminidase (13), an enzyme that disperses the corona radiata (CPE) (14), and hyaluronidase.

The present study concerns biochemical changes in the zona pellucida at the time of fertilization as indicated by a change in the solubility of the zona pellucida in mercaptoethanol and by a change in susceptibility to digestion by trypsin. These changes occur at the time of fertilization and are induced in unfertilized ova by treatment with preparations of certain sperm acrosomal enzymes.

Materials and Methods. Preparation of zona pellucida solutions for protein estimation and electrophoresis. Mature New Zealand white rabbits were superovulated by four subcutaneous injections of 0.25 ml of FSH (Armour) at 12-hr intervals, followed by an intravenous injection of 125 units of HCG 12 hr after the last injection of FSH. Ova were flushed from the oviducts 12 hr after administration of HCG and suspended in sterile physiological saline. The cumulus and corona layers were removed by agitation, and the ova were washed with 1 to 2 ml of either physiological saline or glass distilled water.

The zonae pellucidae were removed from groups of 50 ova by treatment with 1.5 *M* mercaptoethanol (Eastman). The ova were observed continually to ensure that the vitelline membrane did not rupture. The zona-less ova were removed in 5 to 8 μl of saline. The remaining fluid was dialyzed against 3 liters of distilled water for 24 hr at 20° and the residual dialyzed solution was concentrated by lyophilization. Aliquots of the saline used to wash the ova were treated similarly to act as controls for the Ninhydrin assay and gel electrophoresis. Prior to use the samples were resuspended in glass distilled water to a concentration of 150 μg/ml. The weight of the zona was calculated from the formula 1.2 $(4/3\pi R^3 - 4/3\pi r^3)$ where 1.2 is the specific gravity of the zona pellucida and R and r are its outer and inner radius, respectively (15). The weights were found to average 2.9 μg, range 1.6 to 5.5 μg. Zonae were also removed by sonication to determine if the mercaptoethanol removed protein from the zona-less ova. The ova washed with glass distilled water were sonicated with the Blackstone sonicator, at setting 22, for multiples of 3 sec at 20° in 5 to 6 ml of glass distilled water. Zonae pellucidae freed from the ova by each treatment were washed in distilled water and dissolved in 3 *M* mercaptoethanol. The higher molarity was used for the convenience of rapid solution, there being no risk of affecting the vitelline membrane. The solutions were dialyzed and lyophilized as described above.

Electrophoresis of zona solutions. Solutions containing 30 μg of zona material in 6 to 8μl were subjected to disc electrophoresis (16) on polyacrylamide gel using the apparatus shown in Fig. 1 and a microscale modifica-

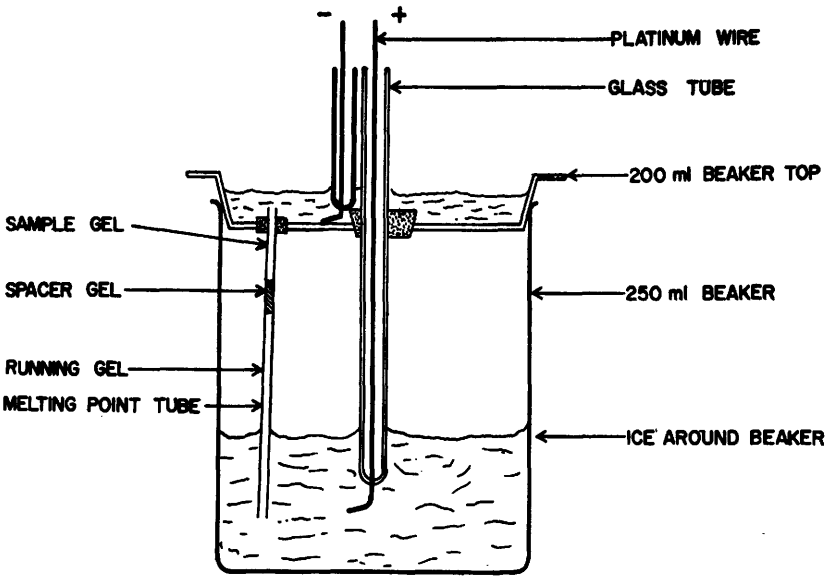

FIG. 1. Micro-disc electrophoresis apparatus.

tion of the procedure of Ornstein and Davis (1964). Gels were stained for protein with Buffalo black and Coumassie blue (17). Periodic acid–Schiff stain was used to demonstrate glycoprotein (18).

Demonstration of biochemical changes in the zona pellucida. The time required for the dissolution of the zona pellucida of fertilized or treated ova was measured with either 0.014 *M* mercaptoethanol in a defined medium containing 3 mg/ml of bovine serum albumin (19) or with bovine pancreatic trypsin in physiological saline containing 10^{-4} *M* $CaCl_2$. In each experiment the dissolution time of the zona pellucida from unfertilized ova was used as control. The dissolution time was measured and expressed as a multiple of the control time.

