The location of acrosin and proacrosin in ram spermatozoa

R. A. P. Harrison, J.-E. Fléchon† and C. R. Brown

A.R.C. Institute of Animal Physiology, Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ, U.K. and† I.N.R.A. Station Centrale de Physiologie Animale, 78350 Jouy-en-Josas, France

Summary. Acrosin and its zymogen form proacrosin were located in various sperm fractions by biochemical and immunocytochemical techniques, using an anti-acrosin serum that cross-reacts strongly with proacrosin.

If activation of proacrosin was prevented, the zymogen was associated almost entirely with the sperm heads, where it was confined to the anterior segment of the acrosome. Electron microscopy revealed that the acrosomal lumen of such heads remained full of matrix material, and the outer acrosomal membrane remained closely apposed to the other head structures. However, if activation had been allowed to take place, the resultant acrosin and the remaining proacrosin were associated with all sperm fractions and no specific location was observed. In these circumstances, the matrix material was almost entirely absent from the heads, and the outer acrosomal membrane, though usually still present, was only loosely attached.

It is concluded that (1) neither acrosin nor proacrosin are truly membrane-bound; they behave as ‘diffusible’ or partly soluble proteins, although they display a non-specific affinity for cell membrane and other surfaces; (2) proacrosin is part of the acrosomal matrix material, and is not located specifically on the inner or the outer acrosomal membrane; (3) the matrix material plays an important mechanical role in stabilizing the position of the outer acrosomal membrane relative to the inner acrosomal membrane; it disperses during proacrosin activation.

Introduction

It has been deduced from biochemical and other studies that acrosin, a trypsin-like proteinase (EC 3.4.21.10), is attached to the inner acrosomal membrane in spermatozoa and acts at fertilization to help the sperm cell cut its way through the zona pellucida surrounding the egg (Stambaugh & Buckley, 1969; Brown & Hartree, 1974; Schill & Wolff, 1974; Zahler & Doak, 1975; Hartree, 1977). If smears of spermatozoa are treated first with anti-acrosin and then with a labelled antibody to immunoglobulin (indirect immunocytochemical technique), an intense and specific staining of the acrosomal region is obtained (Garner, Easton, Munson & Doane, 1975; Morton, 1975; Schill, Schleuning, Fritz, Wendt & Heimbürger, 1975; Garner & Easton, 1977).

However, it is now known that there is no active acrosin in intact spermatozoa: all the acrosin is present as its zymogen form proacrosin (Meizel & Mukerji, 1975; Polakoski & Parrish, 1977; Brown & Harrison, 1978). Conversion to the active form takes place only after

acrosomal disruption (Brown & Harrison, 1978; Green, 1978a). During an earlier study (Fléchon, Huneau, Brown & Harrison, 1977), we were unable to detect acrosin immunocytochemically in smears of disrupted spermatozoa, despite the fact that these preparations contained their normal high levels of acrosin and despite the fact that our anti-acrosin serum has since been shown to react strongly with both acrosin and proacrosin (Harrison, 1982).

We have therefore re-investigated the immunocytochemical staining method for (pro)acrosin, and then used this technique, together with biochemical and other methods, to reassess the location of acrosin and proacrosin in ram spermatozoa.

Materials and General Methods

Spermatozoa: isolation, disruption and fractionation

Semen from Ile-de-France or Suffolk rams was diluted with isotonic saline and washed by the method of Brown & Harrison (1978), usually in the presence of 0.5 mM-*p*-aminobenzamidine (*p*AB, a substance which strongly inhibits the conversion of proacrosin to acrosin). The washed spermatozoa were disrupted with a Stansted Cell Disruptor as described by Brown & Harrison (1978).

The disrupted spermatozoa (aliquots of about 1×10^8 cells) were fractionated by centrifugation on linear sucrose gradients (10–65% (w/v) in H_2O , total volume 25 ml) at 900 g_{max} for 15 min at 4°C, using the Spinco SW25.1 rotor.

Sperm head preparations were obtained by centrifuging the disrupted spermatozoa gently through a sucrose layer containing *p*AB; the resultant pellet was washed by further centrifugation through sucrose at pH 6.0 in the absence of *p*AB. The final pellet (fraction H_w : Brown & Harrison, 1978) consisted of the sperm heads mixed with a small proportion of midpiece fragments; it was used immediately (because *p*AB had been removed in the final wash stage and therefore activation of proacrosin was likely to occur).

Sperm (head) counts were performed by haemocytometry.

