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THE ACTIVATION OF PROACROSIN IN SPERMATOZOA FROM RAM, BULL AND BOAR

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Summary

Acrosin activity was estimated in fractions from washed ram, bull and boar spermatozoa that had been disrupted using a Stansted Cell Disruptor.

When *p*-aminobenzamidine was included in the medium during disruption, all the acrosin (acrosomal proteinase, EC 3.4.21.10) was recovered as its inactive zymogen form, proacrosin. But if spermatozoa were damaged before disruption, or were disrupted in the absence of *p*-aminobenzamidine, considerable amounts of active acrosin were detectable. It was concluded that conversion of proacrosin to acrosin takes place in spermatozoa only after the acrosome has been ruptured.

In a sucrose medium, all the proacrosin was bound to the sperm heads. Conversion to acrosin took place readily with all components in a bound state. Using ram sperm heads, the conversion was found to be relatively insensitive to pH, proceeding rapidly above pH 6.5; the rate of conversion was not affected by physiological levels of Ca^{2+}, Mg^{2+} or Zn^{2+}, although elevated ionic strength caused a solubilization of the acrosin activity and some slowing of the rate. Electrophoretic analysis revealed that several active forms of acrosin were involved, but the final product was a single stable form.

Final levels of the active acrosin (expressed as μmol *N*-α-benzoyl-L-arginine ethyl ester utilised/min per 10^9 heads) were: ram 26.2; bull, 15.9; boar, 133.8. But active site titration revealed that these different levels were not reflected in the numbers of active enzyme molecules on the sperm head; boar acrosin appears to be about three times more active towards benzoyl-arginine ethyl ester than do the acrosins from the other species.

Introduction

Acrosin (acrosomal proteinase, EC 3.4.21.10) is a trypsin-like proteinase associated exclusively with the heads of spermatozoa. It is apparently located

within the acrosome, bound to the inner acrosomal membrane, and it is believed to play an essential role in egg penetration [1,2]. Acrosin is derived from an inactive proenzyme form, proacrosin, which has recently been isolated from several sperm species [3—7]. Various workers have studied the conversion of proacrosin to acrosin in vitro using purified soluble extracts, but no study of the process has been made under conditions thought to approximate to those in vivo, i.e. with the components in a bound state. Moreover it is not known at what point in time in the life of the spermatozoon the conversion occurs in vivo. In this paper, we wish to present data which indicate that activation of proacrosin does not take place until disruption of the acrosome has occurred. We have also studied the activation process as it occurs with the components attached to the sperm head.

Materials and Methods

Spermatozoa. Individual ejaculates were obtained from Friesian bulls, Suffolk rams and crossbred boars; only the sperm-rich fractions of the boar ejaculates were used. Ram epididymal semen was obtained from slaughterhouse material by retrograde flushing of the cauda epididymidis [8]. Precautions were always taken to avoid temperature-shock [9].

The spermatozoa were washed free of seminal plasma by being centrifuged through a sucrose medium. Semen samples were diluted in 0.154 M saline to a concentration of about $7 \cdot 10^8$ cells per ml, and 1-ml portions were layered on top of 7.5 ml of Medium S: 0.264 M sucrose/0.1 mM pAB (*p*-aminobenzamidine hydrochloride)/5 mM MES (2-(*N*-morpholino)-ethane sulphonic acid) pH 6.5. After centrifugation in a swinging bucket rotor at $600 \times g_{max}$ for 5 min followed by $1100 \times g_{max}$ for 10 min, the supernatant layers were removed by aspiration and the loose pellet resuspended in Medium S to a volume of 1 ml.

The washing procedure was carried out at about 20°C. Cell recoveries were in excess of 90% and more than 98.5% of the (diluted) seminal plasma was removed.

Disruption of spermatozoa. Spermatozoa were disrupted using a Stansted Cell Disruptor (Stansted Fluid Power Ltd., Stansted, Essex, U.K.) Model AO 612 fitted with a No. 316 valve, operating with a dial setting of 2.8 bars back pressure (3.2 bars for boar spermatozoa). Samples (3 ml) of washed sperm suspensions were placed in a syringe barrel (5 ml size) attached to the inlet of the Disruptor, and were overlaid with about 5 ml of ice-cold Medium S diluted with water so that the sucrose concentration was 0.2 M. The Disruptor was then operated until nearly all the contents of the syrine barrel had passed through the apparatus; more overlay was added and the apparatus further operated, until a total of 15 ml of sperm suspension plus overlay had passed through. This, termed the homogenate, was collected at 0°C.

The Stansted Cell Disruptor is an excellent new method for disrupting spermatozoa, satisfying all of the criteria mentioned by Harrison [10]. The cell suspension is pumped under pressure through an annular valve controlled by back pressure, and disruption is caused by the resultant liquid shear forces. These forces are related to the back pressure which can be varied over a wide and continuous range, thus a closely controlled range of breakage can be achieved.

