Studies on Ram Acrosin

ISOLATION FROM SPERMATOOZOA, ACTIVATION BY CATIONS AND ORGANIC SOLVENTS, AND INFLUENCE OF CATIONS ON ITS REACTION WITH INHIBITORS

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1. A simple method is given for isolating from ram spermatozoa a water-soluble form of acrosin (a trypsin-like enzyme) which is about 25% pure. It is free from an acrosin inhibitor which is located in the spermatozoa. 2. In the hydrolysis of *N*- α -benzoyl-L-arginine ethyl ester the degree of activation of acrosin by Ca^{2+} , and by some other cations, is dependent on the extent of contamination by the inhibitor. In 50 mM-Tris-HCl buffer (pH 8.2) activation by Ca^{2+} did not exceed 40%, but acrosin that is partially inhibited may be activated by up to 300%: this is due to cation-mediated protection of acrosin against the inhibitor. 3. Increasing concentrations of buffers (e.g. Tris) also activate acrosin but at above certain buffer concentrations Ca^{2+} no longer exerts an activating effect and may become inhibitory. Ca^{2+} is also inhibitory when added to assay systems involving anionic buffers with chelating properties. This is due to a fall in pH. 4. The above results suggest reasons for conflicting conclusions in papers dealing with the effects of Ca^{2+} on acrosin activity. 5. Inhibition of acrosin by the Kunitz pancreatic trypsin inhibitor is increased on addition of Ca^{2+} . Inhibitions of trypsin by the acrosin inhibitor and by the Kunitz inhibitor are insensitive to Ca^{2+} . 6. Like trypsin, acrosin is activated, up to 60%, by 2-methylpropan-2-ol, dimethyl sulphoxide, and some other water-miscible solvents. Effects of cations and solvents tend to be additive and a common maximum acrosin activity can be achieved with various concentrations of solvent, salts and buffer in the assay system. Activation by solvents is increased when low concentrations of the acrosin inhibitor are present. 7. Activations of acrosin by salts and by solvents are more pronounced when the substrate is *N*- α -benzoyl-DL-arginine 2-naphthylamide. 8. K_m values for ram acrosin (about 0.2 mM) are much higher than those for trypsin, and k_{cat} values are slightly higher than those for trypsin. Considerations of the influences of ions and dimethyl sulphoxide on the activities and kinetic constants of acrosin and trypsin suggest that conformational changes are the factors mainly responsible for the reported activations of acrosin. 9. The following conclusions are reached. (a) Acrosin plays a role in the penetration of the sperm cell into the egg without becoming detached from the acrosomal membrane. (b) The enzyme is a peripheral membrane protein which may be classed as a cathepsin. (c) The susceptibility of the activity of soluble acrosin to cations and solvents points to a flexible molecule, i.e. one lacking conformational restraints imposed by association (presumably ionic) with the acrosomal membrane.

A di-isopropyl phosphorofluoridate-sensitive proteinase with many similarities to trypsin is widespread among the spermatozoa of mammals and is generally believed to be located in the acrosome or head-cap (Stambaugh & Buckley, 1969, 1972a; Zaneveld *et al.*, 1972a; Polakoski *et al.*, 1972; Brown & Hartree, 1974). The Committee for Enzyme Nomenclature had considered the term 'acrosomal proteinase' as an official name, with the result that papers have

appeared in which this proteinase is so named (Stambaugh & Buckley, 1972a; Zaneveld *et al.*, 1972a). Such a name is inappropriate, since acrosomes contain more than one proteinase (Allison & Hartree, 1970). In the latest edition of Enzyme Nomenclature (1972) this proteinase (EC 3.4.21.10) is listed as acrosin, a name originating from Zaneveld *et al.* (1971).

Interest in acrosin has been stimulated by the concept that it plays a role in the interaction of spermatozoon and egg: more specifically by assisting the passage of a spermatozoon through the zona pellucida, a thick membrane surrounding the egg cell (Stambaugh *et al.*, 1969; Zaneveld *et al.*, 1970),

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However, conflicting evidence has appeared (Miyamoto & Chang, 1973). Acrosins from rabbit and boar spermatozoa have been purified quite extensively (Polakoski *et al.*, 1972, 1973; Schleuning *et al.*, 1973), although instability of purified fractions has been a barrier to systematic study of the pure protein.

Bovine trypsin is activated by different cations, including Ca^{2+} (Green & Neurath, 1953). Water-miscible solvents also increase the activity of trypsin (Castañeda-Agulló & del Castillo, 1959). The effects of Ca^{2+} on the activity of acrosin have been the subject of considerable disagreement: it has been claimed that this proteinase is activated (Polakoski *et al.*, 1973), unaffected (Fritz *et al.*, 1972; Gilboa *et al.*, 1973), or strongly inhibited (Multamäki & Niemi, 1972) by Ca^{2+} . However, these conflicting conclusions are based on studies of acrosins from different mammalian species and in some cases on a limited range of Ca^{2+} concentrations. Boar acrosin, like trypsin, is both stabilized and activated by Ca^{2+} (Polakoski *et al.*, 1973). These properties are shared by ram acrosin, which is also stabilized by cations other than Ca^{2+} . However, we found that the effects of Ca^{2+} varied among different preparations of ram acrosin. The aim of our investigations was to define the factors responsible for such variations and also to provide a basis for development of a fluorimetric titration of acrosin (Brown *et al.*, 1975).

A preliminary account of the isolation of acrosin from ram spermatozoa has appeared elsewhere (Brown & Hartree, 1974).

Materials

Diazotized *o*-dianisidine (sodium salt), Bz-Arg-OEt (*N*- α -benzoyl-L-arginine ethyl ester hydrochloride) and Bz-Arg-NHNaph (*N*- α -benzoyl-DL-arginine 2-naphthylamide hydrochloride) were supplied by Koch-Light Ltd., Colnbrook, Bucks., U.K.; dimethyl sulphoxide ('puriss') was supplied by Fisons Scientific Ltd., Loughborough, Leics., U.K.; CNBr (Fluka 'purum') was supplied by Fluorochem Ltd., Glossop, Derbys., U.K., and benzamidine hydrochloride (Eastman) was supplied by Kodak Ltd., Kirkby, Liverpool, U.K. Crystalline bovine trypsin (Worthington) was from Cambrian Chemicals, Croydon CR0 4XB, Surrey, U.K.; bovine serum albumin ('cryst. lyophilized'), soya-bean trypsin inhibitor ('type 1-S'), Tos-Arg-OMe (toluene-*p*-sulphonyl-L-arginine methyl ester hydrochloride), Epps [3-(*N*-2-hydroxyethyl)piperazin-*N'*-yl]propanesulphonic acid] and *p*-aminobenzamidine hydrochloride were from Sigma Chemical Co., Kingston-upon-Thames KT2 7BH, Surrey, U.K.; Sepharose 4B and Sephadex G-75 were from Pharmacia Ltd., London W5 5SS, U.K. Trasylol (Kunitz bovine basic pancreatic trypsin inhibitor) was a gift from Bayer Pharmaceuticals Ltd., Haywards Heath, Sussex,

U.K., to Dr. R. A. P. Harrison of this Institute: fluorimetric titration (Brown *et al.*, 1975) indicated that its purity was about 84%. Rententive dialysis tubing 'average pore-radius 24A' was supplied by Viskase Ltd., Croydon CR9 2TT, Surrey, U.K. Tests showed that it was impermeable to Trasylol (mol.wt. 6513).