Electron microscopy

The primary fixative used was 2% (w/v) tannic acid, 1.25% (w/v) glutaraldehyde, 1% (w/v) formaldehyde in 200 mM-sucrose, 100 mM-sodium cacodylate, pH 7.0 and 1.5 mM- $CaCl_2$; treatment was for 1 h at room temperature. After washing in sucrose–cacodylate– $CaCl_2$ overnight at 4°C, secondary fixation was performed at room temperature with 1% (w/v) osmium tetroxide in sucrose (160 mM)–cacodylate– $CaCl_2$. The samples were then dehydrated and embedded in an Epon-based resin using standard procedures. Ultra-thin sections were stained with uranyl acetate and lead citrate.

Assay of acrosin and proacrosin

Acrosin activity was measured either spectrophotometrically using benzoyl arginine ethyl ester as substrate (Brown, Andani & Hartree, 1975), or fluorometrically using benzoyl arginine 2-naphthylamide (BNA) (Brown & Hartree, 1976). Proacrosin was estimated as potential acrosin activity, using thermolysin to activate the zymogen (Harrison & Brown, 1979). Determinations of activity in sperm fractions were always made after acid extraction (see Brown & Harrison, 1978).

Immunocytochemistry

Anti-acrosin serum was raised in rabbits essentially according to Fléchon *et al.* (1977) using as antigen pure ram β -acrosin that had been completely inhibited with di-isopropyl

phosphofluoridate (Brown & Hartree, 1978). The antiserum was characterized in a number of ways and shown to react strongly and specifically with the major naturally occurring forms of both proacrosin and acrosin (Harrison, 1982).

After preliminary experiments (described below), the following staining procedure was adopted.

Air-dried smears of spermatozoa or sperm fragments, stored desiccated at 4°C in the dark before staining, were preincubated unfixed for 15 min with 3% (v/v) normal sheep serum and 0.5 mM-*p*AB in 250 mM-sucrose, 20 mM-phosphate, pH 7.0. They were then incubated for 30 min with 1–2% (v/v) rabbit anti-acrosin serum in sucrose phosphate containing 0.5% (v/v) normal sheep serum (no *p*AB present), and, after rinsing 3 times in serum-free medium, were further incubated for 30 min with 1% (v/v) fluorescein-conjugated sheep anti-rabbit immunoglobulin (Institut Pasteur, Paris) in sucrose phosphate supplemented with 0.5% (v/v) normal sheep serum. All incubations were carried out in a moist chamber, in the dark at room temperature. Finally, the smears were rinsed twice in sucrose phosphate, pH 7.0, and then once in sucrose phosphate, pH 8.0 (fluorescence of fluorescein conjugates is maximal around pH 8.0: Chadwick & Fothergill, 1962). The slides were mounted wet in glycerol.

Experimental Methods and Results

Immunocytochemical staining for proacrosin and acrosin: methodological studies

It is now established that intact sperm cells contain only proacrosin (Brown & Harrison, 1978). Although during the air-drying process proacrosin in smeared spermatozoa may be expected to remain as the zymogen, it seems likely that the proacrosin will activate when the staining solutions are applied because the spermatozoa have been damaged during the drying. Previously published methods for immunocytochemical staining of acrosin have employed saline media (Garner *et al.*, 1975; Morton, 1975; Fléchon *et al.*, 1977), but Brown & Harrison (1978) demonstrated that a considerable proportion of acrosin is released into the medium if proacrosin activation takes place under saline conditions. Such release during staining might be preventable by prior fixation of the spermatozoa (Schill *et al.*, 1975; Garner & Easton, 1977; Fléchon *et al.*, 1977). However, Morton (1977) noted that the reactivity of acrosin with anti-acrosin was very sensitive to fixation.

Experiments were therefore carried out to test the effects of fixation, use of sucrose media for staining, and inclusion of *p*AB in media during staining (to prevent activation of proacrosin). Air-dried smears of ram semen diluted in saline were immersed in 1% (w/v) formaldehyde (in sucrose phosphate) for 5 min, in acetone for 10 min, or in methanol for 30 min, and then rinsed briefly in sucrose phosphate. Staining was carried out on fixed and unfixed smears using as medium 20 mM-phosphate, pH 7.0, containing 250 mM-sucrose or 130 mM-NaCl; the effect of such media was tested at each stage of the staining procedure, in the presence or absence of 0.5 mM-*p*AB. Stained smears were coded and randomized, and then examined for intensity and character of fluorescence.

The effect of ionic strength was dramatic. When a sucrose medium was used throughout the procedure, staining was confined to the anterior region of the acrosome (Pl. 1, Fig. 1), i.e. the equatorial segment remained unstained (see Fléchon *et al.*, 1977). However, use of a saline medium during preincubation and during anti-acrosin treatment led to staining as a halo around the acrosome (Pl. 1, Fig. 2) while the acrosome itself was much less intensely stained; in the large majority of cases, the more intense the halo the less intense the staining of the acrosome itself. Although halo formation in the saline medium was reduced considerably by prior fixation, such fixation (particularly methanol or formaldehyde treatment) also reduced overall intensity of staining. Inclusion of *p*AB during the preincubation step led to increased intensity of staining, but its inclusion during anti-acrosin treatment seemed to reduce attachment of the antibody.