Acrosomal extracts were obtained from rabbit and bull sperm by the method of Hartree and Srivastava (20). The extracts from bull sperm were further purified on DEAE-cellulose and the eluted fraction containing neuraminidase activity was used.

Estimation of the protein content of the zona pellucida. Samples of 0.2 ml containing a known number of zonae and prepared as described previously were hydrolyzed in evacuated sealed tubes at 110° with 2 ml of 6 *N* HCl for 22 hr. The hydrolyzates were adjusted to pH 8.5 and evaporated to dryness at 37° under reduced pressure. This procedure removes any ammonia from hydrolysis of amino sugars and hydrolyzes all peptide bonds (21). The hydrolyzates were dissolved in 0.5 ml of distilled water and the total amino acid nitrogen estimated by a Ninhydrin method (22). The results were expressed as micrograms of Ninhydrin positive material using an equimolar solution of the 20 amino acids occurring in protein as standard (Beckman).

Results. Ninhydrin positive material was 21.0 to 22.5% of the calculated weight of the zona pellucida (Table I). There was no difference in content of Ninhydrin reacting material between fertilized and unfertilized ova. The method of preparation of the solution of

TABLE I. Ninhydrin Positive Material in the Zona Pellucida.

	Fertilized		Unfertilized	
No. of ova	15	4	7	15
Total Ninhydrin reacting material (μg)[a]	9.1	2.6	4.3	9.4
% of zona wt[b]	20.9	22.4	21.2	21.7

[a] Each number represents 3 assays with a maximum range of ± 0.2 μg.

[b] Zona weight averages 2.9 μg.

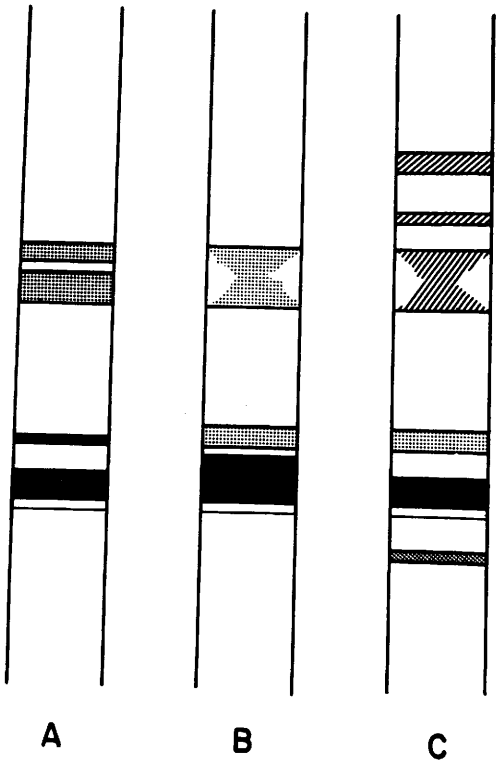

FIG. 2. The separation of the zona pellucida extract achieved by electrophoresis. Tris–glycine buffer, pH 8.5, stained with Buffalo black and Coumassie blue: (A) zona preparation from unfertilized ova; (B) zona preparation from fertilized ova; (C) zona preparation from unfertilized ova, stained with PAS.

zonae pellucidae had no effect on the results.

Four protein components were demonstrated by disc electrophoresis (Fig. 2). The components from fertilized and unfertilized ova did not differ significantly in mobility. Staining with PAS demonstrated two additional components in a few samples examined. Fertilization or treatment of ova with rabbit and bull acrosomal extracts prolonged the dissolution time of zonae pellucidae by mercaptoethanol 5- to 10-fold, the average increase in dissolution time being 6.9-fold (4 expts.) and 11.1-fold (4 expts.) for fertilization and for rabbit acrosomal extracts, respectively (Table II). Bacterial neuraminidase, more purified and at a higher concentration, was considerably less effective, the average increase in dissolution time by mercaptoethanol being 2.1-fold. Fertilization or treatment of ova with a bull sperm neuraminidase preparation prolonged the dissolution time of zonae pellucidae by digestion with bovine pancreatic trypsin an average of 5.1-fold (Table III). Preincubation of ova in either test medium for periods up to 180 min did not alter the dissolution time.

