Immunocytochemical Localization of Acrosin on Both Acrosomal Membranes and in the Acrosomal Matrix of Porcine Spermatozoa

L. A. JOHNSON,* D. L. GARNER,† ANNE J. TRUITT-GILBERT,* AND B. A. LESSLEY†

Immunocytochemical techniques were employed to determine, at the ultrastructural level, the location of acrosin in porcine spermatozoa. Antisera to highly purified porcine acrosin was produced in rabbits. The (Fab')$_2$ fragments of the immunoglobulins were purified and conjugated with horseradish peroxidase (HRP). Washed, formaldehyde-fixed spermatozoa were reacted with the labeled antiacrosin immunoglobulins, utilizing a direct staining technique. Electron microscopy revealed that the peroxidase reaction product of HRP-antiporcine acrosin was distributed evenly over the outer acrosomal membrane of spermatozoa with intact acrosomes. The labeled antibody was also distributed evenly over the inner acrosomal membrane of cells when the overlying acrosomal structures were absent. In some spermatozoa, labeling was noted throughout the acrosomal matrix. No significant labeling was observed in control specimens when spermatozoa were exposed to HRP-antiporcine acrosin immunoglobulins that had been adsorbed previously with excess purified acrosin or exposed to HRP-conjugated rabbit antiporcine immunoglobulins. This pattern of labeling is consistent with the hypothesis that acrosin may function as a zona lysin. The observation that the outer acrosomal membrane and acrosomal matrix are labeled suggests that acrosin is not exclusively located on the inner acrosomal membrane and, thus, could participate in physiologic events other than zona penetration.

Key words: spermatozoa, acrosin, electron microscopy, antibody, immunocytochemical.

*From the *U.S. Department of Agriculture, Beltsville, Maryland, and †Oklahoma State University, Department of Physiological Sciences, Stillwater, Oklahoma*

Acrosin (EC 3.4.21.10), a neutral endopeptidase located in the acrosome of the spermatozoon, has been implicated as a lysin that is necessary for penetration of the zona pellucida by spermatozoa during fertilization (Stambaugh and Buckley, 1969). Several authors, using biochemical extraction techniques on human (Schill and Wolf, 1974), ram (Brown and Hartree, 1974), and bull (Zahler and Doak, 1975) spermatozoa, have concluded that acrosin is associated primarily with the inner acrosomal membrane (IAM). Light microscopic evidence employing fluorescent and peroxidase-labeled antibody techniques have been used for immunohistochemical localization of acrosin in the acrosome of ram (Morton, 1975), boar (Schill et al, 1975), bull (Garner et al, 1975), rabbit, dog, cat and human (Garner and Easton, 1977) spermatozoa. Localization of acrosin on the IAM of denuded rabbit and hamster spermatozoa using a fluorescent synthetic acrosin inhibitor has recently been reported (Bradford et al, 1981). Ultrastructural localization of acrosin in spermatozoa using ferritin-conjugated trypsin inhibitors has also been reported. Green and Hockaday (1978) observed an uneven distribution of ferritin-conjugated acrosin inhibitor on the exposed IAM of acrosome-reacted guinea pig spermatozoa. Shams-Borhan (1979), however, found no ferritin-conjugated inhibitor on the IAM of mechanically disrupted bull sperma-

Anne J. Truitt-Gibert's present address is: Department of Physiology, Emory University, Atlanta, Georgia.

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

Reprint requests: L. A. Johnson, USDA-ARS, Animal Science Institute, Reproduction Laboratory, Building 200, BARC-East, Beltsville, Maryland 20705.

Submitted for publication March 12, 1982; revised version received June 1, 1982; accepted for publication June 24, 1982.

0196-3635/83/0500/0222/$01.20 © American Society of Andrology
(J Androl 1983; 4:222–229)

tozoa. Therefore, the precise ultrastructural location of acrosin within the acrosome is still in doubt.

This paper presents preliminary results concerning the location of acrosin in porcine spermatozoa, obtained by a direct-labeled immunocytochemical technique at the electron microscope level using a specific antiacrosin antibody. These results suggest that acrosin is present on the IAM, on the outer acrosomal membrane (OAM), and in the acrosomal matrix (AM) of porcine spermatozoa.