Following these results, the protocol described in the 'General Methods' section was adopted.

The distribution of (pro)acrosin between sperm fractions before and after activation

Spermatozoa were washed and disrupted in the presence or the absence of *pAB*. The *pAB*-containing sample was then fractionated immediately on a sucrose density gradient, while the *pAB*-free sample was incubated at pH 7.5 for 1 h at 25°C (to allow activation of proacrosin to occur) before being fractionated in a similar fashion. (For details of methodology, see 'General Methods'.) The resultant fractions from the two samples were examined morphologically and analysed for acrosin and proacrosin by the fluorometric assay method.

The results are shown in Text-fig. 1. In the *pAB*-containing sample ('before activation'), almost all the detected acrosin had remained in the zymogen form which was also almost all associated with the sperm heads. In the incubated *pAB*-free sample ('after activation'), much active acrosin had appeared. However, nearly 60% of the total acrosin (including zymogen) in this preparation was associated with midpieces, tail fragments and membrane particles. Specific activity measurements relating total (proacrosin + acrosin) activity to sperm head concentration showed that before activation there were 253 ± 33 milliunits of BNA-splitting activity/ 10^9 heads in the head-containing fractions, whereas after partial activation there were only 115 ± 21 milliunits/ 10^9 heads. The discrepancy was clearly accounted for by the activity associated with the tail and midpiece fractions in the activated sample.

Text-fig. 1. Distribution of proacrosin and acrosin in ram sperm fractions (a) before and (b) after zymogen activation. Spermatozoa washed in the presence of *pAB* were disrupted and fractionated immediately by centrifugation through a sucrose gradient ('before activation'). Spermatozoa washed in the absence of *pAB* were incubated at pH 7.5 for 1 h after disruption, to allow activation of proacrosin to proceed before fractionation. The direction of sedimentation is from left to right.

The outer acrosomal membrane

Spermatozoa were denuded of their outer acrosomal membranes by the method of Brown & Hartree (1974) and the resulting suspension (consisting of denuded cells, acrosomal fragments

Immunocytochemical staining of ram spermatozoa and sperm head preparations using rabbit anti-acrosin and fluorescein-labelled anti-rabbit IgG.

Fig. 1. Unwashed spermatozoa, stained using sucrose based media throughout the procedure. Most anterior segments are strongly stained. $\times 600$.

Fig. 2. Unwashed spermatozoa, stained using saline-based media during preincubation and anti-acrosin treatment. Note the diffuse (halo) staining around the heads. $\times 600$.

Fig. 3. Sperm head preparation before proacrosin activation (acrosin activity ≈ 0.3 units/ 10^9 heads). Only the anterior segments are strongly stained. $\times 1500$.

Fig. 4. Sperm head preparation towards the end of activation (acrosin activity ≈ 17.9 units/ 10^9 heads). Weak staining, mainly on equatorial segments and tail fragments. $\times 1500$.

(Facing p. 352)

Correlation of phase contrast appearance of ram sperm preparations with immunofluorescent staining.

Fig. 5. Head preparation at the start of proacrosin activation (acrosin activity = 1.0 unit/ 10^9 heads). Three heads (arrowed) have darkened equatorial segments, indicating that acrosomal material has been lost from the anterior region. $\times 1500$.

Fig. 6. The same field as in Fig. 5 seen under fluorescence illumination. Only those heads which have not lost acrosomal material are strongly stained. $\times 1500$.

Fig. 7. Head preparation at a late stage of proacrosin activation (acrosin activity = 13.5 units/ 10^9 heads). The anterior acrosomal regions are all severely disrupted and little material remains. One head (arrowed) is completely denuded. $\times 1500$.

Fig. 8. The same field as Fig. 7 seen under fluorescence illumination. There is only faint staining to be seen. $\times 1500$.

Electron microscopical appearance of ram sperm head preparations before and after proacrosin activation.

Fig. 9. Before proacrosin activation ('non-activated' preparation). The acrosomal matrix is present. $\times 32\,000$.

Fig. 10. After proacrosin activation ('activated' preparation). The ruptured outer acrosomal membrane remains loosely attached, but the acrosomal contents have almost completely disappeared. $\times 32\,000$.

and other debris, equivalent to 3.6×10^9 spermatozoa) was submitted to centrifugation through steps of 1.3 M- and 1.75 M-sucrose, to isolate the acrosomal membranes (Zahler & Doak, 1975). Fractions were collected from this separation system and analysed.