Under the disruption conditions we used, more than 99% of heads were removed from tails; principal pieces were also fragmented, but midpieces remained largely intact. Examination by electron microscopy revealed that plasma membranes had been severely disrupted and were mostly absent from all the cell fragments. However the internal structure of the principal piece fragments and of the midpieces and their mitochondria looked the same as in intact spermatozoa. On bull and ram sperm heads (Fig. 1a), the outer acrosomal membrane was largely removed over the anterior region, although it remained over the posterior region (equatorial segment); the inner acrosomal membrane seemed entirely intact and the post-acrosomal material was still present to a

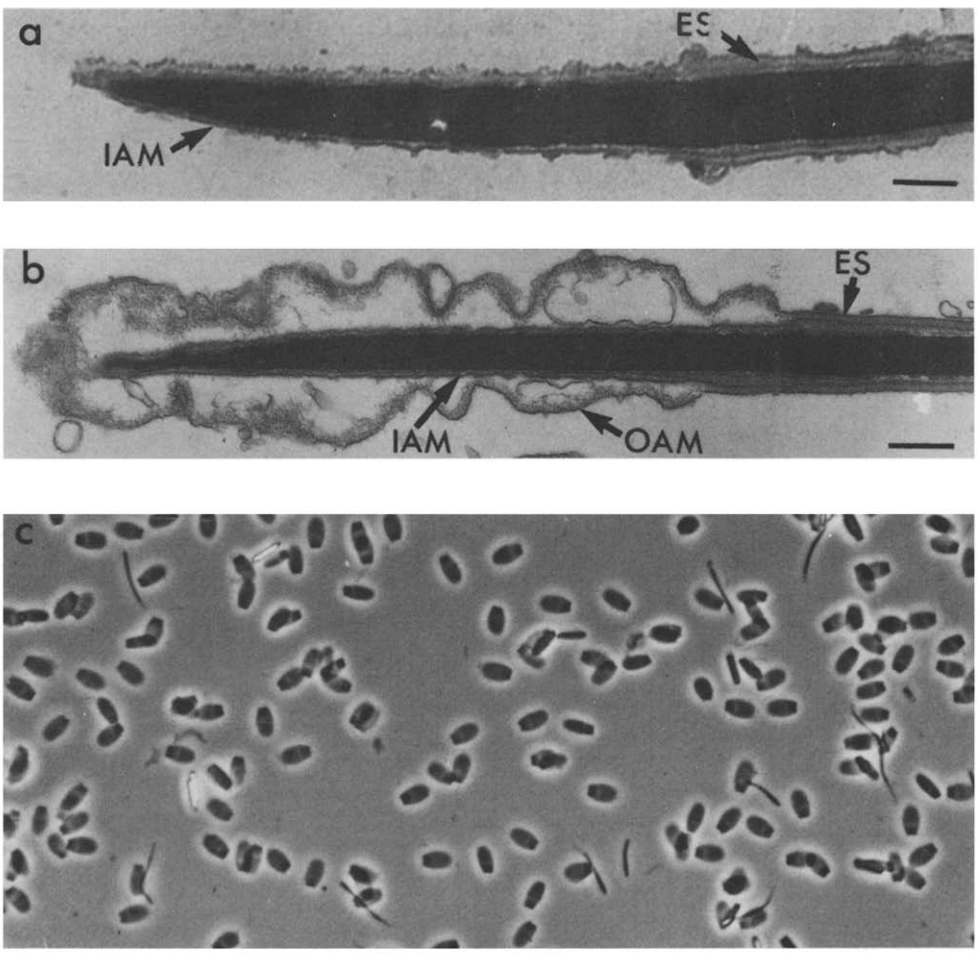

Fig. 1. Appearance of sperm head fractions obtained after cell disruption using the Stansted Cell Disruptor. (a) Electron micrograph of a bull sperm head (fraction H_W). The outer acrosomal membrane (OAM), inner acrosomal membrane (IAM) and equatorial segments (ES) are indicated where present. The bar represents 0.25 μm. (b) Electron micrograph of a boar sperm head (fraction H_W). (c) Phase contrast micrograph of ram sperm heads (fraction H_W), × approx. 500. For electron microscopy, the fractions were fixed for 1 h at 4°C in 4% glutaraldehyde in 0.2 M collidine buffer (pH 7.2) containing 3% (w/v) sucrose. They were post-fixed in 1% osmium tetroxide and processed by standard procedures.

very high degree. Boar sperm heads were even less damaged; on a large proportion, the outer acrosomal membrane was still present, and although the acrosome was greatly swollen, material could be seen remaining within (Fig. 1b).

Fractionation procedures. All fractionation procedures were carried out at 4°C. Portions (7.5 ml) of the homogenate were layered over 2.5-ml steps of 0.5 M sucrose/0.1 mM pAB/5 mM MES (pH 6.0), and centrifuged at 600 × g_{max} for 20 min in a swinging-bucket rotor. The cell cytoplasm, plasma membrane fragments and tail fragments remained in the supernatant layer, midpieces were mostly collected at the interface, while the heads with some midpieces passed through the infranatant layer and formed a pellet (H).

This pellet, resuspended to 2 ml in 0.2 M sucrose containing 0.1 mM pAB, was layered over 7.5 ml of 0.5 M sucrose/5 mM MES (pH 6.0) and centrifuged at 750 × g_{max} for 20 min, to yield a pellet (H_w) which consisted of sperm heads contaminated only by a few midpieces (Fig. 1c). H_w was used immediately (i.e. within 30 s), as the head preparation employed in most of the studies.

Counts of sperm heads showed that recovery of material after disruption was 100% and that overall recovery of heads in H_w was in excess of 70%.

Acrosin assays. The requisite sample (0.4 ml), usually of sperm heads in suspension, was acidified to pH 2.4 with a few μl of 1 M HCl. It was left for 15 min at 4°C and then centrifuged at 12 000 × g_{max} for 15 min. The supernatant contained all the acrosin or proacrosin in the sample and was used for enzyme assays or for electrophoretic analysis. (At pH 2.4 conversion processes are arrested and protein inhibitor · acrosin complexes are dissociated; both proacrosin and acrosin are very stable under these conditions.)

Acrosin activity was measured spectrophotometrically using *N*-α-benzoyl-L-arginine ethyl ester as substrate [11]; a unit of enzyme activity hydrolyses 1 μmol of substrate per min. The operational molarity of the sample (active site titration) was determined fluorimetrically using 4-methyl-umbelliferyl *p*-guanidinobenzoate as the active site titrant [12].