Materials and Methods

Semen was collected from mature boars of proven fertility using the gloved-hand technique (Johnson et al, 1972). Ejaculated spermatozoa were washed and extracted for acrosin with Hyamine 2389 and glycerol (Johnson et al, 1976). Acrosin was purified by a three-step chromatographic procedure consisting of gel filtration on Sephadex G-100 at *p*H 4.0, affinity chromatography on para-amino-benzamidine-agarose (Schleuning and Fritz, 1976), and cation exchange chromatography on SP-Sephadex. The specific activity of the final purified acrosin preparation was 249 μmoles/minute/mg protein using α-N-Benzoyl arginine ethyl ester as the substrate. This value is comparable with the specific activity of 253 μmoles/minute/mg protein reported previously for homogeneous porcine acrosin (Polakoski and Parrish, 1977). The purified acrosin was further analyzed for homogeneity by disc gel electrophoresis at *p*H 4.5. The gels were then stained for both protein and acrosin activity (Garner, 1975). Only single bands that possessed electrophoretic mobilities equivalent to γ-acrosin (25,000 daltons) of Polakoski and Parrish (1977) were observed in the gels stained for protein. Duplicate gels stained for acrosin displayed a single band with the same mobility as the protein band. Portions of the purified acrosin were inactivated with Tosyl lysine chloromethyl-ketone (TLCK) and used to immunize virgin female New Zealand rabbits according to the procedure of Harboe and Ingild (1973). A purified immunoglobulin (IgG) fraction was obtained from the resulting antisera by ammonium sulfate precipitation, dialysis, and ion exchange chromatography (Harboe and Ingild, 1973). When tested by double immunodiffusion against crude sperm extracts, this IgG preparation displayed only a single precipitation line over a wide range of antibody: antigen ratios. The cross-reactivity of the antisera with porcine proacrosin was tested using Ouchterlony immunodiffusion plates. These tests, which were run at *p*H 5.1, indicated by the formation of immunoprecipitate lines that the antisera used in this study also recognized proacrosin. The proacrosin used in these tests was obtained from sperm extracts that were made in the presence of 50 mM benzamidine and partially purified on Sephadex G-100 columns at *p*H 3.0. Fractions eluted from the Sephadex column and possessing molecular weights equivalent to proacrosin (greater than 50,000) showed cross-reactivity with the antisera produced against porcine γ-acrosin. The IgG fraction of the antisera was treated with pepsin under the conditions described by Utsumi and Karush (1965) to produce $(Fab')_2$ fragments which were isolated by gel filtration on Ultra gel AA-44 (LKB Instruments, Rockville, MD). The $(Fab')_2$ fragments were conjugated with horseradish peroxidase (HRP) using the two-step glutaraldehyde procedure (Avrameas and Ternynck, 1971), custom service of Cappel Laboratories (Cochranville, PA). Portions of the HRP-conjugated $(Fab')_2$ fragments of the antiporcine acrosin immunoglobulins [HRP-$(Fab')_2$-APA] were adsorbed with an excess of purified acrosin for use in the control experiments. HRP-conjugated rabbit antiporcine IgG immunoglobulins were purchased from Cappel Laboratories.

Preparation of ejaculated spermatozoa for immunocytochemical staining involved washing 3 × by centrifugation and resuspension in phosphate buffered saline, *p*H 7.2 (PBS), containing 0.01 M ethylene diamine tetraacetic acid (EDTA). The washed spermatozoa were subjected to a preliminary fixation with 0.1% formaldehyde in PBS for 1 minute to stabilize the structural integrity of the cells during subsequent manipulations. The formaldehyde was removed by washing the spermatozoa in PBS, after which the cells were exposed to 0.1 M lysine for 30 minutes to block any residual reactive aldehydes (Kuhlmann, 1977). After two additional washes in PBS, the prefixed spermatozoa were suspended in PBS containing suitably diluted HRP-$(Fab')_2$-APA for 1.5 hours at an ambient (22 to 24 C) temperature. Spermatozoa were processed identically in control experiments, except that the labeled specific antibody was replaced with either HRP-$(Fab')_2$-APA which had been adsorbed with excess purified acrosin or with HRP-conjugated rabbit antiporcine IgG, or the antibody was excluded. The conditions for the adsorption in experiments using HRP-$(Fab')_2$-APA were 4:1 ratio of acrosin to antibody based on protein composition, presence of 50 mm benzamidine, and incubation for 22 hours at an ambient temperature.

The spermatozoa were washed with PBS and then stained by reaction with 3-3'-diaminobenzidine (DAB, immunochemical grade, Polysciences, Inc., Warrington, PA) under the conditions described by Rose et al, 1976. The spermatozoa were then washed with PBS and fixed with 1% glutaraldehyde in 0.15 M cacodylate and 1% osmium tetroxide in 0.15 M cacodylate for 1 hour each. The fixed spermatozoa were dehydrated in ethanol and embedded in Spurr's plastic (Spurr, 1969). Thin sections were cut on an Ultrotome IV (LKB Instruments, Rockville, MD) and viewed with a Phillips 200 or Phillips 400 transmission electron microscope (TEM). Smears of ejaculated porcine spermatozoa were prepared on glass coverslips, dried, fixed in formaldehyde, and washed. The fixed smears were incubated with HRP-$(Fab')_2$-APA, washed, reacted with DAB as described above, and examined by light microscopy.

Results

Under light microscopy, fixed smears of spermatozoa that had been incubated with HRP-$(Fab')_2$-APA and reacted with DAB displayed a dense deposition of the reaction product which was evenly distributed over the entire acrosome (Fig. 1). In

contrast, the major portion of the equatorial segment was only lightly labeled, while the postacrosomal region was virtually devoid of reaction product. Due to the pronounced curvature of the posterior acrosomal margin characteristic of porcine spermatozoa, dense labeling of the acrosome persisted as far as the posterior limit of the equatorial segment at the lateral edges of the sperm head (Fig. 1). Light and unevenly distributed deposits of reaction product were occasionally observed. These were associated with the midpiece and principal piece.