Two bands of material other than spermatozoa were observed and isolated; the fractions were examined by phase contrast microscopy and assayed for acrosin and proacrosin activity. The first band of material contained small unidentifiable particles and 5.3 milliunits (pro)acrosin (BNA-splitting activity); the second contained clearly identifiable acrosomal membrane fragments and 1.9 milliunits (pro)acrosin; and the sperm pellet contained 742 milliunits (pro)acrosin. The outer acrosomal membrane had been removed completely from 30% of the spermatozoa and partly from a further 60%. No other acrosin activity could be detected, and recovery was complete. It was concluded that active acrosin was not associated specifically with the outer acrosomal membrane; the finding confirmed earlier work (Brown & Hartree, 1974; Schill & Woff, 1974).

However, during the isolation process the proacrosin in the heads had largely been activated; attempts were therefore made to 'denude' sperm heads under conditions inhibitory to proacrosin activation. In the presence of *pAB*, neither the procedure of Brown & Hartree (1974) nor the procedure of Zahler & Doak (1975) were in any way effective (on ram or bull spermatozoa). No swelling or lifting of the acrosomal cap was observed, and no detachment could be achieved.

Immunocytochemical localization of (pro)acrosin in sperm head fractions before and after proacrosin activation

Sperm head fractions that had been prepared in the presence of *pAB* and then washed free of this inhibitor were placed immediately in 250 mM-sucrose, 5 mM-Hepes, pH 7.5, and allowed to activate at 25°C as described by Brown & Harrison (1978). At intervals, 0.4 ml aliquots were removed and added to 0.1 ml of 125 mM-Mes, pH 6.0, containing 2.5 mM-*pAB* to halt the activation. Smears were made and stained immunocytochemically for (pro)acrosin, and samples of the mixtures were acidified for acrosin assay.

Staining of the heads was qualitatively inversely proportional to the degree of proacrosin activation. When practically all the acrosin was in the form of proacrosin, staining was bright on most heads and was confined to the anterior region of the acrosome (Pl. 1, Fig. 3), as observed in smears of intact spermatozoa. As activation proceeded, this staining became less intense but more diffuse, over the entire head. By the time activation was complete, only a diffuse staining could be seen which was mainly on the equatorial segment but also to some degree on the post-acrosomal region (Pl. 1, Fig. 4); the anterior region of the acrosome was least stained. After proacrosin activation, considerable staining was also observed on the midpiece fragments which were present as contamination in the fractions. Both the diffuse staining of the 'activated' heads and this staining of the midpieces were specific for acrosin and were not seen in similar smears treated with non-immune serum or with anti-serum that had been absorbed with acrosin. Moreover, 'activated' sperm head fractions from which all acrosin had been extracted by CaCl_2 treatment (Brown & Hartree, 1974) could not be stained at all; but when the extracted heads were further exposed to an acrosin solution and re-isolated, they had re-adsorbed acrosin and were weakly stainable again over the entire head region.

The morphology of sperm head fractions before and after proacrosin activation

The large majority of heads in 'non-activated' preparations (i.e. in which proacrosin activation had been prevented) appeared under phase-contrast optics to have retained their acrosome; i.e. the acrosomal region was darker, the apical ridge apparent and the equatorial segment invisible or slightly paler than its surround. After proacrosin activation (i.e. in 'activated' preparations), however, practically all the heads seemed to have lost their acrosome:

acrosomal region pale, no apical ridge, equatorial segment darker than its surround, lifting or detached membranes. Observation of individual fields in immunocytochemically stained preparations, using first phase-contrast illumination and then fluorescence, showed that only those heads which appeared to have an acrosome were strongly stained for (pro)acrosin (Pl. 2, Figs 5–8). Heads without an obvious acrosome were at best poorly stained.

Electron microscopy revealed that, although the heads in 'non-activated' preparations lacked a plasma membrane, most retained an almost intact acrosome (Pl. 3, Fig. 9). The outer acrosomal membrane showed some degree of 'blebbing' but otherwise seemed generally complete, and the acrosomal matrix was full of material of a granular nature. Although soluble components of the acrosomal lumen had undoubtedly escaped (the matrix of undisrupted spermatozoa had a more uniform appearance), only at the apex of the acrosome had material often clearly been lost (adjacent to a more condensed region). However, in 'activated' preparations, the very large majority of heads, although still possessing an outer acrosomal membrane, were entirely or almost entirely devoid of acrosomal matrix material (Pl. 3, Fig. 10). The outer acrosomal membrane was attached only loosely around the head; ruptured, the membrane was usually arranged in folds, although sometimes it was vesiculated in a fashion reminiscent of an acrosome reaction. The inner acrosomal membrane appeared unchanged.