Electrophoretic analyses. Electrophoresis was carried out on 7.5%T, 5%C polyacrylamide gels (70 mm × 16 mm) using an acid gel system essentially that of Reisfeld et al. [13]. The major modification was the composition of the running buffer, which was 35 mM β-alanine adjusted to pH 4.9 with acetic acid; also, stacking and sample gels were dispensed with. Samples of acrosin were layered onto the gel in the presence of 4 μmol glycine (pH 2.4), to prevent the appearance of a spurious band of proacrosin, and methyl green was included as front marker. Electrophoresis was carried out at room temperature, using 3 mA per gel.

Staining for acrosin and proacrosin was carried out as follows: gels were immersed in 0.1 M Tris/20 mM $CaCl_2$ (pH 8.2), for 30 min at 20°C, with occasional agitation (the buffer was replaced with fresh after 10 min); then the gels were immersed in a solution containing 2 mM *N*-α-benzoyl-DL-arginine-2-naphthylamide, 2 mg Fast Garnet GBC/ml and 5% (v/v) dimethylsulphoxide in the Tris/Ca^{2+} buffer. (The stain solution was made up freshly; the Fast Garnet was dissolved in 10 mM HCl and insoluble material removed, before it was added to the rest of the components.) After incubation at 37°C in the dark for 1—2 h, bands representing acrosin activity appeared; the gels were then fixed, destained and stored in 7% (v/v) acetic acid at 4°C.

Preincubation in the Tris/Ca^{2+} buffer at pH 8.2 allowed activation of proacrosin in the gels, while no appreciable inactivation of acrosin occurred. So when the gels were stained, the locations of both acrosin and proacrosin bands became visible. The position of proacrosin was inferred from gels whose samples were enzymatically inactive before electrophoresis. A similar staining method has been used by Polakoski and Parrish [6].

Determination of sperm (head) concentrations. Sperm head concentrations were estimated in suitably diluted subsamples by haemocytometry; where necessary, sonication (15 s) was used to disperse agglutinated heads in the subsamples beforehand.

Results

Acrosin activity after cell disruption (see Table I)

When spermatozoa were disrupted and heads isolated in the absence of pAB, high levels of acrosin activity were associated with the head fractions as had been described previously [14—17]. However, when pAB was included in the washing and fractionation media, little or no acrosin activity could be detected in any of the fractions. Removal of pAB from the heads (as in preparation of H_w from H) resulted in rapid appearance of acrosin activity in them.

Although pAB is an inhibitor of acrosin [18], failure to detect activity in head preparations that had been prepared in pAB-containing medium was not due to the presence of the inhibitor, because when 0.1 mM pAB was added to already active acrosin samples it did not affect the detectable activity. Also, although there was a natural inhibitor of acrosin in the spermatozoa, this was soluble (see ref. 2) and was therefore completely removed from the heads during the fractionation procedure. No inhibitor could be detected [19] in any head preparations, either when these were already active or when they were being allowed to activate following removal of pAB. As pAB is known to inhibit the conversion of proacrosin (inactive zymogen) to acrosin [20], we have therefore concluded that all the acrosin in intact spermatozoa is in the

TABLE I

ACROSIN ACTIVITY IN SPERM HEADS: EFFECT OF *p*-AMINOBENZAMIDINE

Sperm heads fractions were prepared in the presence or absence (i.e. pAB was omitted from all the washing and fractionation media) of 0.1 mM *p*-aminobenzamidine (pAB), and pAB was subsequently removed, as described in Materials and Methods. Acrosin activity was measured in acid extracts of these fractions, and is expressed as units of enzyme activity per 10^9 sperm heads. Mean values are given ± S.E., with the number of samples shown in parentheses.

Sperm species	Acrosin activity in		
	Heads (fraction H) prepared in absence of pAB	Heads (fraction H) prepared in presence of pAB	Heads (fraction H_w) 60 min after removal of pAB
Ram	11.2 ± 1.6 (3)	0.0 ± 0.0 (3) *	26.2 ± 1.5 (3)
Bull	13.6 ± 1.8 (3)	0.0 ± 0.0 (3)	15.9 ± 1.2 (3)
Boar	106.2 ± 0.7 (3)	0.50 ± 0.10 (3)	133.8 ± 5.4 (3)

* Below the limits of detection (<0.1 unit per 10^9 heads).

inactive zymogen form, but that disruption of the spermatozoa allows rapid conversion to the active form.

The conversion of proacrosin to acrosin

Although head preparations remained inactive in the presence of pAB, acrosin activity appeared rapidly as soon as pAB was removed. This finding was used to study the conversion process in sperm heads from ram.

Sperm heads (fraction H_w) were prepared (the final stage of which involved the removal of pAB). They were immediately dispersed in a buffered medium at 25°C, whose composition varied according to the aims of the experiment. Conversion of proacrosin began at once, and this was monitored by acidifying samples of the suspension at intervals (thus arresting the conversion) and analysing the acid extracts as described in Materials and Methods. These extracts yielded results pertaining to the total proacrosin/acrosin content of the sample. The distribution of the enzymatic activity between heads and incubation medium before acid extraction was determined by centrifuging a sample at 12 000 $\times g_{max}$ for 3 min (using a Misco microcentrifuge, Microchemical Specialities, Calif., U.S.A.); the supernatant and pellet were then acidified and processed as usual.

The time course of the conversion with respect to appearance of activity is shown in Fig. 2.