Trypsin was covalently bound to Sepharose by an unpublished procedure developed by Dr. R. A. P. Harrison. Its basis is the method of Beeley & McCairns (1972) but with modification as follows. The activation reaction (0°C, 6 min) involved 10 ml of Sepharose 4B and 400 mg of CNBr in 25 ml of water. The product was washed extensively with cold 0.1 M- NaHCO_3 . For the coupling reaction the product was stirred overnight at 20°C with 40 ml of 0.1 M- NaHCO_3 , 80 mg of trypsin and 100 mg of benzamidine hydrochloride. The gel was then (a) stirred for 4 h with 1 M-ethanolamine, pH 8.5, and (b) washed four times, alternately, with 1 M-NaCl-0.1 M- NaHCO_3 and 1 M-NaCl-0.1 M-sodium acetate buffer, pH 4. It was stored in 1 mM-HCl.

Semen was collected by artificial vagina from Suffolk rams. Pooled ejaculates from not less than 20 animals were allowed to cool slowly to room temperature (20–22°C).

Buffers and reagent solutions

Unless otherwise stated activity of acrosin was measured in Tris-HCl buffers, pH 8.2. Also used were triethanolamine-HCl and Epps-NaOH. Aqueous solutions (10 mM) of Bz-Arg-OEt and Tos-Arg-OMe were stored for up to 1 week at -20°C. Bz-Arg-NHNaph solution (10 mM) in 2-methylpropan-2-ol-water (1:1, v/v) was stable indefinitely at -20°C.

Methods

Assay systems

Spectrophotometric assay of acrosin. Hydrolysis of Bz-Arg-OEt was followed at 25°C as described by Rick (1963). In 'standard conditions' the buffer was 50 mM-Tris-HCl containing 0.2 M- CaCl_2 , and Bz-Arg-OEt was 1 mM. Two further modifications were made. (a) The assay volume was reduced to 1.6 ml and measurements were made in 5 mm cuvettes. (b) *E* values were recorded with an SP.800 double-beam spectrophotometer (Pye-Unicam, Cambridge, U.K.) at 258.9 nm instead of the customary 254 nm. The former wavelength, being that of a sharp peak in the spectrum of benzene vapour, can be set accurately. The spectrophotometer output was monitored on an external recorder giving full-scale deflexion for $\Delta E = 0.2$. The modifications decrease instrument 'noise' considerably and permit accurate

assays with crude acrosin preparations or with additional u.v.-absorbing factors. These advantages outweigh the 30% decrease in sensitivity resulting from the wavelength change. Hydrolysis of Tos-Arg-OMe was followed similarly at 247 nm.

Assay of acrosin in a pH-stat. This method for following the hydrolysis of Bz-Arg-OEt was used when assays contained dimethyl sulphoxide, which is opaque at 258.9 nm. Assays were performed at 25°C with a Radiometer Titrator (Radiometer, Emdrupvej, Copenhagen, Denmark) (Walsh & Wilcox, 1970). Assay mixtures were as in the preceding paragraph but with 5 mM-Tris-HCl and normally with 2 mM-Bz-Arg-OEt.

Colorimetric assay of acrosin. The procedure is based on that of Riedel & Wunsch (1959). Assay mixtures, in 5 ml test tubes, contained 50 mM-Tris-HCl, pH 8.2, CaCl_2 (0.1 M in 'standard conditions') and enzyme in a total volume of 1.0 ml. The mixtures were brought to 37°C and 0.1 ml of Bz-Arg-NHNaph solution (see above) was added. After 30 min the mixtures were cooled for 30 s in an ice-brine bath, transferred to ice, and treated with 0.2 ml of cold diazotized *o*-dianisidine (0.5% solution in 0.8 M-sodium acetate buffer, pH 4). After 10 min at 0°C the tubes received 0.2 ml of 0.5 M-trichloroacetic acid and 2.0 ml of ethyl acetate. The tubes were shaken to transfer the pigment to the ethyl acetate and centrifuged briefly at 5°C. The upper phases were transferred to 10 mm semi-microcuvettes and E_{525} was determined. Similar treatment of 2-naphthylamine standards established a linear relationship between E_{525} and 0–0.2 μmol of 2-naphthylamine. Highest activity was observed in the presence of 0.2 M- CaCl_2 , but such conditions led to precipitation of substrate (Table 4). Diazotized *o*-dianisidine and 2-naphthylamine are carcinogens and appropriate precautions were taken.

Enzyme units. These are μmol of substrate hydrolysed per min.

Determinations of the operational molarity of acrosin and trypsin solutions. These were made fluorimetrically with 4-methylumbelliferyl *p*-guanidinobenzoate (Brown *et al.*, 1975).

Protein. This was determined by a Folin method with bovine serum albumin as standard (Hartree, 1972).

Isolation of acrosin from ram spermatozoa

By following the procedure outlined by Brown & Hartree (1974) washed spermatozoa are 'denuded', i.e. they are stripped of the plasma membrane and of the greater part of the acrosome, leaving the inner acrosomal membrane and the distal region of the acrosome, the equatorial segment, *in situ* (Fig. 1, Plate 1). The acrosin in the denuded spermatozoa is solubilized at low pH.

Washing of spermatozoa. Ram semen (32 ml) is

diluted with 4 vol. of sucrose-citrate (0.275 M-sucrose, 16 mM-citric acid, +1 M-NaOH to pH 6.5) and centrifuged in a swing-out-type head at room temperature. Conditions are chosen (a) to give fairly loose sperm pellets that may be resuspended by gently rocking the Parafilm-capped centrifuge tubes, and (b) to leave in suspension the cytoplasmic droplets (Dott & Dingle, 1968). The supernatant-diluted seminal plasma should be uniformly opalescent: if the uppermost layer is clearer than the remainder, droplets are being sedimented. Using a Super-Minor centrifuge fitted with a windshield (MSE Scientific Instruments, Crawley, Sussex, U.K.) we found that 600g for 15 min is appropriate. The supernatant solution is drawn off, the spermatozoa are resuspended in sucrose-citrate and centrifuged again (10 min). The wash procedure is repeated but with 0.25 M-sucrose. The spermatozoa are resuspended in 0.25 M-sucrose and stored at -20°C.