Discussion

In a previous study (Fléchon *et al.*, 1977), we were unable to detect acrosin immunocytochemically in a disrupted sperm preparation that apparently contained normal amounts of acrosin activity. The new findings provide a clear explanation for our earlier paradoxical observations. The present study may be considered in two parts: verification of the immunocytochemical technique for locating acrosin, followed by a reinvestigation of (pro)acrosin location using this method in conjunction with others.

Immunocytochemistry. The degree to which an anti-acrosin serum will cross-react with zymogen and with multiple active forms (Brown & Harrison, 1978) is crucial, for upon this will depend the degree to which the enzyme's presence can be revealed immunocytochemically. Although certain anti-acrosin sera do not appear to cross-react with other (pro)acrosin forms (Syner, Kuras & Moghissi, 1979; Mukerji, Scibienski & Meizel, 1980), it has been shown (Harrison, 1982) that the anti-acrosin serum used in the present study reacted strongly with ram proacrosin as well as with forms of active ram acrosin other than the stable β -form to which the antiserum was raised. There was little doubt therefore that all the major forms of acrosin present in ram sperm preparations were being detected immunocytochemically.

Comparison of the immunocytochemical fluorescence of different samples is difficult, and would be further hampered by a variable response to staining. From our results it is clear that the methodology is important if consistent results are to be obtained. Acrosin appears very sensitive to fixatives (see also discussion in Morton, 1977), and therefore it is necessary to carry out the staining under conditions in which the unfixed enzyme is least soluble (i.e. in sucrose media of low ionic strength) so that as little diffusion as possible occurs. Our technique has permitted comparison of samples (e.g. at different stages of activation) with some certainty.

(Pro)acrosin as a soluble enzyme. The precise location of acrosin and other acrosomal enzymes has already been the subject of much research. Following the work of Stambaugh & Buckley (1969, 1970), biochemical investigations have indicated that acrosin is a bound enzyme attached to the inner acrosomal membrane (Brown & Hartree, 1974; Schill & Wolff, 1974; Zahler & Doak, 1975); this conclusion was arrived at via the sedimentation characteristics of acrosin in sperm homogenates taken together with the histochemical or immunochemical localization findings of others, and is currently the generally held opinion. Faltas, Smith & Stambaugh (1975), on the other hand, claimed that proteinase activity was associated with the

outer acrosomal membrane of rabbit spermatozoa. Shams-Borhan, Huneau & Fléchon (1979) demonstrated association of acrosin with the external surface of the outer acrosomal membrane of disrupted bull spermatozoa, but were unable to locate any acrosin on the inner acrosomal membrane. Studies of the gelatinolytic action of spermatozoa (Gaddum & Blandau, 1970; Allen, Bishop & Thompson, 1974) have suggested that an enzyme closely resembling acrosin is readily released from the acrosomal region from many sperm species. Also Schill & Fritz (1975) reported release of acrosin into seminal plasma during ageing of human semen. Some evidence has thus already been presented to suggest that acrosin is not intrinsically a bound enzyme and that it is not attached to the inner acrosomal membrane.

From our investigations, it is now possible to explain the earlier conflicting and confusing reports. Proacrosin produces active acrosin only if the sperm cells are disrupted (Brown & Harrison, 1978). We believe that activation of proacrosin is prevented within the spermatozoon by the presence of locally high concentrations of an acrosin inhibitor within the acrosome, but that following the acrosome reaction *in vivo* the inhibitor disperses and the proacrosin then activates spontaneously (see discussions in Brown & Harrison, 1978; Harrison & Brown, 1979). The results of our localization studies have shown clearly both by biochemical assays and by immunocytochemistry that proacrosin is confined to the anterior region of the acrosome on the sperm head, so long as activation of the zymogen is prevented. If proacrosin is allowed to activate, however, both it and the resultant acrosin tend to disperse either into the supernatant (especially in media of higher ionic strengths: Brown & Harrison, 1978) or onto other cellular components; this 'migration' occurs even in media of very low ionic strength when acrosin and proacrosin behave as though entirely membrane-bound (Brown & Hartree, 1974; Brown & Harrison, 1978).

Acrosin can therefore no longer be regarded as truly a membrane-bound enzyme. Although it has a tendency to bind to biological and other materials (see also Müller-Esterl, Kupfer & Fritz, 1980; Straus, Parrish & Polakoski, 1981), it must be considered at least as a 'diffusible' molecule. The enzyme's ambivalent behaviour can be ascribed to its hydrophobic nature (Brown & Hartree, 1978; Müller-Esterl & Fritz, 1980) and is nicely illustrated by the production of 'halos' of fluorescent stain when immunocytochemical staining of acrosin is carried out in saline media: (pro)acrosin disperses from the acrosomal region, through membranes disrupted by air drying, but tends to adhere to the surrounding glass surface (Pl. 1, Fig. 2).