There was no obvious evidence of any "lag" phase (i.e. of the order of 1—2 h [7,21,22]), and the conversion was virtually complete after 20 min; of the total acrosin activity that had appeared, only a small portion (4.7%) was non-sedimentable after 45 min. Thus, because during preparation of the starting material the sperm heads had essentially been washed twice, it was clear that the

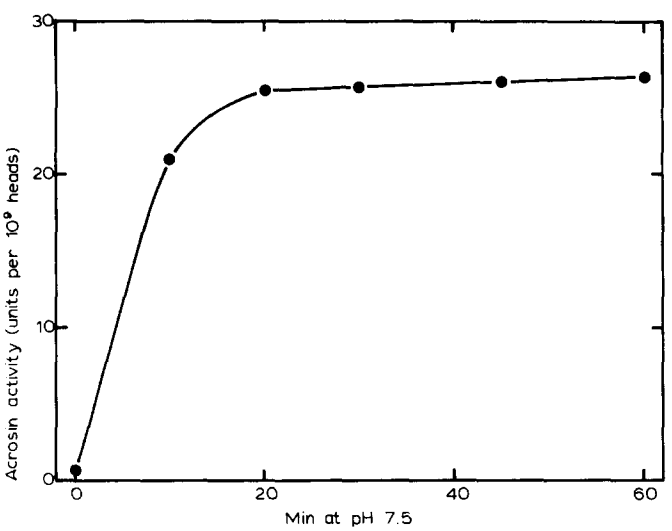

Fig. 2. Time course of proacrosin activation on ram sperm heads. Sperm heads (fraction H_w) were prepared, and were immediately resuspended in 0.25 M sucrose, to which was added 1/10 vol. of 0.2 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 0.2 M Tris (final pH 7.55, final head concentration $6.5 \cdot 10^8$/ml). The suspension was then incubated at 25°C. At intervals, 0.4-ml samples were withdrawn and acid extracts made for acrosin analysis.

entire conversion could proceed effectively with all the components in a bound state.

The effect of pH on the conversion is illustrated in Fig. 3. The experiment involved measurements of activity throughout the activation period (as in Fig. 2), but because the shape of the activation curve with time was essentially similar at all pH values tested, only the activity values at 10 and 60 min are shown. In all cases, conversion (in terms of activity levels) was complete by 60 min. Over the physiological range, pH had little effect, either on the rate of activation (inferred by the activity after 10 min) or on the total activity (after 60 min); virtually all the activity was found to be particulate. Below pH 6.5 both the rate of activation and the total activity produced were clearly lower, and above pH 8.5 they were higher. However, because these observations seemed to correspond only to relatively non-physiological conditions, they were not investigated further.

Electrophoretic analysis of the extracts during these experiments revealed that three enzymic forms were produced as the conversion of proacrosin to acrosin proceeded (Fig. 4). We have designated these forms α, β and γ, in ascending order of their electrophoretic migration distance and in accordance with the nomenclature of Polakoski and Parrish [6]. Although the rate of conversion was slower at pH 6.0 than at pH 8.0, the sequence of events and the products of activation were identical; the α and γ forms were transient and the final stable product of activation was the β form. The precise order of production of the forms was not clear but a possible sequence appeared to be $\gamma \rightarrow \alpha \rightarrow \beta$. A point of interest was that the rate of production of β as judged by electrophoretic analysis seemed to be much slower than the rate of appearance of acrosin activity as judged by enzyme assays; this implies that the transient α and γ forms are enzymatically active forms rather than zymogen intermediates.

It has been reported by other workers [4,5] that divalent metal cations have

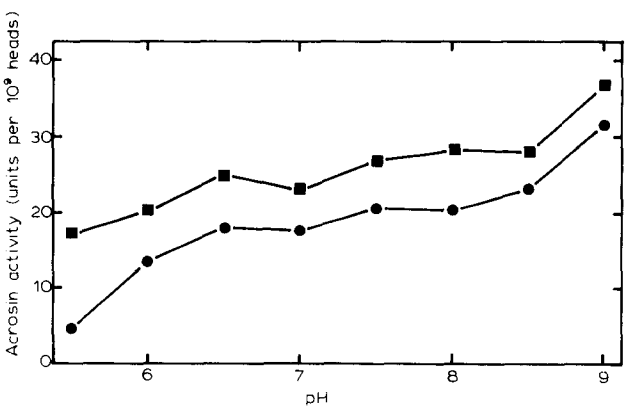

Fig. 3. Effect of pH on proacrosin activation on ram sperm heads. Sperm heads were allowed to activate and the acrosin activity monitored, as described in Fig. 2. However, the composition of the buffer added to the head suspension varied: 0.2 M MES, 0.2 M Tris (pH 5.5), or 0.2 M HEPES, 0.2 M Tris (pH 7.0, 7.5, 8.0, 8.5, or 9.0). The pH of each suspension was measured after mixing. The activities observed at 10 min (●) and 60 min (■) are shown.

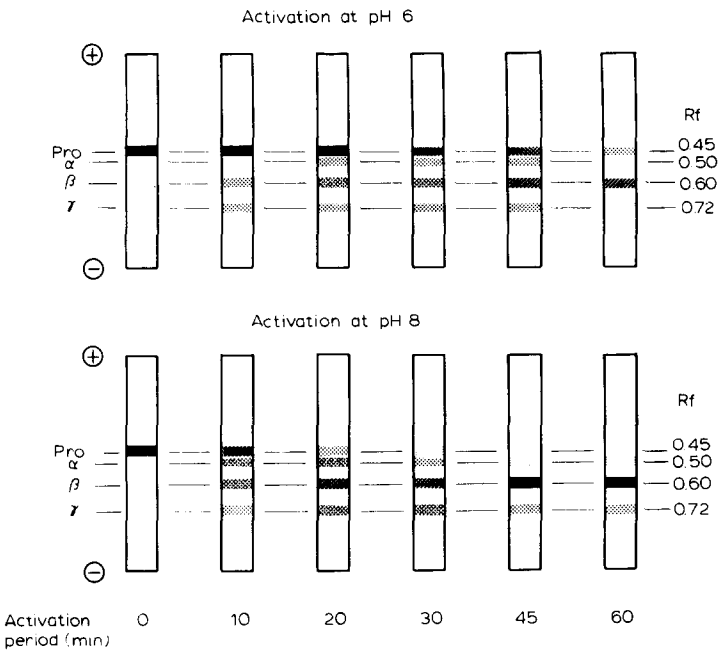

Fig. 4. Electrophoretic analysis of the conversion of proacrosin to acrosin on ram sperm heads. Acid extracts obtained during the course of the experiment described in Fig. 3 were subjected to electrophoresis on polyacrylamide gels. The gels were then stained for acrosin activity, as described in Materials and Methods.