Preparation of denuded spermatozoa. Washed spermatozoa are kept at -20°C for at least 16 h, thawed, diluted with 0.25 M-sucrose to 10 times the original semen volume and kept at room temperature for 2 h. Phase-contrast microscopy now reveals that

Fig. 1. Diagrammatic representation of sagittal sections of the acrosomal region of a typical mammalian spermatozoon (not to scale)

(a) Rostral part of sperm head (acrosome stippled). (b) Changes occurring during denudation. The entire plasma membrane is lost, as is also the part of the outer acrosomal membrane that is rostral to the equatorial segment. (c) The resealing process (fusion of plasma membrane with outer acrosomal membrane, arrow) which occurs during the 'acrosome reaction' *in vivo* (Bedford, 1968).

acrosomal membranes are lifting from the sperm-heads (Plate 1). All subsequent manipulations are at 0–5°C. Portions (10ml) of the suspension in 25ml test tubes are agitated vigorously with a vortex mixer for a period, usually 20–30s, which results in about 50% of the spermatozoa becoming denuded (Plate 1, Fig. 1). The pooled suspensions are centrifuged for 15 min at 900g (swing-out head), resuspended in 0.25M-sucrose to 10 times semen volume, and again subjected to vortex-mixing. After this treatment virtually all spermatozoa are denuded. The suspension is centrifuged again at 900g and the pellet of denuded spermatozoa is resuspended in 0.25M-sucrose. (This material may be stored at –20°C for 6 months without loss of acrosin activity.) The supernatant fractions are kept at –20°C as a source of the acrosin inhibitor in spermatozoa.

Isolation of acrosin in a stable freeze-dried form. (All manipulations are at 0–5°C. Unless otherwise stated centrifugation is at 9000g for 15 min.) Denuded spermatozoa are diluted to 4 times semen volume in 0.25M-sucrose, stirred, and cautiously acidified with 1M-HCl to pH2.7. This pH is maintained during 10min and the suspension is centrifuged (4000g, 10min). The supernatant solution S1 is collected, the residue is resuspended in 0.25M-sucrose, pH2.7, to 4 times semen volume and centrifuged together with fraction S1. The pooled supernatant fractions (S1/2) are stirred and adjusted to pH7.5 by dropwise addition of 1M-Tris. The resulting precipitate, which contains all the acrosin, is centrifuged, resuspended in 0.25M-sucrose, and centrifuged again. The pellets are suspended evenly in 20mM-ammonium citrate buffer, pH4 (0.5 times semen volume) and the pH is brought back to 4 with citric acid. After 5min the suspension is centrifuged, the supernatant solution S3 is removed, and the residue is re-extracted with ammonium citrate (0.5 times semen volume) to give solution S4. The pooled extracts (S3/4) contain most of the acrosin that was present in fraction S1/2: acrosin remaining in the residue is solubilized by adding

water (0.25 times semen volume) followed by HCl to pH2.7. Centrifugation yields a slightly opalescent fluid (solution S3/4R). The clear fraction S3/4 is dialysed for 24h, first against two portions of 1mM-HCl and then against 0.1mM-HCl. This solution (S5) is freeze-dried and stored in a desiccator at 5°C (fraction FD). Analytical data on the fractions are in Table 1. In experiments with trypsin a value of 1.4 was found for the quotient Bz-Arg-OEt units/nmol of enzyme and 2.7 for the quotient Tos-Arg-OMe units/nmol of enzyme.

Titration of acrosin (and trypsin) with acrosin (trypsin) inhibitors

A series of colorimetric assays was set up in which the only variable was the concentration of added inhibitor. Bz-Arg-NHNaph was added after time had been allowed for combination of enzyme and inhibitor. The linear portion of the plot of enzyme activity against inhibitor concentration was extrapolated to zero activity. The intercept gives the quantity of inhibitor that is stoichiometrically related to the enzyme taken for assay (Rhodes *et al.*, 1960; Fritz *et al.*, 1970.) Approximate titrations were made with a single concentration of inhibitor (as in Fig. 2). Thus if uninhibited and inhibited activities are x and y respectively and if $y > 0.5x$ the stoichiometric ratio inhibitor/enzyme is $(x-y)/x$.

Separation of acrosin from an endogenous acrosin inhibitor

Detection of inhibitor in acrosin preparations. The complex of acrosin and inhibitor dissociates at pH less than 3 and reassociates at pH8 (Zaneveld *et al.*, 1972a; Brown & Hartree, 1974). If an acrosin solution is kept at low pH for 5min and then added to buffered Bz-Arg-OEt the initial rate of hydrolysis equals that of the acrosin in the absence of inhibitor ('total activity'). However, addition of Bz-Arg-OEt to enzyme

Table 1. *Analysis of acrosin fractions derived from 32ml of ram semen*

Units of activity are derived from assays by standard procedures.

Fraction	Volume (ml)	Protein (mg/fraction)	$E_{280}^{1\text{cm}}$	Acrosin activity (units/fraction)		Acrosin (nmol/fraction)	Bz-Arg-OEt (units/nmol of acrosin)
				Bz-Arg-NHNaph	Bz-Arg-OEt		
S1/2	172	236	1.26*	21.1	1750	220	7.9
S3/4	32.6	37.2	1.55	24.8	1253	198	6.3
S3/4R	15.8	82.8		1.0	81		
S5	34.1	29.9	1.42	24.0	1468	193	7.6
FD	Weight of product: 33.2mg			25.7	1277	180	7.1

* After centrifuging at 100000g: the resulting pellet was devoid of acrosin.

EXPLANATION OF PLATE I

(a) Sagittal section of the acrosomal region of a ram spermatozoon ($\times 25\,000$); (b) the same as (a) after denudation ($\times 25\,000$). (Compare *a* and *b* with Fig. 1.) (c) Phase-contrast micrograph of intact ram spermatozoa ($\times 1800$); (d) ram spermatozoa after freeze-thawing, which loosens acrosomal membranes ($\times 1200$); (e) denuded spermatozoa ($\times 1800$). Comparison of heads in (e) and (c) shows loss of the dark crescent at the frontal border and change to a more 'angular' outline after loss of acrosomal material.

Fig. 2. Chromatography on Sephadex G-75 of 42mg of acrosin fraction FD

The column ($4\text{cm}^2 \times 57\text{cm}$) was run at 5°C with 0.2M-KCl plus HCl to $\text{pH}2.3$. Rate of flow, 23ml/h ; fraction size, 6ml . Enzyme assay (●): fractions ($10\mu\text{l}$) were assayed with Bz-Arg-NH-Naph by the standard method. Inhibitor assay (■): mixtures of $10\mu\text{g}$ of acrosin and 0.2ml of fraction were stored for 10min at $\text{pH}8.2$ and assayed in the same way. The activity of the enzyme alone is indicated by the arrow. ----, E_{280} ; —, enzyme activity.

buffered to $\text{pH}8$ will assay only the free acrosin remaining after formation of the inhibitor-enzyme complex ('net activity'). In the former case recombination leads to a progressive decrease in reaction rate after the first minute. Low concentrations of inhibitor may escape detection by this procedure but can be detected by gel chromatography (see next paragraph).

Removal of residual inhibitor from acrosin fraction FD. This was effected on a column of Sephadex G-75 (Fritz *et al.*, 1972), as described in Fig. 2. Fractions 16–18 were pooled, dialysed against HCl (1mM , 0.1mM) and freeze-dried. This inhibitor-free product contained 50% of the acrosin that was applied to the column, and unless otherwise stated this fraction was used for the experiments described below. Approx. 30% of the applied enzyme was recovered from adjacent fractions. The specific activity of the inhibitor-free fraction did not differ appreciably from that of the acrosin applied to the column. The acrosin activity of fraction 24 is apparent in the uppermost curve of Fig. 2, which illustrates the distribution of inhibitor. The excess of activity over the control, indicated for fraction 24 in the upper curve, approximates to the depression of the control activity exhibited by fractions (29, 30) that are richest in acrosin inhibitor. Thus in stoichiometric terms the acrosin

in fraction 24 was approximately equivalent to the inhibitor in fractions 29 or 30. It follows that in the solution applied to the column acrosin was present in very large excess over inhibitor.