The discovery of acrosin's 'diffusibility' resolves two puzzles, at least. Our earlier apparent inability to locate acrosin immunocytochemically in disrupted spermatozoa (Fléchon *et al.*, 1977) must have been due partly to the dispersion of (pro)acrosin from the acrosomal region following the activation resultant from cell disruption, and partly to the saline staining medium having eluted much of the dispersed acrosin from the cell fragments. The gelatinolytic activity of smeared spermatozoa (Gaddum & Blandau, 1970), so apparently in contradiction to the believed behaviour of acrosin, is also explained; the 'halos' produced in the gelatin film are indeed due to diffusion of acrosin from the sperm heads.

Intracellular location of proacrosin. With respect to the precise intracellular location of proacrosin and acrosin, it is clear from our results that in intact spermatozoa the proacrosin is confined to the anterior region of the space bounded by the acrosomal membranes: the immunocytochemical appearance (Pl. 2, Fig. 6) of sperm heads lacking a plasma membrane (Pl. 3, Fig. 9) is identical to that of untreated spermatozoa (Pl. 1, Fig. 1), i.e. proacrosin is not associated with the plasma membrane.

We can find no evidence that after activation acrosin is located primarily on the acrosomal membranes. Immunocytochemical staining of 'activated' heads (all of which possess an inner acrosomal membrane at least: Pl. 3, Fig. 10) reveals that the anterior acrosomal region is less stained than the rest of the head whereas the equatorial segment is often quite strongly stained (Pl. 1, Fig. 4). The equatorial segment, though, can hardly be regarded as the location of active acrosin because in 'non-activated' heads this region remains unstained (see also Fléchon *et al.*,

1977). And although under certain conditions (usually while activation is proceeding) the outer acrosomal membrane may appear strongly stained, our biochemical fractionation experiments confirmed previous observations that acrosin is not specifically associated with it (c.f. Brown & Hartree, 1974; Zahler & Doak, 1975). It seems to us more likely that any apparent association of acrosin with the equatorial segments, or with the outer acrosomal membrane (as reported by Faltas *et al.*, 1975; Garner, Reamer, Johnson & Lessley, 1977; Green & Hockaday, 1978; Shams-Borhan *et al.*, 1979), is due to a temporary initial concentration of (pro)acrosin on those surfaces as the protein starts to diffuse away from its true location, the acrosomal matrix.

The suggestion that acrosin was located in the acrosomal matrix was made by Fléchon in 1973, though at that time no firm evidence was available. Our morphological observations and our other findings now provide such evidence. The majority of sperm heads in non-activated preparations stain intensely for (pro)acrosin in the anterior region of the acrosome; these intensely stained heads always appear under phase-contrast microscopy to possess an acrosome (displaying a characteristic darker, thicker anterior region) whereas the heads which remain weakly stained always appear as if they have been denuded (pale anterior region). The heads in activated preparations are all only weakly stained and all appear denuded. In fact, electron micrographs show that most heads in both types of head preparation possess an outer as well as an inner acrosomal membrane. However, whereas in the majority of heads in the non-activated preparations the acrosomal matrix material is still very largely present, filling the anterior acrosomal lumen, in the activated preparations this material is almost entirely absent, with only a few remnants attached to the outer acrosomal membrane. Thus the presence and location of the acrosomal matrix as seen by electron microscopy coincides with the presence and location of proacrosin deduced from immunocytochemistry and biochemistry, and would account for the specific darkened appearance of the stainable heads. It seems very probable therefore that, in ram spermatozoa at any rate, proacrosin constitutes part of the acrosomal matrix material in the anterior segment of the acrosome. Direct evidence, such as positive location by labelling at the electron microscopic level, is still needed.

Acrosin and the acrosome reaction. Involvement of acrosin in the acrosome reaction has been reported by Meizel & Lui (1976), Lui & Meizel (1979) and Green (1976, 1978a). Meizel (1978) proposes a central role for acrosin in the reaction whereas Green (1978b) assigns only the dispersal of the acrosomal matrix to acrosin. While obviously the absence of a plasma membrane precluded any kind of an acrosome reaction taking place in our experiments, the retention of very large elements of the outer acrosomal membranes, albeit ballooned, after activation of proacrosin did seem to show that acrosin is not directly responsible for disintegrating and dispersing this membrane. The ruptures observed in the outer acrosomal membrane after activation were almost certainly caused by the mechanical preparation procedures which removed the plasma membrane; more obvious signs of general disintegration would have been expected from a biochemical susceptibility to acrosin. On the other hand, there was a distinct impression that the dispersal of the acrosomal matrix material 'loosened' the outer acrosomal membrane from the head leaving it free to balloon. Our observations therefore support the ideas of Green (1978b). We suspect that the acrosomal matrix is a rather solid structure which maintains close apposition of the acrosomal membranes. The dispersal of the matrix through the activation of proacrosin (perhaps an integral part of the matrix structure) induces loosening of the outer acrosomal membrane in our artificial system of heads denuded of plasma membrane, and would lead to a loosening of the 'acrosomal cap' (outer acrosomal membrane, overlying plasma membrane and associated matrix material) in a true acrosome reaction. Prevention of proacrosin activation would prevent such loosening and explains our inability to prepare an outer acrosomal fraction in the presence of *pAB*, described above. This 'mechanical' role of (pro)acrosin is completely unrelated to any possible biochemical involvement of acrosin in the fusion processes involved in the physiological acrosome reaction (see Meizel, 1978), a consideration of which is beyond the scope of the present work.

