ENZYMIC ACTION OF ACROSOMAL PREPARATIONS ON THE RABBIT OVUM *IN VITRO*

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Summary. Cell-free enzyme preparations consisting of lipoglycoprotein were obtained from acrosomes of ram, bull and rabbit spermatozoa. The ram and bull preparations showed proteolytic and hyaluronidase activities. Preparations from the three species brought about dispersal of the cumulus oophorus and corona radiata of newly ovulated rabbit eggs. In some cases the zona pellucida was also removed. Iodoacetate and a polyanionic hyaluronidase inhibitor each reduced the denuding activity of the ram preparation and to a lesser extent of the bull preparation. Heating to 100° C also reduced the activity of the ram and bull preparations. It is concluded that proteolytic enzymes in spermatozoa contribute to the denudation of rabbit eggs and probably facilitate penetration of the zona pellucida.

INTRODUCTION

The cumulus oophorus of rabbit eggs is dispersed *in vitro* by suspensions of rabbit spermatozoa (Pincus, 1930; Yamane, 1930). According to McLean & Rowlands (1942) this action is due to the enzyme hyaluronidase which is released from the spermatozoa. The corona radiata, on the other hand, is not dispersed either by sperm or by hyaluronidase (Swyer, 1947; Chang, 1950; Braden, 1952; Dickmann, 1962). The role of hyaluronidase in the fertilization process has been reviewed by Austin (1961) and by Mann (1964).

The feasibility of using cationic detergents to detach acrosomes from spermatozoa was demonstrated by Hathaway & Hartree (1963). Using the cationic detergent Hyamine 2389 (methyl dodecyl benzyl trimethyl ammonium chloride and methyl dodecyl xylene bis trimethyl ammonium chloride), Hartree & Srivastava (1965) developed a method for obtaining suspensions of ram and bull acrosomes which are virtually free from sperm. These acrosomal materials are lipoglycoprotein complexes. In the present paper we describe the effects of such preparations upon newly ovulated rabbit eggs with particular reference to denudation.

MATERIALS AND METHODS

Acrosomal lipoglycoproteins of ram, bull and rabbit spermatozoa were obtained by Method B of Hartree & Srivastava (1965) except that removal of lipid from the complexes by means of chloroform and methanol was omitted. After the acrosomal suspensions, obtained by treating spermatozoa with Hyamine

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2389, had been diluted with an equal volume of ethanol the resulting precipitate was suspended in calcium-free Ringer solution (Mann, 1946) and dialysed overnight at 4° C against the same solution. The protein content of the product was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) and adjusted, by addition of Ringer solution, to 6 mg/ml.

Human plasma Ba- α_2 glycoprotein (Schmid & Burgi, 1961) was supplied by Dr Schmid, and a guinea-pig serum glycoprotein (fraction 1 of Simkin, Skinner & Seshadri, 1964) by Dr Simkin. These two glycoproteins will be referred to as A and B respectively. Luteinizing hormone (Lutormone) was obtained from Burroughs Wellcome, hyaluronic acid (human umbilical cord) from Light & Co, Hyamine 2389 from British Drug Houses, hyaluronidase (Hyalase, ovine) from Bengers Laboratories Ltd, and *p*-chloromercuriphenylsulphonic acid from Sigma Chemical Co. Sialic acid (*N*-acetylneuraminic acid) was prepared from boar semen gel using the method described by Zilliken, Braun & Györgyi (1956). Hyaluronidase inhibitor 53DK (Parkes, Rogers & Spensley, 1954) was provided by Dr Rogers.

Estimation of Hyamine 2389

Since this anionic detergent was used to detach acrosomes from spermatozoa it was necessary to determine residual Hyamine in lipoglycoprotein solutions in order that the necessary control experiments could be set up. The method takes advantage of the fact that the extinction of Hyamine is much higher at 275 m μ than at 295 m μ . A suitable volume of lipoglycoprotein solution is diluted to 5 ml with Ringer solution, treated with 5 ml of 10 % (w/v) perchloric acid, heated 10 min at 80° C, cooled and shaken with 5 ml of *n*-butanol. The Hyamine passes quantitatively into the butanol layer. This layer is clarified by centrifuging and its extinctions (*E*) at 275 and 295 m μ are determined using 10 mm silica cuvettes. The reference cuvette contains *n*-butanol. The concentration of Hyamine in the 5 ml of diluted lipoglycoprotein solution is 0.072 ($E_{275} - E_{295}$) % (w/v).