Recovery of the acrosin inhibitor from wash fluids of denuded spermatozoa. Pooled fluids (1 litre) was adjusted to $\text{pH}2.5$ (HCl) and centrifuged at $36000g$ for 20min . The clarified fluid was adjusted to $\text{pH}4.5$ with sodium acetate and percolated at 10ml/h through a column ($2\text{cm}^2 \times 18\text{cm}$) of Sepharose 4B -trypsin at 5°C . The column was washed successively with 0.5M-NaCl , $\text{pH}2.4$, and 0.4M-NaCl , $\text{pH}2.0$ (pH being adjusted with HCl). Fractions were assayed for inhibitor as described in Fig. 2. Three fractions ($\text{pH}2.0$) containing most of the inhibitor were pooled and dialysed in retentive dialysis tubing for 3 days against 1mM-HCl , then for 1 day against 0.1mM-HCl . When freeze-dried the solution yielded 9mg of inhibitor. The product is not homogeneous, since the inhibitor undergoes slow proteolysis during retention on the column.

This acrosin inhibitor, being present in both epididymal and ejaculated ram spermatozoa, is distinct from inhibitors present in seminal plasma. It is widely regarded as being of acrosomal origin, but rigorous proof of its location is lacking. However, to avoid confusion with other inhibitors of acrosin it will be referred to in this paper as 'acrosomal inhibitor'.

Electron microscopy

Loosely pelleted spermatozoa were fixed for 1 h in sodium phosphate-buffered 1.5% (w/v) glutaraldehyde, $\text{pH}7.3$, and, after two washes in phosphate buffer, were transferred to phosphate-buffered 1% (w/v) OsO_4 . After two washes in 0.9% NaCl , the pellets were dehydrated in ascending concentrations of ethanol, followed by immersion for 2h in propylene oxide. The pellets were then embedded in Epon 812 and silver-dark-grey sections were cut with a LKB Ultratome III fitted with a diamond knife. Sections were stained with aqueous uranyl acetate (3%) and lead citrate (0.2%), and examined in a AEI 801 electron microscope at 60kV .

Results

Stability of acrosin in acid solutions and its reversible inactivation by desiccation

Activity (units per fraction) estimated with Bz-Arg-OEt remains approximately proportional to molar content of acrosin during the conversion of fraction S1/2 into fraction FD (Table 1), except for fraction S3/4. However, activity per fraction, measured with Bz-Arg-NH-Naph , increases significantly as the amount of enzyme decreases, in molar

Fig. 3. Spontaneous reactivation of extensively desiccated acrosin after its dissolution in 1 mM-HCl

Of this solution 5 μ l portions (10 μ g of acrosin) were periodically assayed under standard conditions, and titrated fluorimetrically. $\square$, Bz-Arg-OEt assays and $\blacksquare$, titrations of enzyme molarity in solutions stored at 5°C. $\circ$, Bz-Arg-OEt assays and $\bullet$, titrations of solutions stored at -20°C. Frozen samples were assayed immediately after thawing and were not re-used for subsequent assays.

terms, by 19%. We have no evidence that this is due to removal of inhibitor. The low Bz-Arg-OEt-hydrolysing activity of fraction S3/4 arises from contact with ammonium citrate. When a sample of fraction S1/2 was dialysed for 16 h against the ammonium citrate buffer its activity fell by 30%: a parallel dialysis of fraction S3/4 against 1 mM-HCl led to a comparable increase in activity. A more marked reversible inactivation can be obtained by extreme desiccation. When inhibitor-free acrosin is freeze-dried by standard methods and is redissolved in 1 mM-HCl the activity of the solution may increase by 10–30% during 24 h at 5°C. On the other hand, when a freeze-dried product was stored in a high vacuum over P_2O_5 for 4 weeks and then dissolved in 1 mM-HCl, immediate assay revealed very low activity. However, during the next 48 h the activity of this solution rose considerably (Fig. 3) and approached that of the original undried solution. (The peak in the -20°C curve was more pronounced in parallel assays with Bz-Arg-NHNaph.) The increase in Bz-Arg-OEt-hydrolysing activity was proportional to the increase in titratable active sites. The extent of this reversible inactivation was variable and with some samples it was as low as 30%. Activities of solutions of freeze-dried acrosins recorded elsewhere in this paper refer to reactivated enzyme.

Fig. 4. Effect of $CaCl_2$ on the activity (Bz-Arg-OEt) of acrosin fraction S1/2 (two preparations)

Assays of 25 μ l portions were performed in standard conditions except for variations in $CaCl_2$ concentration. $\circ$, Net activity of preparation 1; $\square$, net activity of preparation 2; $\bullet$, total activity of preparation 1. For measuring net activity enzyme was stored in buffer for 20 min.

Lack of influence of glass surfaces on catalysis by ram acrosin at I not less than 0.05

Johnson & Whateley (1972) showed that dissolved trypsin is adsorbed on glass surfaces at I 0.01 but not at I 0.05. However, investigators of acrosin of the boar (Schiesler *et al.*, 1972) and of man (Fritz *et al.*, 1972) have stressed the necessity of using plastic vessels and pipettes, as well as silicone-treated cuvettes, at I about 0.1 to prevent adsorption and inactivation. We failed to observe this phenomenon with ram acrosin at I not less than 0.05 (e.g. in 50 mM-Tris buffer). While using 20–30 mM-Tris buffer we found that rates of hydrolysis of Bz-Arg-OEt were less reproducible than with 50 mM-Tris and that use of silicone-treated cuvettes led to increased activities. However, this increased activity was still below that observed with 50 mM-Tris and untreated cuvettes.

Activation of acrosin preparations by cations

Experiments with S1/2 fractions. When ram spermatozoa are denuded by vortex-mixing and then centrifuged the supernatant contains acrosomal inhibitor. In early experiments we were unaware that a small proportion of this inhibitor was particulate and was sedimented in the conditions then chosen (4000g). This inhibitor was solubilized at pH 2.7 and so carried over into fraction S1/2.

The influence of $CaCl_2$ on the acrosin activities of such fractions is illustrated in Fig. 4. When assayed

Fig. 5. Effects of cations on the total activity (Bz-Arg-OEt) of inhibitor-free acrosin

The standard assay was used but 0.2M-CaCl₂ was replaced by different concentrations of (a) CaCl₂, (b) BaCl₂, (c) MgCl₂, (d) MnCl₂, (e) LiCl, (f) NaCl, (g) KCl. Formation of Mn(OH)₂. (ai) Experiment as (a) but with 10 μ g of acrosin fraction FD.

for net activity (see the Methods section) some 'early' S1/2 fractions showed very low activity in the absence of Ca²⁺ but were activated 4–5-fold by 0.2M-CaCl₂ (curve a). Other early fractions exhibited a higher (net) activity in the absence of Ca²⁺, inhibition by approx. 1 mM-CaCl₂, but activation by higher Ca²⁺ concentrations (curve b). When early S1/2 fractions were assayed for total activity there was activation at all concentrations of CaCl₂ (curve c). Curves a and c were obtained with a single S1/2 preparation and the zero-Ca²⁺ points on these two curves illustrate the effect of contaminating acrosomal inhibitor. The difference in activities was not due to autodigestion of acrosin at pH8.2, a process that can occur rapidly with rabbit acrosin (Zaneveld *et al.*, 1972b). In fact, the net activity was independent of the time of storage at pH8.2 (before addition of Bz-Arg-OEt) for periods between 1 and 25 min. Purification of early S1/2 fractions, as in Table 1, did not eliminate the inhibitor and did not significantly change the extent of activation by Ca²⁺.