We are very grateful to many of our colleagues for help and advice given during the course of this work, in particular to M. Daniel Huneau for considerable help with the electron microscopy.

References

- Allen, G.J., Bishop, M.W.H. & Thompson, T.E. (1974) Lysis of photographic emulsions by mammalian and chicken spermatozoa. *J. Reprod. Fert.* **36**, 249–252.
- Brown, C.R. & Harrison, R.A.P. (1978) The activation of proacrosin in spermatozoa from ram, bull and boar. *Biochim. Biophys. Acta* **526**, 202–217.
- Brown, C.R. & Hartree, E.F. (1974) Distribution of a trypsin-like proteinase in the ram spermatozoon. *J. Reprod. Fert.* **36**, 195–198.
- Brown, C.R. & Hartree, E.F. (1976) Identification of acrosin in mouse spermatozoa. *J. Reprod. Fert.* **46**, 249–251.
- Brown, C.R. & Hartree, E.F. (1978) Studies on ram acrosin. Activation of proacrosin accompanying the isolation of acrosin from spermatozoa and purification of the enzyme by affinity chromatography. *Biochem. J.* **175**, 227–238.
- Brown, C.R., Andani, Z. & Hartree, E.F. (1975) Studies on ram acrosin. Isolation from spermatozoa, activation by cations and organic solvents, and influence of cations on its reaction with inhibitors. *Biochem. J.* **149**, 133–146.
- Chadwick, C.S. & Fothergill, J.E. (1962) Fluorochromes and their conjugation with proteins. In *Fluorescent Protein Tracing*, pp. 4–30. Ed. R. C. Nairn. Livingstone Ltd, Edinburgh.
- Faltas, S., Smith, M. & Stambaugh, R. (1975) The organized distribution of acrosomal proteinase within the acrosomes of rabbit spermatozoa. *Fert. Steril.* **26**, 1070–1074.
- Fléchon, J.-E. (1973) Modifications ultrastructurales et cytochimiques des spermatozoïdes de lapin au cours du transit epididymaire. In *Transport, survie et pouvoir fécondant des spermatozoïdes chez les vertébrés*, pp. 115–140. INSERM (Colloques No. 26), Paris.
- Fléchon, J.-E., Huneau, D., Brown, C.R. & Harrison, R.A.P. (1977) Immunocytochemical localization of acrosin in the anterior segment of the acrosomes of ram, boar and bull spermatozoa. *Annls Biol. anim. Biochim. Biophys.* **17**, 749–758.
- Gaddum, P. & Blandau, R.J. (1970) Proteolytic reaction of mammalian spermatozoa on gelatin membranes. *Science, N.Y.* **170**, 749–751.
- Garner, D.L. & Easton, M.P. (1977) Immunofluorescent localization of acrosin in mammalian spermatozoa. *J. exp. Zool.* **200**, 157–162.
- Garner, D.L., Easton, M.P., Munson, M.E. & Doane, M.A. (1975) Immunofluorescent localization of bovine acrosin. *J. exp. Zool.* **191**, 127–130.
- Garner, D.L., Reamer, S.A., Johnson, L.A. & Lessley, B.A. (1977) Failure of immunoperoxidase staining to detect acrosin in the equatorial segment of spermatozoa. *J. exp. Zool.* **201**, 309–315.
- Green, D.P.L. (1976) The effect of trypsin inhibitors on the *in vitro* acrosome reaction of guinea-pig spermatozoa. *J. Physiol., Lond.* **263**, 281P–282P.
- Green, D.P.L. (1978a) The activation of proteolysis in the acrosome reaction of guinea pig sperm. *J. Cell Sci.* **32**, 153–164.
- Green, D.P.L. (1978b) The mechanism of the acrosome reaction. In *Development in Mammals*, vol. 3, pp. 65–81. Ed. M. H. Johnson. North-Holland, Amsterdam.
- Green, D.P.L. & Hockaday, A.R. (1978) The histochemical localization of acrosin in guinea-pig sperm after the acrosome reaction. *J. Cell Sci.* **32**, 177–184.
- Harrison, R.A.P. (1982) The interaction of ram proacrosin and acrosin forms with antiserum raised against ram m_p acrosin. *J. Reprod. Immunol.* (in press).
- Harrison, R.A.P. & Brown, C.R. (1979) The zymogen form of acrosin in testicular, epididymal, and ejaculated spermatozoa from ram. *Gamete Res.* **2**, 75–87.
- Hartree, E.F. (1977) Spermatozoa, eggs and proteinases. *Biochem. Soc. Trans.* **5**, 375–394.
- Lui, C.W. & Meizel, S. (1979) Further evidence in support of a role of hamster sperm hydrolytic enzymes in the acrosome reaction. *J. exp. Zool.* **207**, 173–185.
- Meizel, S. (1978) The mammalian sperm acrosome reaction, a biochemical approach. In *Development in Mammals*, vol. 3, pp. 1–64. Ed. M. H. Johnson. North-Holland, Amsterdam.
- Meizel, S. & Lui, C.W. (1976) Evidence for the role of a trypsin-like enzyme in the hamster sperm acrosome reaction. *J. exp. Zool.* **195**, 137–144.
- Meizel, S. & Mukerji, S.K. (1975) Proacrosin from rabbit epididymal spermatozoa: partial purification and initial biochemical characterization. *Biol. Reprod.* **13**, 83–93.
- Morton, D.B. (1975) Acrosomal enzymes: immunocytochemical localization of acrosin and hyaluronidase in ram spermatozoa. *J. Reprod. Fert.* **45**, 375–378.
- Morton, D.B. (1977) Immunoenzymic studies on acrosin and hyaluronidase in ram spermatozoa. In *Immunobiology of Gametes*, pp. 115–155. Eds M. Edidin & M. H. Johnson. Cambridge University Press.
- Mukerji, S.K., Scibienski, R.J. & Meizel, S. (1980) Rabbit testis proacrosin: immunological similarities between testis, sperm proacrosins and the initially formed testis acrosin. *Gamete Res.* **3**, 241–246.
- Müller-Esterl, W. & Fritz, H. (1980) Interactions of boar acrosin with detergents. *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1673–1682.
- Müller-Esterl, W., Kupfer, S. & Fritz, H. (1980) Purification and properties of boar acrosin. *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1811–1821.
- Polakoski, K.L. & Parrish, R.F. (1977) Boar proacrosin. Purification and preliminary activation studies of proacrosin isolated from ejaculated boar sperm. *J. biol. Chem.* **252**, 1888–1894.
- Schill, W.-B. & Fritz, H. (1975) N^{α} -benzoyl-L-arginine