Collection and treatment of eggs

Rabbits (sixteen in all) were injected intravenously with 25 i.u. of luteinizing hormone to induce ovulation. About 13 hr later, at autopsy, the fallopian tubes were excised and flushed with 0.9 % NaCl (saline). At recovery, the eggs were invariably found embedded in a cumulus clot from which individual eggs were dissected out (Pl. 1, Fig. 1). Each egg, still surrounded by cumulus, was placed in a small tube with 0.5 ml of saline followed by the test substance(s) dissolved in 0.5 ml of Ringer solution. The stoppered tubes were incubated at 37° C for 24 hr and the eggs were examined microscopically (25 $\times$ and 100 $\times$) at 6 hr and 24 hr.

Enzyme assays

Hyaluronidase activity was determined, as turbidity reducing units (TRU), by the method of Tolksdorf (1954). Proteolytic activity was measured by incubating solutions of lipoglycoprotein either alone (autoproteolysis) or with three times recrystallized egg albumen. Activity was measured at 37° C in

water, Ringer solution, or phosphate buffer, pH 6.8. Incubations were carried out in open test tubes or under nitrogen in Thunberg tubes. The concentration of amino groups (as glycine) was estimated periodically by the method of Yemm & Cocking (1955).

RESULTS

DENUATION OF RABBIT OVA BY ACROSOMAL LIPOGLYCOPROTEINS

The effects of the lipoglycoproteins are summarized in Table 1. To describe the extent of denudation of eggs the following arbitrary scale is used.

Cumulus still present	0
Cumulus removed, thick layer of corona cells present	1
Thin layer of corona cells	2
Complete removal of corona	3
Zona pellucida removed	4

We shall refer to state 3 as denudation.

TABLE 1

EFFECTS OF ACROSOMAL LIPOGLYCOPROTEINS (LGP) FROM RAM AND BULL SPERMATOOZOA UPON RECENTLY OVULATED RABBIT EGGS. INFLUENCE OF PROTEIN-BINDING REAGENTS AND OF A HYALURONIDASE INHIBITOR (53DK)

<i>Incubation medium</i>	<i>No. of experiments</i>	<i>Effect*</i>
Ringer-saline	5	0
Ringer-saline + 0.01% Hyamine	3	0
Ram LGP	5	3-4
Ram LGP (15 min at 100° C)	2	1
Ram LGP + 0.01 M-iodoacetate	2	1-3
Ram LGP + 0.01 M- <i>p</i> -chloromercuri-phenylsulphonate	2	3
Ram LGP + 53 DK (0.1 mg/ml)	2	1
Bull LGP	4	3-4
Bull LGP (15 min at 100° C)	2	1
Bull LGP + 0.01 M-iodoacetate	2	3
Bull LGP + 0.01 M- <i>p</i> -chloromercuri-phenylsulphonate	2	4
Bull LGP + 53 DK (0.1 mg/ml)	2	2-3

Each experiment was carried out with three eggs which were incubated at 37° C and examined at 6 and 24 hr.

* See text.

Each experiment was carried out with three rabbit eggs: thus the results in the last column of Table 1 are each based upon the observation of at least six eggs. The final concentration of lipoglycoprotein was 3 mg of protein/ml in all cases. After eggs had been incubated in Ringer-saline or in Ringer-saline containing 0.01 % Hyamine varying amounts of cumulus were still attached to the eggs (Pl. 1, Figs. 2 and 3). Experiments with the Ringer-saline-Hyamine medium serve as controls for all the following experiments in Table 1 since the residual Hyamine in the lipoglycoprotein preparations contributed not more than 0.01 % of Hyamine to incubation media containing either ram or bull lipo-

glycoprotein. Treatment with up to 1500 units/ml of hyaluronidase did not remove the corona (Pl. 1, Fig. 4).

Ram, bull and rabbit lipoglycoproteins brought about extensive denudation of eggs (Pl. 1, Fig. 5): cumulus and corona cells were invariably removed while the zona pellucida was removed in some instances and the vitellus attacked (Pl. 1, Fig. 6). After the lipoglycoproteins had been heated to 100° C their effects were limited to the cumulus layer. The two protein-binding reagents, iodoacetate and *p*-chloromercuriphenylsulphonate, had little or no effect upon the action of bull lipoglycoprotein but the former had a detectable, though variable, effect upon ram lipoglycoprotein. Iodoacetate (0.067 M) did not inhibit the activity of the commercial hyaluronidase preparation. The denuding activity of ram lipoglycoprotein, unlike that of bull lipoglycoprotein, was also sensitive to the hyaluronidase inhibitor 53DK. The two protein-binding reagents and 53DK each had no effect upon eggs in absence of lipoglycoprotein. Our preparations of acrosomal lipoglycoprotein from rabbit spermatozoa contained much non-acrosomal material. Of two such preparations one was active and removed the corona completely.