The effect of Ca²⁺ was not specific: similar large activations (approx. 300%) were obtained with BaCl₂, MgCl₂, KCl, NaCl and LiCl. Optimum concentrations were approx. 0.2M for bivalent and 0.8M for univalent cations.

Table 2. Design of experiments to illustrate stabilization of acrosin at pH8.2 by Ca²⁺, Mg²⁺ and Na⁺

Acrosin (20 μ g/ml in 50mM-Tris buffer) was stored at 20°C in the presence of additional salt as in column 1. At different times 0.5 ml of stored solution was diluted with 1.1 ml of the Tris buffer containing 1.6 μ mol of Bz-Arg-OEt and sufficient salt to give the concentration shown in column 2. Assays were run at 25°C. Experiments involving Ca²⁺ are recorded in Fig. 6.

Additions to stored solutions of buffered acrosin	Concentrations of salts during assays
—	—
0.05M-CaCl ₂	0.36M-CaCl ₂
0.1M-CaCl ₂	
—	—
0.05M-MgCl ₂	0.1M-MgCl ₂
0.1M-MgCl ₂	
—	—
0.5M-NaCl	1.0M-NaCl
1.0M-NaCl	

Experiments with other acrosin fractions. Demonstrations (i) that activation by inorganic cations is a general phenomenon, and (ii) that Tris, and also other buffers, activate acrosin (Fig. 7) raised the question of an appropriate buffer for routine assays. The choice of 50mM-Tris followed from observations that with lower concentrations assays were not reproducible, whereas with higher concentrations the activation produced by Tris tended to mask activation by other cations, particularly by Ca²⁺. In 50mM-Tris buffer the activities (total) of acrosin fraction FD and of the inhibitor-free enzyme were enhanced by inorganic cations, but the degrees of activation were considerably lower than was the case with fractions of the type used in Fig. 4. Fig. 5 shows the effects of Ca²⁺, Ba²⁺, Mg²⁺, Mn²⁺, Li⁺, Na⁺ and K⁺ on inhibitor-free acrosin. Maximum activation was obtained with Mg²⁺. Activations by alkali-metal cations were low, especially that by Na⁺. Replacement of CaCl₂ by calcium acetate did not change the extent of activation.

Fraction FD gave almost identical results in assays for total and net activities, and fractionation on Sepharose-trypsin (Fig. 2) detected very little inhibitor. Nevertheless, this fraction exhibited a higher degree of activation by Ca²⁺ (Fig. 5, curve ai) than did inhibitor-free acrosin, and also a different optimal CaCl₂ concentration. Routine assays of acrosin with Bz-Arg-OEt were therefore made in the presence of 0.2M-CaCl₂, a concentration intermediate between the maxima of curves (a) and (ai).

Stabilization of acrosin at pH8.2 by cations

The three salts that were studied (CaCl₂, MgCl₂, NaCl) showed both activating and stabilizing

Fig. 6. Stabilization of acrosin by CaCl_2 at pH 8.2

Experimental details are given in Table 2. ■, Storage in absence of CaCl_2 ; ●, storage with 0.05M- CaCl_2 ; ▲, storage with 0.1M- CaCl_2 .

effects. The enzyme was therefore stored for various periods with different concentrations of a given salt, but the substrate (Bz-Arg-OEt) was added simultaneously with a solution of the salt that raised its concentration to the value for maximum activity (Table 2). Stabilization by CaCl_2 was complete during 5h (Fig. 6) and the subsequent decline in activity was slower in the presence of Ca^{2+} . Experiments with MgCl_2 yielded very similar results. Improved stabilization was afforded by NaCl: inclusion of 1M-NaCl during storage stabilized acrosin completely for 50h, and 0.5M-NaCl was almost as effective.

Measurement of acrosin activity in different buffers

Three buffers were compared: Tris-HCl, triethanolamine-HCl, and the zwitterion Epps-NaOH (all pH 8.2). Activities of acrosin towards Bz-Arg-OEt in these buffers are shown in Fig. 7. Analytical-grade triethanolamine is not available and the noisy recorder traces obtained when assays contained more than 0.6M-triethanolamine signified u.v.-absorbing impurities. All the buffers activated acrosin, especially Tris, which at 1.2M was more effective than inorganic cations (Fig. 5). However, rates of hydrolysis of Bz-Arg-OEt in all buffers slowly declined after 1min. With buffers at 50mm, added Ca^{2+} gave rise to significant activations: in Epps buffer activation was comparable with that obtained in Tris buffer (Fig. 5), but

Fig. 7. Relative activities of acrosin in different buffers at pH 8.2

Hydrolysis of Bz-Arg-OEt was followed in triethanolamine-HCl (○), Epps-NaOH (□) and Tris-HCl (▽). ■, Effects of adding CaCl_2 to experiment (a).

in triethanolamine there was less activation (10–15%). As buffer concentrations were raised above 50mm the percentage activation by Ca^{2+} fell, becoming insignificant with 0.3–0.6M-triethanolamine and also with 0.6–1.2M-Tris. With Epps at higher concentrations Ca^{2+} had a small inhibitory action (Fig. 7). Whenever Ca^{2+} was present rates of hydrolysis of Bz-Arg-OEt remained linear for 3–4min. Unlike CaCl_2 , concentrations of Tris up to 1M did not stabilize acrosin at pH 8.

Apparent inhibitions by Ca^{2+} may be observed with buffers that react with Ca^{2+} . When the zwitterion buffer *NN*-(bis-2-hydroxyethyl)glycine-NaOH was used for experiments as in Fig. 7 chelation with added Ca^{2+} caused falls in pH of up to 2.5 units. The universal buffer used by Multamäki & Niemi (1972), which contains phosphate and borate (Britton & Robinson, 1931), also exhibited a fall in pH on addition of CaCl_2 ; subsequently an acrosin-binding precipitate appeared.

Effects of Ca^{2+} and Na^+ on the reactions of acrosin and trypsin with inhibitors

Inhibition of acrosin by acrosomal inhibitor: influence of Ca^{2+} and Na^+ . Figs. 4 and 5 suggest that Ca^{2+} decreases the affinity of this inhibitor for acrosin. At the present stage, when neither component is available in pure form, measurements of affinity constants would be premature. However, we are reporting experiments that illustrate conditions in which Ca^{2+} and Na^+ influence the affinity.