- ethyl ester splitting activity (acrosin) in human spermatozoa and seminal plasma during aging *in vitro*. *Hoppe Seyler's Z. Physiol. Chem.* **356**, 83–90.
- Schill, W.-B. & Wolff, H.H.** (1974) Ultrastructure of human sperm acrosome and determination of acrosin activity under conditions of semen preservation. *Int. J. Fert.* **19**, 217–223.
- Schill, W.-B., Schleuning, W.-D., Fritz, H., Wendt, V. & Heimbürger, N.** (1975) Immunofluorescent localization of acrosin in spermatozoa by boar acrosin antibodies. *Naturwissenschaften* **62**, 540.
- Shams-Borhan, G., Huneau, D. & Fléchon, J.E.** (1979) Acrosin does not appear to be bound to the inner acrosomal membrane of bull spermatozoa. *J. exp. Zool.* **209**, 143–149.
- Stambaugh, R. & Buckley, J.** (1969) Identification and subcellular localization of the enzymes effecting penetration of the zona pellucida by rabbit spermatozoa. *J. Reprod. Fert.* **19**, 423–432.
- Stambaugh, R. & Buckley, J.** (1970) Comparative studies of the acrosomal enzymes of rabbit, rhesus monkey, and human spermatozoa. *Biol. Reprod.* **3**, 275–282.
- Straus, J.W., Parrish, R.F. & Polakoski, K.L.** (1981) Boar acrosin. Association of an endogenous membrane proteinase with phospholipid membranes. *J. biol. Chem.* **256**, 5662–5668.
- Syner, F.N., Kuras, R.A. & Moghissi, K.S.** (1979) Radioimmunoassay study of immunologic changes associated with the conversion of rabbit testicular proacrosin to acrosin. *Biol. Reprod.* **21**, 857–866.
- Zahler, W.L. & Doak, G.A.** (1975) Isolation of the outer acrosomal membrane from bull sperm. *Biochim. Biophys. Acta* **406**, 479–488.

Received 22 February 1982