Discussion. The data presented above demonstrate the occurrence of a biochemical change in the zona pellucida of the rabbit ovum at the time of fertilization. A similar change is induced by treatment of the zona pellucida with extracts from sperm acrosomes which have neuraminidase activity. Sperm trypsin-like enzyme and corona penetrating enzyme (11–14), do not cause this change. The exact nature of the change remains to be determined, but the evidence available strongly suggests a change in the quaternary and tertiary structure of the zona pellucida. The digestion of the zona pellucida by trypsin demonstrates the structural importance of the

TABLE II. Dissolution Time of Ova Treated with Mercaptoethanol.[a]

Treatment	Unfertilized	Treated	Ratio treated:unfertilized
Fertilization	14.0 ± 3	65 ± 7	4.6
	11.5 ± 2	70 ± 5	6.1
	15.5 ± 3	>150	9.7
	12.5 ± 4	87 ± 7	7.0
Rabbit acrosomal extract[b]	9.5 ± 2	95 ± 8	10.0
	11.0 ± 2	>150	13.6
	12.0 ± 3	60 ± 5	5.0
	9.5 ± 2	>150	15.8
Bull sperm neuraminidase[c]	11.0 ± 4	60 ± 5	5.4
Bacterial neuraminidase[d]	11.0 ± 4	23 ± 4	2.1
	13.5 ± 3	45 ± 5	3.3
	25.0 ± 5	26 ± 4	1.0

[a] Each assay is the average dissolution time of 6 ova in 0.014 *M* mercaptoethanol.

[b] Sixty-min preincubation at 37°, 15 to 25 μg of protein/ml.

[c] Purified on DEAE, 25 μg of protein/ml of incubation medium; preincubated 60 min at 37°; specific activity, 0.18.

[d] Worthington Biochemical Co., *Clostridium perfringens,* Type VI.

TABLE III. Dissolution Time of Zona Pellucida by Bovine Pancreatic Trypsin.[a]

Treatment	Unfertilized ova (min)	Treated ova (min)	Ratio treated:unfertilized
Fertilized	25 ± 4	85 ± 6	3.4
	15 ± 3	70 ± 4	4.6
Bull sperm neuraminidase[b]	12 ± 4	60 ± 4	5.0
	20 ± 4	>120	6.0
	12 ± 3	70 ± 5	5.8
	15 ± 5	70 ± 7	4.6
Bull acrosomal extract heated 100° for 5 min	25 ± 5	20 ± 3	0.8
	17 ± 4	25 ± 4	1.5
	20 ± 3	35 ± 5	1.7

[a] Sigma Chemical Co.; dissolution time is the average time of 5 ova. Medium was saline plus 10^{-4} *M* $CaCl_2$ and 880 units/ml of trypsin at 37°.

[b] As footnote *c* in Table II.

protein moiety, and evidence that disulfide bonds are essential to the integrity of the protein is provided by the dissolution of the zona pellucida by mercaptoethanol which readily reduces disulfide bonds in proteins. The dissolution of the zona pellucida is not attributable to a change in pH or to mercaptoethanol acting as a chelating agent since 0.2 *M* EDTA between pH 7.1 to 7.4 does not dissolve the zona. A change in structure of sufficient magnitude to render peptide bonds involving lysine and arginine inaccessible to trypsin and disulfide bonds inaccessible to mercaptoethanol is feasible (23, 24). This theory is supported by the electrophoretic evidence. If the change at fertilization involved cleavage of peptide bonds it is likely that the electrophoretic pattern of the products of mercaptoethanol treatment would differ between fertilized and unfertilized ova. Such a difference was not demonstrated. The reproduction of this change by treatment with relatively pure preparations of sperm acrosomal neuraminidase strongly suggests the involvement of this enzyme in the biochemical changes in the zona induced by fertilization. Sperm neuraminidase is much more active in prolonging the dissolution time of the zona pellucida than is bacterial neuraminidase. This may be related to the demonstrated difference in specificity of the two enzymes (13). Bacterial neuraminidase (*Cl. perfringens*, Type VI) cleaves the 2→3 linked sialic acid while sperm neuraminidase preferentially cleaves the 2→6 ketosidic linkage and has relatively little action on the 2→3 link. By reducing the susceptibility of the zona pellucida to lysis by trypsin, sperm neuraminidase may reduce the number of sperm penetrating at fertilization. After entry of the first spermatozoon, the zona pellucida of sheep, dog, and hamster ova becomes impervious to further sperm penetration. This change is less complete in the rat, mouse, and pig. In the rabbit it is evident that this is not an effective means of blocking polyspermy, but it is possible that a similar but more effective reaction occurs in those species exhibiting a block to polyspermy at the level of the zona and is one of the factors involved in the zona reaction.

The research upon which this publication is based was performed pursuant to Contract No. NIH 69-2103 and Career Development Award 2K3 GM4831-09 with the National Institutes of Health, Department of H.E.W. Partial research support was also received from the Ford Foundation.

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Received July 15, 1970. P.S.E.B.M., 1971, Vol. 136.