After routine fixing and embedding of porcine spermatozoa for viewing with a TEM, it was observed that the morphologic integrity of the spermatozoa generally remained intact (Fig. 2). However, even under the best of preparation conditions, the plasma membrane (PM) overlying the acrosome may become partially detached.

The morphologic integrity of spermatozoa processed for ultrastructural localization according to procedures described earlier are shown after completion of the fixation, washing, and staining steps (Fig. 3). However, antibody was not included in this series. In a significant proportion of the spermatozoa from each sample, it was observed that the PM was absent, and only the OAM remained. The acrosomal contents were also missing in some cases. With the exception of the PM and, in some cases, the AM, the major morphologic aspects of spermatozoa were satisfactorily preserved.

Spermatozoa incubated with the HRP-$(FAB')_2$-APA and reacted with DAB revealed three distinct patterns of labeling. In spermatozoa that appeared structurally intact, the reaction product was generally limited to the OAM (Fig. 4). Such cells possessed reaction product along the perimeter of the OAM, while underlying structures, including the acrosomal matrix and the IAM, were not labeled. The membranes overlying the equatorial segment of some intact spermatozoa also showed some labeling (Fig. 4). This phenomena was also evident on spermatozoa at the light microscope level (Fig. 1). There was no labeling of the postacrosomal cap (Fig. 4). In some spermatozoa, labeling was observed on the OAM, throughout the AM, and on the IAM (Fig. 5). Intact spermatozoa possessed reaction product on the OAM only (Fig. 6), while obviously disrupted spermatozoa had label on both the OAM and IAM (Fig. 6). This observation represents a third staining pattern. Spermatozoa displaying the described staining patterns were observed in replicate experiments. Occasional very light reaction product was also observed associated with midpiece and principal piece cross-sections, as well as occasional fragments of noncellular debris. The staining of these structures was markedly uneven and granular in appearance, suggesting that this staining may have been due to solubilization and redistribution of acrosin.

When spermatozoa were incubated with HRP-$(Fab')_2$-APA that had been previously adsorbed with excess purified acrosin, no label was observed on any acrosomal structures, regardless of whether the acrosomal membranes were disrupted or intact (Fig. 7). Similar results were obtained when the HRP-$(Fab')_2$-APA was replaced with HRP-conjugated rabbit antiporcine IgG (Fig. 8). In both control experiments, however, some reaction product was observed associated with particulate debris of unidentified origin.

Discussion

These experiments provide evidence that acrosin is associated with the OAM, IAM, and AM of boar spermatozoa. Several lines of evidence support the conclusion that these observations represent a valid localization of acrosin.

A direct-labeled antibody technique using prefixation staining was chosen for these studies primarily to circumvent potential problems associated with denaturation of the antigen during fixation and embedding procedures entailed by postfixation staining techniques. The antiporcine acrosin antibody employed in these experiments was elicited using a highly-purified preparation of acrosin and was specific for acrosin and its proenzyme form. Preparation of $(Fab')_2$ fragments from the antiporcine acrosin IgG fraction was performed to prevent the nonspecific binding of antibody to spermatozoa mediated through the F_c portion of whole immunoglobulins (Allen and Bourne, 1978).

The staining pattern observed at the light microscope level after the reaction of dried and fixed porcine spermatozoa with HRP-$(Fab')_2$-APA was virtually identical to the results reported previously for the localization of acrosin using indirect peroxidase- or fluorescent-labeled immunohistochemical techniques (Morton, 1975; Schill et al, 1975; Garner et al, 1975; Garner and Easton 1977; Garner et al, 1977; Flechon et al, 1977). In control experiments for the ultrastructural studies employing HRP-conjugated rabbit antiporcine IgG immunoglobulins and HRP-$(Fab')_2$-APA that had been adsorbed with excess purified acrosin, no staining was observed associated with any of the acrosomal struc-