profound effects upon the kinetics of the conversion of proacrosin to acrosin. The effect of these cations and of ionic strength was investigated using H_w as the source of proacrosin, essentially with the same methodological approach as in earlier experiments.

The effect of ionic strength (NaCl) is shown in Fig. 5. At pH 6.0 appreciable acrosin activity was non-sedimentable even at low concentrations of NaCl, whereas at pH 8.0 only NaCl concentrations above 50 mM had any effect on the degree of solubilization. At higher levels of NaCl, the rate of conversion tended to be slower and may not have been complete after 40 min. As in the experiments on the effect of pH, lower final levels of acrosin activity resulted at pH 6.0. A comparative electrophoretic analysis (not shown) of the total, non-sedimentable and sedimentable fractions of the proacrosin/acrosin population revealed no difference between them, other than a tendency for proacrosin to be preferentially retained in the sedimentable fractions.

The effect of some divalent cations on the conversion was investigated at pH 7.5, both at low and at high ionic strengths. The results are illustrated in Figs. 6 and 7. At low ionic strengths, physiological levels (3 mM or less) of Ca^{2+} and Zn^{2+} had no effect; 10 mM Ca^{2+} caused considerable solubilization (cf. ref. 18), and there was an overall inhibitory action of 10 mM Zn^{2+}. Mg^{2+} (results not shown) had absolutely no effect on any of the parameters. At high ionic strengths, neither 1 mM Ca^{2+} nor 1 mM Zn^{2+} had any effect; 10 mM Ca^{2+} slowed the rate of conversion, while 10 mM Zn^{2+} appeared to reduce the amount of acrosin produced. The proportion of soluble activity was high in all

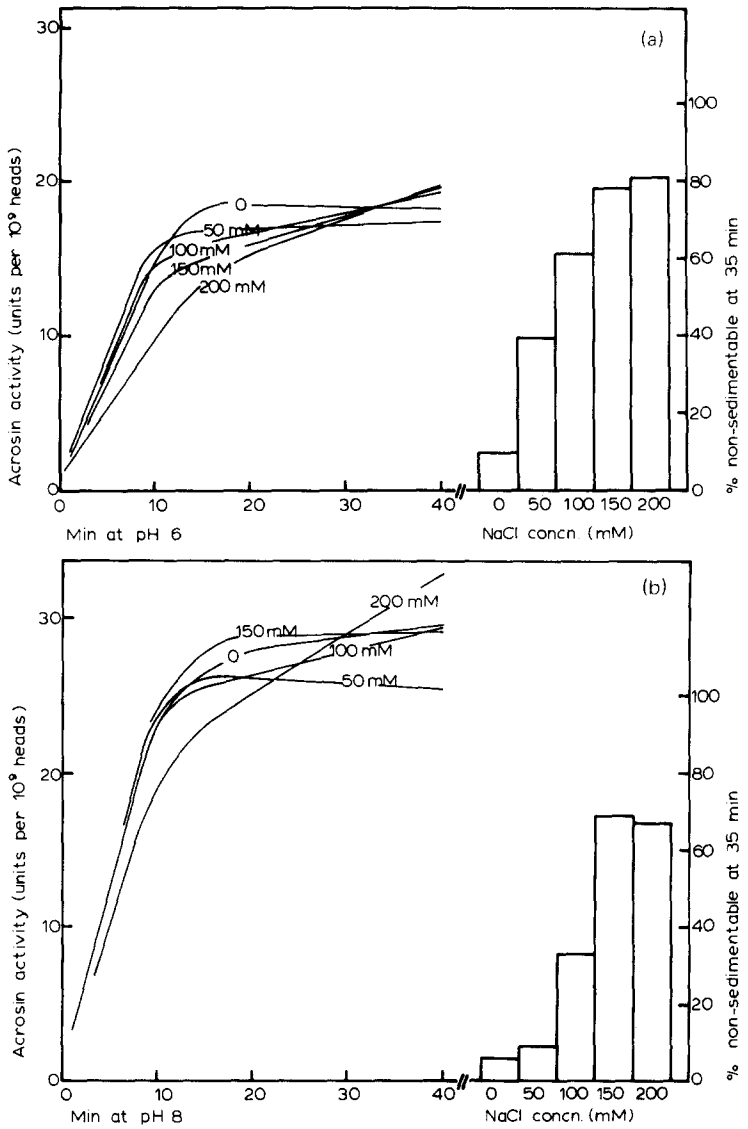

Fig. 5. Effect of ionic strength on proacrosin activation on ram sperm heads. Sperm heads (fraction H_W) were prepared, and resuspended in sucrose. Immediately portions were mixed with buffer and saline to yield suspensions containing various salt concentrations at pH 6 and pH 8. The suspensions were then incubated at 25°C. After 0, 10, 20 and 40 min, acid extracts were made for acrosin analysis. After 35 min, a sample was centrifuged to provide a non-sedimentable fraction, which was then acidified; from this was estimated the proportion of active acrosin that was non-sedimentable at 35 min. Final conditions were: 0, 50, 100, 150 and 200 mM NaCl in (a) 0.25 M sucrose/20 mM MES/20 mM Tris (pH 6.0); (b) 0.25 M sucrose/20 mM HEPES/20 mM Tris (pH 8.0). Final head concentrations were approx. $4 \cdot 10^8$/ml. The bar chart indicates the proportion of activity at 35 min that was non-sedimentable under the given conditions.

the saline media, as would be expected from the results shown in Fig. 5; it was noted that Zn^{2+} tended to reduce the degree of solubilization. As in the earlier experiments, there was no sign of a "lag" phase under any conditions.