Substitution of acrosomal preparations by blood plasma glycoproteins A or B (see Materials and Methods) plus 0.01 % Hyamine gave results which were indistinguishable from those of experiments with Ringer-saline-Hyamine. Incubation of eggs in Ringer-saline containing 1 mM concentrations of lactic, pyruvic or sialic acids gave virtually negative results: in some cases a partial removal of cumulus cells was observed.

ENZYME ACTIVITIES OF ACROSOMAL LIPOGLYCOPROTEINS

The hyaluronidase activity of ram lipoglycoprotein was 940 TRU/mg while that of bull lipoglycoprotein was 43 TRU/mg. The less pure acrosomal preparation from rabbit spermatozoa contained 35 TRU/mg of protein. Masaki & Hartree (1962) found that when bull semen is stored at room temperature there is a transfer of hyaluronidase from the spermatozoa to the seminal plasma which is virtually complete in 6 hr. Under similar circumstances we have found that losses of hyaluronidase from ram spermatozoa are negligible. Thus with the latter species a valid comparison is possible between the level of hyaluronidase in spermatozoa and the level in the derived acrosomal lipoglycoprotein. In Table 2 the hyaluronidase activity of ram semen (i.e. of the spermatozoa in the semen) is derived from Hathaway & Hartree's (1963) examination of the activity of extracts obtained by treating ram spermatozoa with cetyltrimethylammonium bromide. The activity of ram lipoglycoprotein is calculated from the yield of lipoglycoprotein: 480 mg/100 ml of semen (Hartree & Srivastava, 1965). Table 2 includes similar figures for bull semen but it must be remembered that a significant proportion of the hyaluronidase originally present in the bull spermatozoa had no doubt passed into the plasma before isolation of acrosomes could be carried out.

Proteolytic activities are given in Table 3. Ram lipoglycoproteins showed appreciable activity in water or in Ringer solution. In water, activity is enhanced by addition of albumen or by absence of oxygen. This lipoglycoprotein was inactive in phosphate buffer except when both oxygen and albumen were

PLATE 1

FIG. 1. Rabbit ovum, recovered shortly after ovulation, 13 hr after LH injection ($\times 190$).

FIG. 2. Rabbit ovum, after incubation in Ringer-saline for 24 hr at 37°C ($\times 75$).

FIG. 3. Another rabbit ovum, treatment similar to that in Fig. 2 ($\times 120$).

FIG. 4. Rabbit ovum treated with hyaluronidase, 1500 units/ml, for 6 hr ($\times 190$).

FIG. 5. Rabbit ovum treated for 24 hr with a rabbit acrosomal lipoglycoprotein preparation ($\times 300$).

FIG. 6. Part of the vitellus of a rabbit ovum treated for 24 hr with ram acrosomal lipoglycoprotein ($\times 300$).

(Facing p. 64)

absent. Bull lipoglycoprotein showed some activity in air but this was largely abolished by the addition of albumen. These experiments suggest that the acrosomal preparation can catalyse the oxidation of amino acids. A similar enzymic oxidation of amino acids has been demonstrated in bull semen by Tosic & Walton (1950). In one case albumen caused an initial lag in proteolysis and in others an inhibition throughout the experiments. Since albumen also

TABLE 2

HYALURONIDASE ACTIVITY, AS TURBIDITY REDUCING UNITS, OF SEMEN
AND OF ACROSOMAL LIPOGLYCOPROTEINS

	<i>Ram</i>	<i>Bull</i>
Whole semen	≤ 21,000/ml of semen*	500 to 1000/ml of semen†
Lipoglycoprotein (LGP)	940/mg of LGP 4500/ml of semen	43/mg of LGP ~70/ml of semen

* Hathaway & Hartree (1963).

† Masaki & Hartree (1962).

TABLE 3

PROTEOLYTIC ACTIVITY OF RAM AND BULL LIPOGLYCOPROTEINS (LGP)

<i>Solvent:</i>	<i>Water</i>				<i>Ringer solution</i>			0.06 M phosphate buffer, pH 6.8			
<i>Gas phase:</i>	<i>Air</i>			<i>Nitrogen</i>	<i>Air</i>			<i>Air</i>		<i>Nitrogen</i>	
<i>Time of incubation (hr):</i>	1	2	3	0.5	1	2	3	1	2	1	2
Ram LGP	0.23	0.31	0.43	0.38	0.32	0.70	1.21	0	-0.05	0.61	0.72
Ram LGP + albumen	0.38	0.62	0.70	0.58	0.08	0.41	0.74	-0.06	-0.20	0.03	-0.03
Bull LGP	0.25	(0.08)	0.33		No significant changes						
Bull LGP + albumen	0.04	0.09	-0.07		No significant changes						