Table 3. Influence of Ca^{2+} on the inhibition by acrosomal inhibitor of the hydrolysis of Bz-Arg-OEt: design of experiments

[For results see Fig. 8(a).] All assays contained Tris-HCl buffer, pH 8.2 (80 μmol). Other components were: 10 μg of acrosin (in 10 μl of 1 mM-HCl), 0.3 ml of 2.67 M- CaCl_2 , 0.15 ml of 10 mM-Bz-Arg-OEt, inhibitor as in Fig. 8; order of addition as in the Table. S, Time of storage at 25°C.

Expt.	Water+buffer±inhibitor to a volume of: (ml)	CaCl_2	Acrosin	S (min)	Water (0.3 ml)	CaCl_2	Bz-Arg-OEt
i	1.15	—	+	5	+	—	+
ii	1.15	—	+	5	—	+	+
iii	0.85	+	+	5	+	—	+
			Bz-Arg-OEt		Acrosin		
iv	1.45	—	+	0	+		
v	1.15	+	+	0	+

Fig. 8. Results of experiments carried out as described in Table 3

(a) Influence of 0.5M- CaCl_2 on the acrosin-catalysed hydrolysis of Bz-Arg-OEt in the presence of different amounts of acrosomal inhibitor: —, CaCl_2 present; ----, CaCl_2 absent. Curves are numbered to correspond to Table 3. All reactions proceeded linearly for 4 min except in Expt. iv, where initial rates ($\odot$) usually exceeded rates after 3 min ($\bullet$). Curve (ii) is omitted (see the text), but two experimental points ($\times$) are given. (b) Experiments as in iii and v of Table 3, but with NaCl (1 M final concentration) replacing CaCl_2 .

Effects of Ca^{2+} were studied by the procedure of Table 3. In experiments i, ii and iii acrosin was stored briefly at pH 8.2 before the addition of Bz-Arg-OEt, whereas in experiments iv and v the reaction was started by adding acrosin to buffered Bz-Arg-OEt. Ca^{2+} was either absent, present throughout, or added

after the storage period. The most marked effect of the inhibitor was obtained in the absence of Ca^{2+} and with the substrate added last (Fig. 8a, curve i). Degrees of inhibition expressed by curve i were considerably decreased if acrosin was added to buffered inhibitor- Ca^{2+} (curve iii). However, if Ca^{2+} was added after prior contact of acrosin and inhibitor (curve ii) the inhibitory action was almost as strong as in the absence of Ca^{2+} . For the sake of clarity curve (ii) is omitted from Fig. 8(a); with not more than 1 μg of inhibitor present experimental points lay close to those of curve (iii), but with higher amounts the points ($\times$) were close to curve (i). The branched curve (iv) summarizes experiments in which acrosin was added to buffered mixtures of Bz-Arg-OEt and inhibitor. Rates measured during the first minute (upper limb of curve iv) were those of uninhibited acrosin, but within 3 min combination of acrosin and inhibitor had occurred (lower limb of curve iv). Curve (v) was obtained in the same way as curve (iv) except that Ca^{2+} was mixed with buffered inhibitor before acrosin was added. In these conditions the effect of the inhibitor was almost abolished. Thus although Ca^{2+} may largely prevent the interaction of acrosin with acrosomal inhibitor it can dissociate the already formed complex only to a limited extent.

Fig. 8(b) shows experiments similar to those of curves (iii) and (v) of Fig. 8(a) except that 0.5M- CaCl_2 was replaced by 1 M-NaCl. Protection of acrosin against inhibition was again obtained. Further, the initial fall in the curves of Fig. 8(a) (within the range 0–0.2 μg of inhibitor) is absent from Fig. 8(b). This fall is perhaps due to a form of acrosin which, in its reaction with the inhibitor, is unaffected by Ca^{2+} .

Inhibition of acrosin by Trasylol: potentiation by Ca^{2+} . Trasylol has a very high affinity for bovine trypsin (Vincent & Lazdunski, 1972) but a low affinity for acrosin. Thus addition of Bz-Arg-OEt causes rapid dissociation of the acrosin-Trasylol complex. Such dissociation occurs to a lesser extent in the

Table 4. *Effect of CaCl_2 on the inhibition by Trasylol of hydrolysis of Bz-Arg-NHNaph by acrosin*

Acrosin ($10\mu\text{g}$) was stored for 30 min at 25°C with 1.0 ml portions of 50 mM-Tris buffer containing different concentrations of CaCl_2 . A parallel series was set up with 0.32 mg of Trasylol present. Bz-Arg-NHNaph (0.1 ml) was added and the assays were run at 37°C .

CaCl ₂ in assay (mM)	Acrosin activity (munits)	
	-Trasylol	+Trasylol
0	2.96	2.12
2	3.17	2.71
8	4.60	2.43
40	5.38	1.32
200	6.86*	0.64*

* Partial precipitation of substrate occurred. Activation by Ca^{2+} is greater than in assays with Bz-Arg-OEt.

Bz-Arg-NHNaph assay system. In the experimental conditions of Table 4 addition of Ca^{2+} accentuated the inhibitory activity from 28% in the absence of Ca^{2+} to about 90% in the presence of 0.2 M- CaCl_2 .

Proteinase-inhibitor systems that are unaffected by Ca^{2+} . Inhibition of acrosin by benzamidine is insensitive to CaCl_2 concentrations up to 0.4 M. Also insensitive are inhibitions of trypsin by benzamidine, Trasylol and acrosomal inhibitor.

Titration of acrosin and trypsin with inhibitors

Because Ca^{2+} decreases the affinity of acrosin for acrosomal inhibitor, titrations are best carried out in the absence of Ca^{2+} (Fig. 9, curve *a*). The $10\mu\text{g}$ of acrosin used for this experiment contained 47 pmol of enzyme. Thus from the ordinate intercept (1.65) it follows that the inhibitor preparation contained 28.5 pmol/mg. Titration in the presence of Ca^{2+} yielded an atypical curve (*b*) suggestive of the presence of more than one type of acrosin. The affinity of trypsin for acrosomal inhibitor is uninfluenced by Ca^{2+} and a linear titration curve was obtained in the presence of this ion (curve *c*). The assays on which curve *c* was based contained 190 pmol of trypsin. From this value, and extrapolation of curve *c*, a value of 27.0 pmol of acrosomal inhibitor per mg was obtained. A third estimate can be derived from curve *i* of Fig. 8(a), namely 28.5 pmol/mg.

Titration of acrosin with Trasylol yielded curves with no linear regions. However, logarithmic plots (Fig. 10) revealed interesting results. In the presence of Ca^{2+} such plots were linear, but in its absence the curve was biphasic, showing an intermediate section ($1\text{--}30\mu\text{M}$ -Trasylol) within which the percentage inhibition remained constant. This implies the presence of forms of acrosin with very different affinities for Trasylol in Ca^{2+} -free conditions.

Fig. 9. *Titration of acrosin and trypsin with acrosomal inhibitor by using the colorimetric assay*

Enzyme+buffer+inhibitor were kept at 20°C for 20 min before addition of Bz-Arg-NHNaph. ●, $10\mu\text{g}$ of acrosin in 50 mM-Tris-HCl buffer; ○, $10\mu\text{g}$ of acrosin in Tris-HCl-0.1 M- CaCl_2 ; □, $7.5\mu\text{g}$ of trypsin in the latter buffer.