In preliminary experiments, 1 mM EDTA, and 0.4 mM Zn^{2+} were added

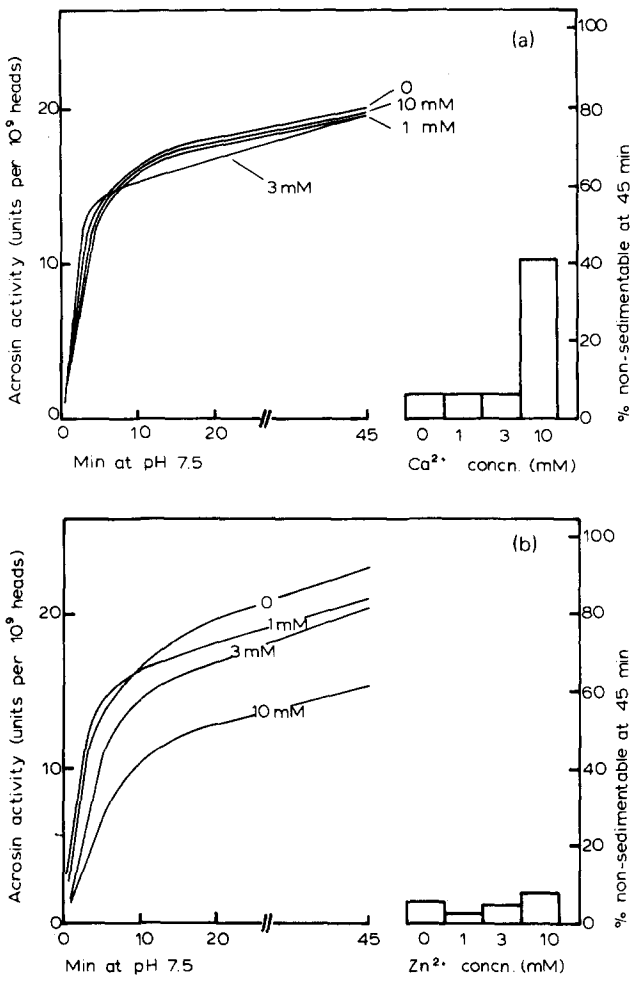

Fig. 6. Effect of Ca^{2+} and Zn^{2+} on proacrosin activation on ram sperm heads. Sperm heads were allowed to activate at pH 7.5 and the acrosin activity monitored, essentially as described in Fig. 2. The medium contained 0.25 M sucrose and various concentrations of Ca^{2+} (as $CaCl_2$) (a) or Zn^{2+} (as zinc citrate) (b), buffered at pH 7.5 with 20 mM HEPES. At 45 min, samples were centrifuged to provide a non-sedimentable fraction, from which the proportion of active acrosin that was non-sedimentable was estimated. Final head concentrations were approx. $3.5 \cdot 10^8$/ml. The bar chart indicates the proportion of activity at 45 min that was non-sedimentable under the given conditions.

individually to the homogenization medium, in the absence of pAB, but neither ion had any effect on the activity of acrosin in the sperm fractions. The later experiments, on the effect of ions on the model system, were in accord with these early observations.

Acrosin activity on the sperm heads of different species

Acrosin activity values for sperm head preparations that were fully activated have been collected for various species and presented in Table II. It will be noted that the activity varied considerably between species; boar sperm heads were especially active. However simultaneous determination of the operational molarity (of the acid extracts) revealed that the species variation was not

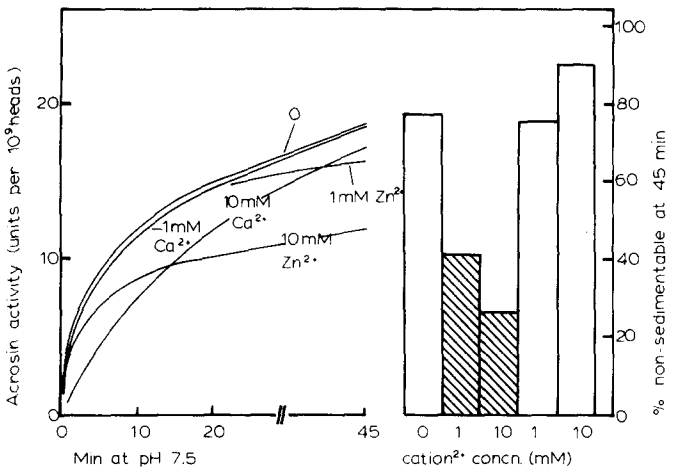

Fig. 7. Effect of Ca^{2+} and Zn^{2+} on proacrosin activation on ram sperm heads in a saline-containing medium. Sperm heads were allowed to activate at pH 7.5 exactly as described in Fig. 6, except that the medium also contained 0.154 M NaCl. Final head concentrations were approx. $4 \cdot 10^8$/ml. The bar chart indicates the proportion of activity at 45 min that was non-sedimentable in the presence of the different concentrations of Ca^{2+} (□) or Zn^{2+} (▨).