Reaction mixtures contained LGP (0.3 to 1.2 mg protein) and, where indicated, 100 μ g egg albumen in a total volume of 1.2 ml. Activities are expressed as changes in the content of free $-\text{NH}_2$ groups, measured by a ninhydrin method (Yemm & Cocking, 1955) against glycine standards, and expressed as μ -equiv. of $-\text{NH}_2$ /mg of LGP.

gave rise to increases in activity no interpretation of these effects can be suggested. Complete hydrolysis of 1 mg of lipoglycoprotein would give rise to 6 to 8 μ -equiv. of amino groups: therefore the observed proteolytic activities are rather low.

DISCUSSION

Our cell-free acrosomal lipoglycoprotein preparations from ram, bull and rabbit spermatozoa are able to disperse both cumulus and corona cells of rabbit eggs whereas the enzyme hyaluronidase only disperses the cumulus (Chang, 1950; Braden, 1952; Dickmann, 1962). The concentration of hyaluronidase in the

bull preparation is much lower than that in the ram preparation yet they are equally effective as agents for dispersal of both cumulus and corona cells. It is clear, therefore, that some factor other than hyaluronidase is involved in the denuding activity of these lipoglycoproteins. Furthermore, while the hyaluronidase inhibitor 53DK partially blocked the activity of ram lipoglycoprotein it had no significant effect upon dispersal by bull lipoglycoprotein. However, clear interpretation of experiments with 53DK is made difficult by its lack of specificity as an inhibitor of hyaluronidase and by the fact that its inhibitory action can be reduced by addition of protein (Parkes *et al.*, 1954). Our experiments support the views of Austin (1960) and Hathaway & Hartree (1963) that at least part of the hyaluronidase of spermatozoa is present in, or associated with, the acrosome. Since the extent to which detergents can extract hyaluronidase from acrosomes is not known, the levels of hyaluronidase in acrosomes of intact ram and bull spermatozoa may well be higher than the values given in Table 2 for the corresponding lipoglycoproteins.

The absence of any denuding effect by two plasma glycoproteins and the lack of activity of boiled acrosomal lipoglycoproteins suggest that one or more specific enzymes are responsible for the denudation that we have observed. Steric factors may be responsible for the lack of inhibitory action of such a large molecule as *p*-chloromercuriphenylsulphonic acid. The negligible effects of lactic, pyruvic and sialic acids are consistent with the hypothesis of an enzymic mechanism. Sialic acid was tested since acrosomal preparations contain bound sialic acid (Hartree & Srivastava, 1965) while the uterus of the rabbit contains neuraminidase, which liberates bound sialic acid, and an aldolase which converts sialic acid to pyruvic acid (Hartree & Srivastava, unpublished results).

In vivo, physical factors, such as ciliary movement and muscular activity in the fallopian tube, undoubtedly contribute to the denudation of eggs (Swyer, 1947). However, in the experiments listed in Table 1 the eggs remained undisturbed and it is almost certainly for this reason that denudation occurred at a slower rate than *in vivo* and that periods up to 24 hr were necessary for the full effect to be obtained. In the course of a few experiments in which tubes containing eggs were gently shaken, we did observe that denudation in the presence of either ram or bull lipoglycoprotein was considerably accelerated. In similarly treated controls the corona layer remained undisturbed. Denudation of rodent eggs, and also removal of the zona pellucida, can be brought about by different proteolytic enzymes (Yamane, 1930; Braden, 1952; Gwatkin, 1964; Edwards, 1964). Austin & Bishop (1958) have postulated the existence of a 'zona lysin' which dissolves the zona pellucida of rodent eggs. Since in certain rodents the acrosome is detached before penetration of the zona occurs these authors suggested that the lysin originates in the perforatorium, a structure which they observed between the acrosome and the sperm head. Attempts to isolate from spermatozoa a proteolytic enzyme which will dissolve or disperse the hyaluronidase-resistant layers around the ovum have hitherto been unsuccessful (Austin, 1961). In some experiments with acrosomal lipoglycoproteins we observed removal of the zona pellucida. These lipoglycoproteins also showed proteolytic activity. It is possible that proteolytic activity lies not in the acrosome but in an adjacent structure, and proteolytic activity of the lipo-

glycoproteins may be only a contaminant and therefore unrepresentative of the potential activity of spermatozoa.

Our investigations provide the first experimental evidence that proteolytic enzymes of spermatozoal origin contribute to the denudation of rabbit eggs and probably facilitate penetration of the zona pellucida.

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