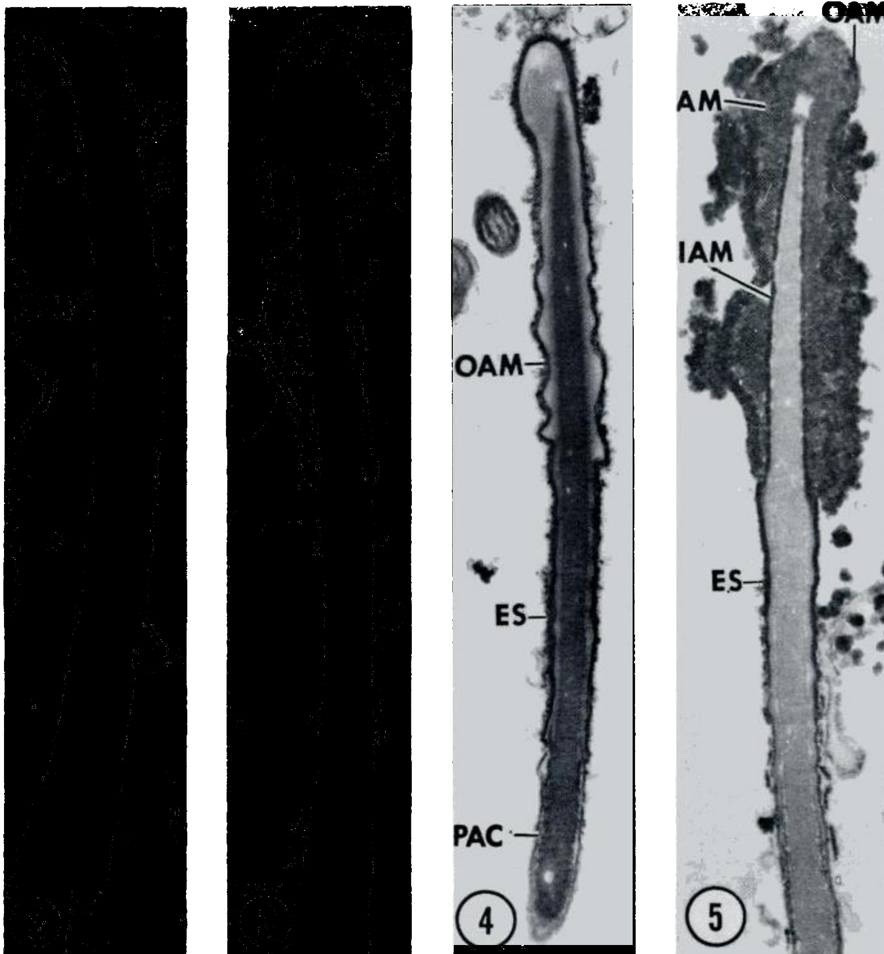

Fig. 1. Fixed smears of porcine spermatozoa which have been incubated with horseradish peroxidase conjugated antiporcine acrosin [HRP-(Fab')$_2$-APA], stained with diaminobenzidine (DAB), and examined under light microscopy. Reaction product is evenly distributed over the acrosomal cap (AC). There is also light labeling of reaction product on the equatorial segment (ES). (Magnification ×3,400). **Fig. 2.** Midsaggital section of a porcine sperm head which has been fixed in glutaraldehyde (GL) and embedded. Note the close adherence of the plasma membrane (PM) over most of the AC and ES. (Magnification ×17,500). **Fig. 3.** Section of sperm, prefixed in formaldehyde (FO), incubated in the absence of specific antibody, fixed in GL, and embedded. Note the loss of PM and acrosomal matrix (AM) and the disorientation of outer acrosomal membrane (OAM). (Magnification ×16,500). **Fig. 4.** An oblique saggital section of sperm which has been prefixed in FO, incubated with HRP-(Fab')$_2$-APA, fixed in GL, reacted with DAB, and embedded. There are dense deposits of reaction product on the OAM and a lesser amount of reaction product on the ES, with no reaction product on the postacrosomal cap (PAC). (Magnification ×17,400). **Fig. 5.** Saggital section of sperm which has been prefixed in FO, incubated with HRP-(Fab')$_2$-APA, fixed in GL, reacted with DAB, and embedded. There are dense deposits of reaction product on the OAM, throughout the AM, and on the inner acrosomal membrane (IAM). Some reaction product is also evident on the ES. (Magnification ×16,500).

tures. Some staining was observed in both controls, but this was associated with unidentified particulate debris rather than with the spermatozoa, and probably represents precipitated DAB.

As originally noted by Morton (1977a), no staining is observed when viable spermatozoa are incubated with Fab fragments of antiacrosin immunoglobulins, suggesting that the intact plasma