TABLE II

A COMPARISON OF THE ACTIVITIES AND OPERATIONAL MOLARITIES OF FULLY ACTIVATED ACROSIN IN DIFFERENT SPERM SPECIES

Enzyme activity is expressed as μmol substrate cleaved/min per 10^9 spermatozoa, operational molarity [12] as nmol enzyme per 10^9 spermatozoa, and molecular activity as μmol substrate cleaved/min per μmol enzyme. Values are given ± S.E., with the number of samples in parentheses. The molecular activity was calculated from the other two parameters.

Species	Enzymic activity	Operational molarity	Molecular activity
Ram	26.2 ± 1.5 (3)	3.08 ± 0.19 (3)	8 500
Bull	15.9 ± 1.2 (3)	1.64 ± 0.08 (3)	9 700
Boar	133.8 ± 5.4 (3)	4.74 ± 0.14 (3)	28 200
Guinea pig	53.6 (1)	6.46 (1)	8 300

necessarily reflected in the molar content of active acrosin on the sperm heads: it is clear that the high acrosin activity observed in boar spermatozoa was due partly to boar acrosin being unusually active towards benzoyl-arginine ethyl ester.

Discussion

Meizel and Mukerji [4] reported that virtually all the acrosin in washed rabbit epididymal sperm was in a zymogen form which they called proacrosin. Since that time, they and others have extended this observation to the spermatozoa of hamster [5], human [7,23], bull [24,25], and boar [6,20]. We have now extended this list further to include ram, and have confirmed the observations of others with respect to bull and boar. We conclude that there is no active

acrosin in intact ejaculated spermatozoa: all the enzyme is in the zymogen form.

There are two main consequences of this finding. Firstly, experiments have apparently demonstrated that active acrosin is essential for the penetration of eggs by spermatozoa [26,27]. Thus conversion of proacrosin to acrosin must take place in vivo. The question now arises, at what stage in the spermatozoon's life does the conversion occur? One possibility is that contact with seminal plasma triggers activation (cf. ref. 28). However, Table III shows that there was essentially no difference in the levels of active acrosin in pAB-inhibited heads between epididymal and ejaculated spermatozoa, or between freshly ejaculated spermatozoa and spermatozoa that had been in contact with seminal plasma for 5 h. On the other hand, Polakoski and Parrish [6] have implied that removal of seminal plasma triggers activation. But this hypothesis is not borne out by the results of the experiment shown in Table IV: acrosin activity was very low in extracts of washed spermatozoa, provided the spermatozoa had not been damaged previously; however, cold shock or deep freezing caused the appearance of considerable acrosin activity. Because such treatments are known to cause disruption of the acrosome (e.g. ref. 29), in which the acrosin is believed to be located (see ref. 30 and refs. therein), this finding, coupled with the rapid activation observed following cell disruption in the Stansted Cell Disruptor, leads us to the conclusion that proacrosin is converted to acrosin following physical disruption of the acrosome. The event in vivo that would correspond to such a situation is, of course, the acrosome reaction (see ref. 2). We therefore suggest that proacrosin is converted to acrosin in vivo during or as a result of the acrosome reaction; such a possibility has also been suggested by Zahler and Polakoski [25].

The question remains, how is proacrosin activation triggered, or how is activation prevented within the acrosome? Spermatozoa contain an acrosin inhibitor which is intrinsic to the cells [2,31] and which we believe to be located within the acrosome (although this is not yet strictly proven). Our experiments,

TABLE III

ACROSIN ACTIVITY IN SPERMATOZOA FOLLOWING CONTACT WITH SEMINAL PLASMA (EJACULATION

Sperm head fraction H was prepared from spermatozoa in the presence of pAB. The spermatozoa were either form the epididymis (ram only), or from fresh ejaculates, or from ejaculates incubated at 25°C for 5 h. Acrosin activity was measured in acid extracts of the head fractions, and is expressed as units per 10^9 sperm heads ± S.E., with the number of samples in parentheses.

Sample		Acrosin activity
Ram:	epididymal spermatozoa	0.0 * ± 0.0 (3)
	freshly ejaculated spermatozoa	0.0 ± 0.0 (3)
	ejaculated spermatozoa after 5 h in seminal plasma	0.30 ± 0.17 (3)
Bull:	freshly ejaculated spermatozoa	0.0 ± 0.0 (3)
	ejaculated spermatozoa after 5 h in seminal plasma	0.03 ± 0.03 (3)
Boar:	freshly ejaculated spermatozoa	0.50 ± 0.10 (3)
	ejaculated spermatozoa after 5 h in seminal plasma	0.47 ± 0.09 (3)

* Below the limits of detection (<0.1 unit per 10^9 heads).

TABLE IV

ACTIVATION OF PROACROSIN IN RAM SPERMATOZOA FOLLOWING COLD SHOCK OR DEEP FREEZING

Ram spermatozoa were washed and were then diluted with 0.154 M NaCl to about $0.3 \cdot 10^9$ cells/ml ('washed' cells). A sample of untreated semen was similarly diluted with 0.154 M NaCl ('unwashed cells'). The two suspensions were incuated at 25°C for 10 min, after which one aliquot of each was frozen rapidly to −79°C and kept there for 10 min ('deep frozen') while another was cooled as rapidly as possible to 0°C and kept there for 10 min ('cold shocked'); a third aliquot was maintained at 25°C ('control'). After the 10 min period, the cooled aliquots were warmed up as quickly as possible to 25°C and all aliquots were incubated at 25°C for a further 10 min. The spermatozoa in each aliquot were then washed in Medium S (containing 0.1 mM pAB) as described in Materials and Methods, and afterwards frozen and thawed twice (−79°C) to disrupt them. They were layered onto 3 ml of 0.5 M sucrose/0.1 mM pAB/5 mM MES (pH 6.0), centrifuged at $1000 \times g_{max}$ for 10 min followed by $12\,000 \times g_{max}$ for 10 min using an angle rotor, and the supernatant layers discarded. Finally the pellets were extracted with 0.1 M glycine buffer (pH 2.4). The extracts were assayed for acrosin as described in Materials and Methods. Activity is expressed as units per 10^9 cells.