Fig. 10. *Inhibition of 40 pmol of acrosin with different quantities of Trasylol*

Assays were as in Fig. 9 but with the following buffers (pH 8.2): ●, 50 mM-Tris-HCl; ○, 50 mM-Tris-HCl-0.1 M- CaCl_2 ; □, 0.1 M-sodium barbiturate-HCl-0.2 M- CaCl_2 diluted with 0.2 vol. of dimethyl sulphoxide (Brown *et al.*, 1975).

ACTIVATION OF RAM ACROSIN BY CATIONS

Table 5. *Effects of 2-methylpropan-2-ol and dimethyl sulphoxide on the total activity of acrosin in different buffers*

The volumes of solvent present in the 1.6 ml assay volume were 0–0.3 ml (at intervals of 0.05) and 0.4 ml. Assay: Bz-Arg-OEt and 8 μ g of acrosin. The volume of solvent for maximum activity is given in parentheses.

Concentration of buffer constituents (mm)			Enzyme activity in absence of solvent (unit/assay)	Maximum activation (%) by solvent	
Tris	NaCl	CaCl ₂		2-Methylpropan-2-ol	Dimethyl sulphoxide
5	100	—	0.278	23 (0.2)	82† (0.4)
5	100	200	0.400	16 (0.1)	24† (0.4)
5	100	20	0.314	21 (0.1)	63† (0.4)
50	—	—	0.274	*	
50	100	—	0.304	31 (0.4)	
50	—	200	0.366	11 (0.25)	
50	100	200	0.392	14 (0.2)	
50	—	20	0.323	62† (0.25)	
50	100	20	0.365	40† (0.3)	
250	—	—	0.315	55† (0.25)	
1000	—	—	0.390	22 (0.1–0.3)	

* Reaction rates were not reproducible with 0.05–0.20 ml of solvent. Higher solvent concentrations gave rise to not more than 10% activation.

† These degrees of activation resulted in maximum activities of 0.49–0.52 unit/assay.

Table 6. *Kinetic constants for bovine trypsin and inhibitor-free ram acrosin at 25°C*

Values for trypsin (pH 8.0) are taken from Walsh & Wilcox (1970). In experiments with Tris the pH was adjusted to pH 8.1 with HCl. Constants were determined from plots of $V/[S]$ against $[S]$ and fluorimetric titration of enzyme molarity.

	Substrate	Medium for determination of constants	K_m (app.) (mm)	k_{cat} (s ⁻¹)
Trypsin	Bz-Arg-OEt	0.1 M-NaCl–2 mm-CaCl ₂	0.0043	14.6
	Tos-Arg-OMe	0.2 M-KCl–50 mm-CaCl ₂	0.0125	100
Acrosin	Bz-Arg-OEt	50 mm-Tris	0.28	115
	Bz-Arg-OEt	50 mm-Tris–0.2 M-CaCl ₂	0.12	120
	Bz-Arg-OEt	50 mm-Tris–20 mm-CaCl ₂ –0.1 M-NaCl	0.16	134
	Bz-Arg-OEt	5 mm-Tris–20 mm-CaCl ₂ –0.1 M-NaCl + Me ₂ SO (25% v/v)	0.16	162
	Tos-Arg-OMe	50 mm-Tris–0.2 M-CaCl ₂	0.021	49

Influence of water-miscible solvents on the activity of acrosin

Our experimental findings are summarized in Table 5. At the higher ionic strengths, where cation activation was marked, activation by solvents tended to be low. In some cases, especially with dimethyl sulphoxide, activation increased progressively as solvent concentration rose to 25% (v/v). In other cases maximum activities were found with lower solvent concentrations. In excess of 25% (v/v) both solvents caused progressive inactivation of the enzyme. When assays included acrosomal inhibitor sufficient to cause about 50% inhibition the solvent effects were augmented: in 50 mm-Tris and 20 mm-CaCl₂ partially inhibited acrosin was activated by 95%. This explains our observations that solvent

effects were more marked with 'early' S1/2 preparations than with inhibitor-free acrosin.

Lower alcohols were less effective activators than 2-methylpropan-2-ol, and butan-1-ol, at near-saturation, was inhibitory.

Kinetic constants for trypsin and acrosin

Since commercial trypsin is a mixture of active forms and since there is evidence that the inhibitor-free acrosin preparation is also heterogeneous the values of K_m and k_{cat} in Table 6 can have no absolute validity. We have therefore not used statistical or computer-aided methods for minimizing experimental error. Nevertheless, there are clear discrepancies between our results and some published values for K_m (Bz-Arg-OEt). Schleuning & Fritz (1974) give

0.27 mM for boar acrosin (assayed in the absence of Ca^{2+}), whereas Polakoski *et al.* (1973) give 0.05 mM (plus Ca^{2+}) for the same species and Stambaugh & Buckley (1972b) give 0.005 mM (plus Ca^{2+}) for rabbit acrosin. The last two groups used detergents to solubilize acrosin.

Discussion

Isolation procedures for acrosin

Hartree & Srivastava (1965) developed a method for detachment of ram acrosomes by means of a cationic detergent: Hyamine 2389. Solubilization of the resulting acrosome-rich fraction provided a medium which exhibited marked activity in the removal and digestion of the cellular and membranous investments of the freshly ovulated rabbit egg (Srivastava *et al.*, 1965). Such detergent extracts contain hyaluronidase (Srivastava *et al.*, 1965), a trypsin-like proteinase, i.e. acrosin (Stambaugh & Buckley, 1969), and several lysosomal-type hydrolases (Allison & Hartree, 1970). The last-named authors put forward the view that the acrosome is a lysosome that has evolved to aid penetration of the spermatozoon into the egg (see also Hartree, 1975). The disadvantages of detergent extraction are (i) the incompleteness of solubilization of acrosomal enzymes, (ii) the presence in such extracts of hydrolases, in particular of proteinases, which could conceivably degrade acrosin, and (iii) the presence of lipids and detergent which decrease the stability of acrosin and hinder purification.

Fink *et al.* (1972) and Fritz *et al.* (1972) extracted acrosin from spermatozoa with acetic acid-HCl, pH 1.8, concentrated the solution, and separated acrosomal inhibitor from acrosin by gel chromatography. The neutralization step in our procedure achieves a similar result in a simpler manner. For success this step requires a very low ionic strength. Thus if ram spermatozoa are extracted by the acetic acid-HCl method, and the extract is neutralized, the acrosin remains in solution. Extracts (pH 2.7) of denuded ram spermatozoa exhibit greater acrosin stability than do comparable extracts of whole spermatozoa. This may be related to the removal during denudation of acid hydrolases, in particular of an acid proteinase. Whereas this proteinase would be less active at pH 1.8 the yield of soluble ram acrosin (fraction S1/2) is highest at pH 2.7.

Factors influencing the catalytic activity of acrosin: comparisons with trypsin

Green & Neurath (1953) discussed the activation of trypsin by Ca^{2+} in terms of equilibria between active and reversibly denatured trypsin (Northrop *et al.*,

1948) and the digestion of the latter by the former. Postulates that Ca^{2+} binds to the active form (or has a higher affinity for the active form) were put forward to explain both activating and stabilizing influences of this cation at neutral pH. Yon (1959) applied similar arguments to the decrease in the rate of autolysis of trypsin by 1 M-NaCl, i.e. maintenance of tertiary structure of the native enzyme was invoked. Direct optical evidence for a Ca^{2+} -induced conformational change has been provided by Lazdunski & Delaage (1965).