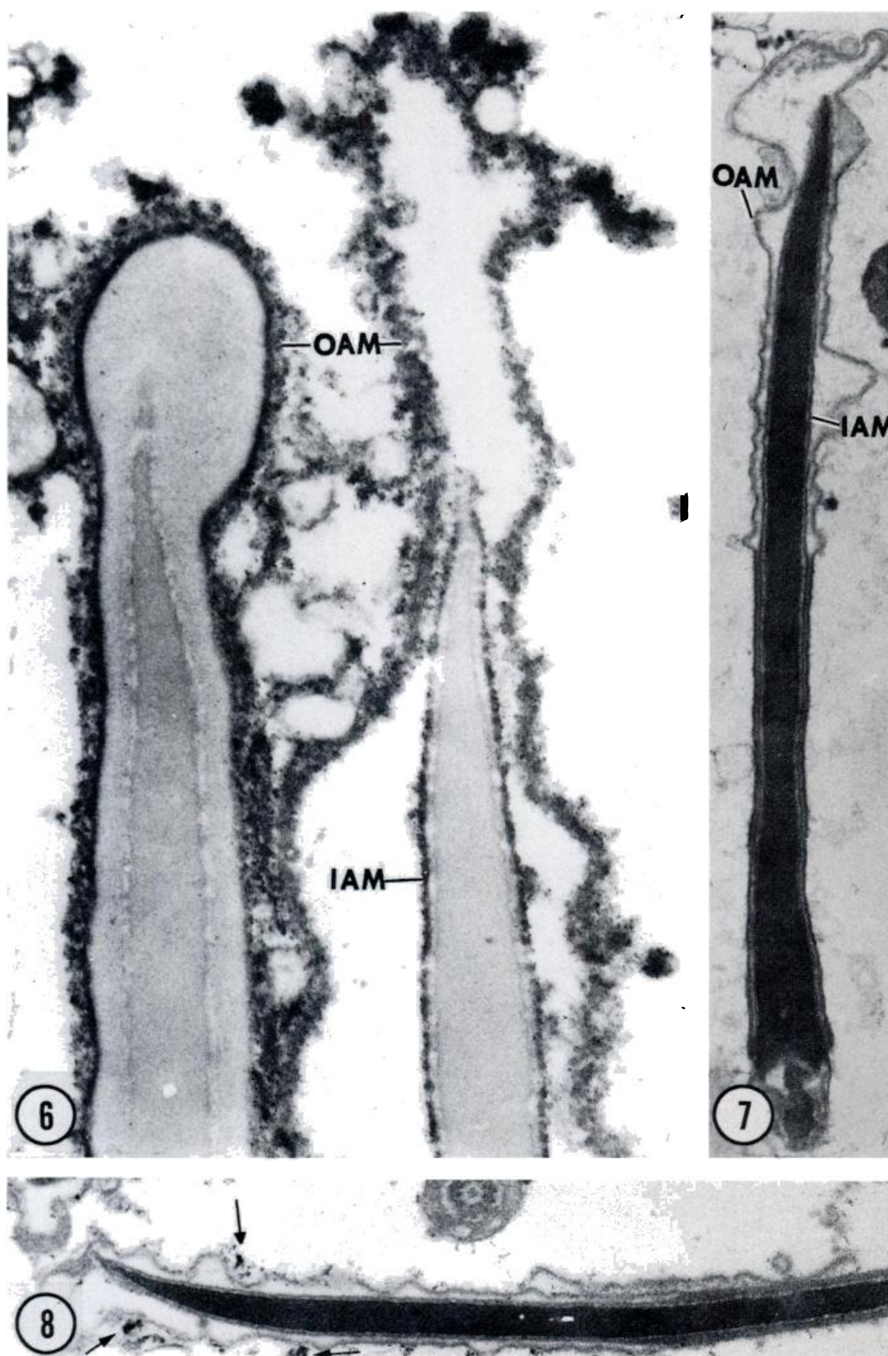

Fig. 6. A high magnification section of the anterior sperm heads of porcine sperm. The sperm have been prefixed in FO, incubated with HRP-(Fab')$_2$-APA, fixed in GL, reacted with DAB, and embedded. Dense reaction product is present on the OAM of the intact sperm and on the IAM and OAM of the disrupted sperm. (Magnification ×65,000). (Abbreviations defined in legend to Figs. 1–5). **Fig. 7.** A midsaggital section of sperm which has been prefixed in FO, incubated with HRP-(Fab')$_2$-APA that has been adsorbed with excess purified acrosin, fixed in GL, reacted with DAB, and embedded (control). There is a complete absence of reaction product on the OAM, IAM, ES, and PAC. (Magnification ×16,500). **Fig. 8.** Saggital section of sperm prefixed in FO, incubated with antiporcine immunoglobulin conjugated to horseradish peroxidase, fixed in GL, reacted with DAR, and embedded (control). Reaction product is not adherent to OAM, IAM, or ES. Some spurious reaction product is present (arrows). (Magnification ×13,000).

membrane precludes access of antibody to the acrosin associated with the underlying acrosomal structures. Thus, in any attempt at subcellular localization of acrosin within the acrosomal complex, consideration of antibody accessibility to antigen is essential. Due to the prefixation and vigorous washing procedures employed during the preparation of the spermatozoa in this study, the plasma membrane was disrupted or absent in the majority of spermatozoa at the time that they were exposed to HRP-(Fab')$_2$-APA. It may be assumed, therefore, that the antibody had access to the exterior surface of the OAM. In spermatozoa that retained an intact OAM, access of the antibody to the underlying structures would presumably be severely restricted. This hypothesis accounts for the predominant labeling pattern observed in this study; that is, spermatozoa in which dense reaction product was found on the OAM only. Similarly, loss of integrity of the OAM should allow access of con-

jugated antibody to acrosomal structures beneath this membrane. Indeed, some spermatozoa displayed dense reaction product within the AM and on the IAM. In cases wherein most of the superficial acrosomal components were absent, labeling was seen only on the IAM. One interpretation of these observations is that acrosin is located throughout the acrosome, and that the specific pattern observed for an individual spermatozoon depends on the degree of membrane integrity and consequent accessibility of the antibody. Unfortunately, removal of particular acrosomal membranes and the presumed increased access of antibody to the deeper acrosomal contents was not under direct experimental control.