Sperm sample	Acrosin activity
Washed	
control	0.66
cold shocked	3.2
deep frozen	5.4
Unwashed	
control	0.84
cold shocked	2.4
deep frozen	4.5

as well as those of others [6,7,20,23—25], show that proacrosin activation is inhibited by synthetic inhibitors of acrosin. Although the acrosomal inhibitor is a relatively poor inhibitor of bound acrosin, its effective concentration within the acrosome could be very high [18], perhaps sufficiently high for it to inhibit the activation of bound proacrosin. We should like to propose, therefore, that proacrosin activation is inhibited within the intact acrosome by the sperm acrosin inhibitor. When the acrosome is disrupted, the inhibitor will be released into the medium (see ref. 2, and our experiments) and its effective concentration will be drastically lowered; inhibition will be relieved and activation will begin. The feasibility of our hypothesis is indicated by recent evidence for the interaction of the zymogen trypsinogen with a protein trypsin inhibitor [32].

A second consequence of the finding that active acrosin is only produced following cell (acrosomal) disruption is that estimations of acrosin activity in sperm extracts are rendered more complicated. Several studies have demonstrated variable levels of active acrosin in sperm extracts prepared in different ways [28,33—35]. It now seems likely that most, if not all, of the variation stems from differences in the amount of activation that had occurred under the various extraction conditions; the degree of acrosome disruption, the pH of the environment, the presence of sperm and seminal plasma acrosin inhibitors, and the state of (pro)acrosin (membrane-bound or soluble) must alls interact in this respect. Values for acrosin activity that have been reported previously must therefore be reviewed in this light. Our data are the first estimates for acrosin

content of spermatozoa specifically made from a fully activated system. The values obtained for ram agree well with values calculated from the data of Brown and Hartree [16], and the values for bull agree well with values calculated from the maximal activity found by Zahler and Polakoski [25]; on the other hand, the values for boar are greatly in excess of those found by Johnson et al. [35], whose extracts, however, are unlikely to have been fully activated.

All detailed studies of proacrosin activation described hitherto have involved solubilized extracts which had been at least partially purified. As far as we are aware, ours is the first study to demonstrate conclusively that proacrosin is converted to acrosin efficiently when all components involved are in a bound state on the sperm head. It is believed that acrosin acts in vivo as a membrane-bound enzyme (see ref. 2), thus our activation system may be similar to that occurring in vivo.

The characteristics of proacrosin activation are not at all clear despite many studies [4—6,20,22,25]. One obvious reason for differences observed is that proacrosins from different sperm species have different properties. But there are other possible reasons, among them the wide variation in experimental conditions, and differences in the purity of the proacrosin preparations studied. Thus differences between the characteristics of our membrane-bound proacrosin activation system and the previously reported characteristics of other systems can only be pointed out; the reason for the differences may not be the membrane-bound nature of our relatively impure system.

The effect of pH upon the membrane-bound system is not pronounced between pH 6.5 and 8.5. Some workers have found a marked effect of pH upon proacrosin activation [4,20,22], while others have found little effect [5]. Our observations resemble most closely those of Zahler and Polakoski [25], who found little difference in final levels between pH 6.5 and 9.5, but considerable difference in initial rate.

We observed little evidence for a sigmoid activation curve (cf. refs. 4, 6 and 22), although the initial phase of the sigmoid either may have occurred during the final centrifugation stage of H_w preparation after heads had passed out of the pAB-containing medium or was not detected because of the rather wide sampling intervals used in our activation experiments. Nevertheless, there was certainly no extended "lag" phase before activation began (such as described by Meizel and Huang-Yang [21], Mukerji and Meizel [22], and Tobias and Schumacher [7]). This extended lag period appears to be due to impurities [21], possibly acrosin inhibitors (which were not present in our system).

Ca^{2+} and Zn^{2+} have been reported [4,5,20] to have a pronounced effect upon proacrosin activation, the former stimulatory, the latter inhibitory. However, we were unable to detect any stimulatory effect of calcium at levels up to 10 mM, and in other experiments (unpublished) have repeatedly found a depressive effect of calcium (at levels up to 200 mM) on the activation of soluble proacrosin preparations. Zn^{2+} was somewhat inhibitory but the effect appeared to be on the acrosin produced rather than on the activation process; it is of interest that Mukerji and Meizel [36] have found that zinc will inactivate rabbit acrosin.

Several forms of acrosin have been found in sperm extracts from human [37], boar [20], bull and ram [24], and the molecular basis for the multiple

forms in boar spermatozoa have been proposed in some detail by Polakoski and Parrish [6]. Our findings for ram spermatozoa resemble those of Polakoski and Parrish [6] for boar, in that three acrosin forms, α, β, and γ, are produced from proacrosin. But in ram the sequence of appearance is not in the order $\alpha \rightarrow \beta \rightarrow \gamma$; it may be that the α and γ forms are not intermediates but are labile species produced as alternatives to the stable predominant β form. The existence of several active acrosin species adds a new dimension to the elucidation of properties, especially characteristics of the activation process described above; some enzyme forms may be more readily produced or be more active than others under certain conditions; are these the forms produced under conditions pertaining to those in vivo? Our studies indicate that the production of the various acrosin forms proceeds in an essentially similar manner in our proacrosin activation system in all the different conditions we have tested so far, and that one major species is produced, the β-form. It is this form which has been purified and characterized by Brown and Hartree [38] (see also ref. 2).

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