The almost complete stabilization of acrosin by Ca^{2+} (Fig. 6), Mg^{2+} and Na^+ at pH 8 is more striking than the partial stabilization of trypsin by cations. This suggests a more mobile structure for acrosin, as does also the reversible inactivation by desiccation. The latter phenomenon finds a parallel in the reversible denaturation of haemoglobin by dehydration (Keilin & Hartree, 1952). We have been unable to effect reversible inactivation of trypsin by desiccation, although other means of doing so have been described (Northrop *et al.*, 1948). The extent of activation of acrosin by cations depends on the type and concentration of buffer as well as on the presence of solvents. Table 5 shows that there is an apparent maximum enzyme activity that can be realized in media containing various concentrations of Tris, Na^+ , Ca^{2+} and solvent. Thus the effects of ions and solvents are not additive but appear to be complementary. These results are in accord with the views expressed below that the effects of dimethyl sulphoxide on acrosin, like those of Ca^{2+} , are in the main expressions of configurational changes. They also illustrate the principle that one cannot observe any intrinsic influence of a non-enzyme additive to an assay system but only a change in activity that reflects a perturbation by that component of a complex equilibrium between water, enzyme and other solutes. In other words conformation, and therefore activity, of an enzyme is governed by the total environment. Similar statements of this principle have been made (e.g. in Fink, 1974) and it appears self-evident. However, the effects of the environment can vary. At a given pH value the activity of glucose oxidase from *Penicillium notatum* is unaffected to a considerable degree by changes in buffer composition, a tendency that is consistent with an uncharged substrate (E. F. Hartree, unpublished work).

It is reasonable to assume by analogy with trypsin that stabilization of acrosin by cations is due to maintenance of a native configuration. What is not clear at present is why such stabilization (by Ca^{2+}) should inhibit the reaction of acrosomal inhibitor with acrosin yet promote the reaction of Trasylol with acrosin. Lack of effects of Ca^{2+} on inhibition of acrosin by benzamidinone indicates that Ca^{2+} influences the affinities of acrosin for protein-type inhibitors by action at secondary binding sites.

Now that a very sensitive method is available for determining the molarity of acrosin solutions (Brown *et al.*, 1975) it becomes feasible to express concentrations of acrosin inhibitors in absolute terms instead of in the arbitrary units so far used.

Possible mechanisms for the solvent-induced changes in acrosin activity

Addition of up to 20% (v/v) of different water-miscible solvents to reactions catalysed by trypsin and chymotrypsin causes considerable increases in K_m , whereas k_{cat} is relatively unaffected (Kaufmann & Neurath, 1949; Inagami & Sturtevant, 1960; Clement & Bender, 1963; Mares-Guia & Figueiredo, 1972). Dimethyl sulphoxide is a potential perturbant of protein structure owing to its influence on hydrophobic bonding and to its own hydrogen-bond-accepting propensities (Rammner, 1971; Fink, 1974). However, the limited effects of solvents on k_{cat} appear to rule out major conformational changes, at least in the region of the active site. Large solvent-induced changes in K_m have been ascribed to dielectric changes and hence to weakening of electrostatic interactions between enzyme and substrate (Inagami & Sturtevant, 1960) or to the combined influences of dielectric changes and competition between solvent and substrate for the enzyme (Mares-Guia & Figueiredo, 1972). Fink (1973, 1974) has studied the hydrolysis of *p*-nitrophenyl esters by chymotrypsin and trypsin. In these experiments $\log K_m$ increased approximately linearly with percentage dimethyl sulphoxide, whereas k_{cat} decreased only in proportion to the fall in water concentration. However, effects on k_{cat} may vary with pH, temperature and possibly with substrate (Fink, 1974); thus a generalized comparison of the influence of solvents on acrosin and trypsin is difficult.

For acrosin and Bz-Arg-OEt (Table 6) we have found that addition of 25% (v/v) of dimethyl sulphoxide has no effect on K_m but leads to a significant increase in k_{cat} . In this instance it appears that conformational change, rather than dielectric and competitive phenomena, is the predominating influence on activity. In fluorimetric titrations of acrosin and trypsin with 4-methylumbelliferyl *p*-guanidinobenzoate (Brown *et al.*, 1975) the rate of formation of acyl-trypsin is retarded by dimethylsulphoxide whereas that of acyl-acrosin is not. This is consistent with the contrasting influences of this solvent on K_m values for the two enzymes obtained with Bz-Arg-OEt.

Should acrosin be classed as a cathepsin?

The denudation process involves removal of the plasma membrane and the greater part of the acrosome, but allows retention of almost the entire

complement of acrosin (Brown & Hartree, 1974; Plate 1 of this paper). Since acrosin is limited to the acrosomal region (Stambaugh & Buckley, 1972a) it has been concluded that the intracellular distribution of acrosin must be restricted to those parts of the acrosome that are not dislodged during the denudation process, i.e. to the inner acrosomal membrane and to the residual acrosomal membranes that constitute the equatorial segment (Brown & Hartree, 1974). The denuded ram spermatozoon is similar in form to a rabbit spermatozoon that has undergone the 'acrosome reaction'. This reaction occurs *in vivo* as the spermatozoon approaches the egg: it involves loss of membranes from the rostral region of the head and results in the inner acrosomal membrane becoming a limiting membrane (Fig. 1). The essential difference between this process and denudation is a sealing process (Fig. 1), which leaves the 'reacted' spermatozoon with a continuous limiting membrane and which results in preservation of its internal milieu. There is no evidence that acrosin becomes soluble during penetration of the spermatozoon through the egg investments; there are indeed indications that lytic agents at, or close to, the acrosomal surface (i.e. that persisting after the acrosome reaction) remain *in situ* after penetration of the zona pellucida and become functional at the stage of fusion of the spermatozoon with the egg (Bedford, 1970). It is thus reasonable to classify acrosin as a cathepsin.

Acrosin may be solubilized *in vitro* not only by acidification but also by raising the ionic strength. Reversing either change in the medium leads to re-adsorption of acrosin. Inhibitor-free acrosin does not aggregate and precipitate at neutral pH. It thus meets the main criteria for a peripheral membrane protein, i.e. one that plays no essential role in maintaining membrane structure (Singer & Nicholson, 1972). Binding of acrosin to a membrane must impart a degree of rigidity to the molecule. The lower K_m values for acrosin solubilized with detergents may arise from a degree of conformational stabilization by solubilized membrane components.

We have devised a simple method for preparation of soluble ram acrosin as a stable water-soluble product. Preliminary experiments involving affinity chromatography indicate that it may contain about 25% enzyme. With this material we have resolved certain contradictions between earlier publications. Although soluble acrosin must be regarded as an artifact it retains biochemical interest. Further purification should provide material suitable for quantitative study of proteolysis of different protein classes, a subject which has so far received no attention apart from a paper by Polakoski & McRorie (1973). It would also allow determinations of the primary structure of ram acrosin and detection of possible homologies with trypsins.

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