Although labeling of the AM and the IAM was consistently observed, it was noted only in a few spermatozoa within a given sample. Therefore, the origin of the spermatozoa that labeled in this fashion is an important consideration. All samples of ejaculated semen contain some spermatozoa in various stages of degeneration. As reported by Brown and Harrison (1978) and Goodpasture et al, (1981), damaged spermatozoa release a significant portion of their total complement of acrosin in soluble form. Presumably, the formaldehyde fixation step would arrest further degeneration or release of acrosin. However, it is possible that at least some of the observed labeling is an artifact attributable to solubilization and redistribution of acrosin by resorption to sites on the spermatozoa (midpiece and tail) unrelated to the original location.

The spermatozoa with labeling on the OAM predominated numerically in all samples and were generally intact. In these cells, a dense uniform layer of reaction product was noted over the entire exterior surface of the OAM. This label was mainly limited to the acrosomal region and, to a much lesser extent, to the equatorial segment. The observed labeling of the equatorial segment in some electron micrographs probably represents sections cut well off of the central axis of the spermatozoan. This labeling pattern may be related to the curvature of the posterior margin of the porcine acrosome. The postacrosomal regions were generally not labeled. Overall, it is difficult to account for the uniformity of labeling and specificity of location by postulating that acrosin from some degenerating spermatozoa was solubilized and redistributed by nonspecific adsorption along exposed acrosomal surfaces of other spermatozoa.

The association of labeled antibody with deeper acrosomal structures, particularly the IAM, is more difficult to interpret. Presumably the OAM must be disrupted before antibody can gain access to the AM or IAM. Cells labeled along the IAM probably represent spermatozoa that were originally intact but sustained damage during the washing procedures. If this was the case, it is likely that labeling of the IAM represents a valid localization of acrosin. One cannot discount the possibility, however, that a few of these spermatozoa represent degenerating cells that existed in the original ejaculate.

An additional factor which makes interpretation of the results difficult is uncertainty as to the molecular form of acrosin that the antibody recognized. It has been demonstrated for several species that, in the intact spermatozoon, more than 90% of the total potential acrosin activity is present as the inactive precursor, proacrosin (Meizel and Mukerji, 1975; Zahler and Polakoski, 1977; Goodpasture et al, 1981). Since the antibody employed in this study was developed with purified acrosin, one may question whether sufficient immunologic similarity exists between acrosin and proacrosin to allow binding of the antibody to spermatozoa if a significant portion of the antigen was present as proacrosin. However, we have evidence that the antiporcine acrosin antibody used in this study also recognized the porcine proacrosin (unpublished results). In addition, immunologic cross-reactivity between acrosin and proacrosin has been reported for other species (Mukerji et al, 1980). We have no direct evidence to indicate what proportions of the antigen present on the spermatozoa in this study were proacrosin or acrosin. Apparently, activation of proacrosin to acrosin does not occur within the intact acrosome but is initiated after physical disruption of the acrosome (Brown and Harrison, 1978; Goodpasture et al, 1981). Since exogenous acrosin inhibitors were not included in the washing solution employed in this study, it is possible that at least part of the original proacrosin had undergone activation to acrosin. However, the formaldehyde fixation may have prevented completion of the activation process in partially disrupted spermatozoa, leaving substantial quantities of proacrosin unactivated. Thus, if the HRP-$(Fab')_2$-APA reacts weakly with membrane-bound proacrosin, failure to detect labeled antibody associated with the AM and IAM of relatively intact spermatozoa is reasonable.

Comparison of the results described in this report with other attempts at ultrastructural localization of acrosin using ferritin-conjugated soybean trypsin inhibitor is difficult, since these studies

produced contradictory results. Using guinea pig spermatozoa in which the acrosome reaction had been artificially induced, Green and Hockaday (1978) concluded that acrosin was present within the AM and on the surface of the exposed IAM. In contrast, Shams-Borhan et al (1979) did not observe any ferritin-conjugated inhibitor on the exposed IAM of bull spermatozoa after disruption of the acrosome by a combination of osmotic shock and mechanical shear forces. Similar results were reported by Sakai and Yasuda (1981) using peroxidase-labeled trypsin inhibitor and silver proteinase for the localization of acrosin in guinea pig spermatozoa. These studies suffer from the technical deficiencies noted by Morton (1977b) which are inherent with labeled trypsin inhibitors. In addition, labeled trypsin inhibitors are unlikely to bind proacrosin, and thereby fail to detect the major source of potential acrosin activity. Of these two reports, the conclusions of Green and Hockaday (1978) are most comparable with the results of this study.

Several reports employing biochemical techniques for subcellular fractionation or selective extraction of spermatozoa have provided evidence that removal of the superficial acrosomal components does not result in significant losses of acrosin activity from the spermatozoa (Schill and Wolf, 1974; Brown and Hartree, 1974; Zahler and Doak, 1975). Such reports imply that acrosin is present on the IAM. However, it must be noted that these earlier reports did not take into account the contribution of proacrosin activation. More recent studies have separately determined the proacrosin, acrosin, and acrosin inhibitor levels in the same sample. Goodpasture et al (1981) demonstrated that approximately 50% of the total acrosin activity is readily lost from acrosome-reacted guinea pig spermatozoa, suggesting that it was associated with superficial acrosomal structures. The remaining acrosin activity was associated with reacted spermatozoa, suggesting that this portion was more tightly bound. Similar results were obtained using biochemical procedures on human spermatozoa (Bhattacharyya and Zaneveld, 1978), supporting the hypothesis that acrosin is present on both the OAM and IAM. The observation described in this report of labeled antiacrosin antibodies associated with both the OAM and IAM is entirely consistent with the results of these more recent biochemical studies. Furthermore, during the process of spermatid differentiation, the acrosome is formed from a spherical granule that flattens and spreads over the spermatid nucleus (Bane and Nicander, 1966). Such evidence does not suggest an exclusive developmental association of acrosomal contents with either the OAM or IAM.

The major interest in identifying the precise subcellular location of acrosin in the spermatozoa is acrosin's proposed physiologic function as a zona lysin essential for fertilization (McRorie and Williams, 1974). One essential property of a putative zona lysin is that it be situated on the IAM of acrosome reacted spermatozoa during penetration of the zona (for detailed discussion, see Morton, 1977b). The observation in this study that labeled antiacrosin antibodies are associated with the IAM of partially disrupted porcine spermatozoa is consistent with the proposed function of acrosin as a zona lysin. It is interesting to note, however, that other physiologic functions for acrosin have been proposed. These functions include liberation of kinins (Palm et al, 1976) and participation in the acrosome reaction (Meizel and Lui, 1976; Lui and Meizel, 1979). If acrosin influences the molecular events preceding zona penetration, it is possible that the acrosin located in the more superficial aspects of the acrosome is involved. Thus, our observation of acrosin on the OAM and AM is compatible with the various physiologic functions proposed by others.

In summary, these investigations still have not completely resolved the questions regarding the exact subcellular location of acrosin. The conclusion that acrosin may be associated with both IAM and OAM as well as the AM can be advanced only tentatively due to the many complications involved in interpreting the results. These observations, however, do merit careful consideration, particularly in light of the recent biochemical studies on the distribution of acrosin. It is anticipated that future experiments incorporating refinements in technique will provide unambiguous, immunocytochemical localization of acrosin and proacrosin in spermatozoa.

Acknowledgments

A special thanks to Norita Chaney and Doug Caldwell for technical assistance in this study and to Linda Neuenhahn and Jane Fleming for typing the manuscript.

Note Added in Proof

A recent study (Harrison, Journal of Reproductive Immunology 1982; 4:231–249) demonstrated that rabbit antibodies elicited against ram m_{β}-acrosin, the principal form obtained by affinity chromatog-

raphy on Sepharose-linked-P-(P′-aminophenoxy)benzamidine, cross-reacted strongly with native proacrosin of the ram. It was concluded that antibodies to m_β-acrosin could be used for location of both proacrosin and acrosin in ram spermatozoa. Our results with rabbit antiporcine acrosin support similar conclusions.

References

Allen GJ, Bourne FJ. Interaction of immunoglobulin fragments with the mammalian sperm acrosome. J Exp Zool 1978; 203:271–276.

Avrameas S, Ternynck T. Peroxidase labeled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry 1971; 8:1175.

Bane A, Nicander L. Electron and light microscopical studies on spermateliosis in a boar with acrosome abnormalities. J Reprod Fertil 1966; 11:133–138.

Bradford MM, Dudkiewicz AB, Penny GS, Dyckes DF, Burleigh BD, Wooley RE, McRorie RA. Localization of proacrosin on the inner acrosomal membrane of spermatozoa in rabbits and hamsters. Am J Vet Res 1981; 42:1082–1083.

Brown CR, Harrison RAP. The activation of proacrosin in spermatozoa from ram, bull and boar. Biochem Biophys Acta 1978; 526:202–217.

Brown CR, Hartree EF. Distribution of a trypsin-like proteinase in the ram spermatozoon. J Reprod Fertil 1974; 36:195–198.

Bhattacharyya Ak, Zaneveld LJD. Release of acrosin and acrosin inhibitor from human spermatozoa. Fertil Steril 1978; 30:70–78.

Flechon JE, Huneau D, Brown CR, Harrison RAP. Immunocytochemical localization of acrosin in the anterior segment of the acrosomes of ram, boar and bull spermatozoa. Ann Biol Anim Biochim Biophys 1977; 17:749–758.

Garner DL. Improved zymographic detection of bovine acrosin. Anal Biochem 1975; 67:688–694.

Garner DL, Easton MP. Immunofluorescent localization of acrosin in mammalian spermatozoa. J Exp Zool 1977; 200:157–162.

Garner DL, Easton MP, Munson ME, Doane MA. Immunofluorescent localization of bovine acrosin. J Exp Zool 1975; 191:127–131.

Garner DL, Reamer SA, Johnson LA, Lessley RA. Failure of immunoperoxidase staining to detect acrosin in the equatorial segment of spermatozoa. Exp Zool 1977; 201:309–315.

Goodpasture JC, Reddy JM, Zaneveld LJD. Acrosin, proacrosin and acrosin inhibitor of guinea pig spermatozoa capacitated and acrosome reacted in vitro. Biol Reprod 1981; 25:44–55.

Green DPL, Hockaday AR. The histochemical localization of acrosin in guinea-pig sperm after the acrosome reaction. J Cell Sci 1978; 32:177–184.

Harboe N, Ingold A. Immunization, isolation of immunoglobulin, estimation of antibody titre. In: Axeleson NA, Kroll H, Weeke B, eds. Manual of quantitative immunoelectrophoresis, methods and application. Oslo, Norway: Universitetssorliget, 1973; 161–164.

Johnson LA, Pursel VG, Gerrits RJ, Thomas CH. Free amino acid composition of porcine seminal epididymal and seminal vesicle fluids. J Anim Sci 1972; 34:430–434.

Johnson LA, Pursel VG, Chaney N, Garner DL. Comparison of extraction procedures for boar spermatozoal acrosin. Biol Reprod 1976; 15:79–83.

Kuhlmann WD. Ultrastructural immunoperoxidase cytochemistry. In: Progress in Histochemistry and Cytochemistry. New York: Gustav Fischer Verlag, 1977; 10:1–57.

Lui GW, Meizel S. Further evidence in support of a role for hamster sperm hydrolytic enzymes in the acrosome reaction. J Exp Zool 1979; 207:173–186.

McRorie RA, Williams WL. Biochemistry of mammalian fertilization. Rev Biochem 1974; 43:777–803.

Meizel S, Lui CW. Evidence for the role of a trypsin-like enzyme in the hamster sperm acrosome reaction. J. Exp Zool 1976; 195:137–144.

Meizel S, Mukerji SK. Proacrosin from rabbit epididymal spermatozoa: partial purification and initial biochemical characterization. Biol Reprod 1975; 13:83–93.

Morton DB. Acrosomal enzymes: immunochemical localization of acrosin and hyaluronidase in ram spermatozoa. J Reprod Fertil 1975; 45:375–378.

Morton DB. Immunoenzymic studies on acrosin and hyaluronidase in ram spermatozoa. In: Edidin M, Johnson MH, eds. Immunobiology of gametes. Cambridge, England: Cambridge Press, 1977a; 115–155.

Morton DB. The occurrence and function of proteolytic enzymes in the reproductive tract of mammals. In: Barrett AJ, ed. Proteinases in mammalian cells and tissues. Amsterdam: Elsevier/North-Holland Biomedical Press, 1977b; 445–500.

Mukerji SK, Scibienski RJ, Meizel S. Rabbit testis proacrosin: immunological similarities between testis, sperm proacrosins and the initially formed testis acrosin. Gamete Res 1980; 3:241–246.

Palm S, Schill WB, Wallner O, Prinzen R, Fritz, H. Occurrence of components of the Kallikrein-Kinin system in human genital tract secretions and their possible function in stimulation of sperm motility and migration. In: Sicuteri S, Back N, Haberland GL, eds. Kinins: pharmacodynamics and biological roles. New York: Plenum Publishing, 1976; 271–279.

Polakoski KL, Parrish RF. Purification and preliminary activation studies of proacrosin isolated from ejaculated boar sperm. J Biol Chem 1977; 252:1888–1894.

Rose NR, Jhort T, Rumke P, Harper MJ, Vyazov O. Technique for detection of iso- and auto-antibodies to human spermatozoa. Clin Exp Immunol 1976; 23:175–199.

Sakai Y, Yasuda K. Localization of acrosomal proteinase after vesiculation of the acrosome. Dev Growth Differ 1981; 23:487–494.

Schill WB, Scheluning WD, Fritz H, Wendt V, Heimburger N. Immunofluorescent localization of acrosin in spermatozoa by boar acrosin antibodies. Die Naturwissenschaften 1975; 11:540.

Schill WB, Wolff HH. Ultrastructure of human sperm acrosome and determination of acrosin under conditions of semen preservation. Int J Fertil 1974; 19:217–223.

Schleuning WD, Fritz H. Sperm acrosin. In: Colowick SP, Kaplan N, Lorand L, eds. Methods of Enzymology. New York: Academic Press, 1976; 35:330–342.

Shams-Borhan G, Huneau D, Flechon JF. Acrosin does not appear to be bound to the inner acrosomal membrane of bull spermatozoa. J Exp Zool 1979; 209:143–149.

Spurr AR. A low viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 1969; 26:31–43.

Stambaugh RL, Buckley J. Identification and subcellular location of the enzymes effecting penetration of the zona pellucida by rabbit spermatozoa. J Reprod Fertil 1969; 19:423–432.

Utsumi S, Karush F. Peptic fragmentation of rabbit G-immunoglobulin. Biochem 1965; 4:1766–1779.

Zahler WL, Doak GA. Isolation of the outer acrosomal membrane from bull sperm. Biochem Biophys Acta 1975; 406:479–488.

Zahler WL, Polakoski KL. Benzamidine as an inhibitor of proacrosin activation in bull sperm. Biochem Biophys Acta 1977; 480:461–468.