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THE HISTOCHEMICAL LOCALIZATION OF ACROSIN IN GUINEA-PIG SPERM AFTER THE ACROSOME REACTION

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SUMMARY

The protease acrosin is widely considered to be an essential component of a zona lysin which enables sperm to penetrate the zona pellucida of the egg. Sperm form a characteristic penetration slit little wider than the sperm head itself and this has long suggested that any zona lysin is attached to the sperm surface after an acrosome reaction. This paper provides the first ultrastructural evidence that this is the case.

The protein acrosin inhibitor, Kunitz soybean trypsin inhibitor, has been covalently attached to the electron-dense marker, ferritin, and the conjugate incubated with guinea-pig sperm which have undergone an A23187-induced acrosome reaction. Electron microscopy shows that ferritin is distributed unevenly over the outer surface of the newly exposed inner acrosomal membrane but does not extend to the equatorial segment. This is further evidence that acrosin can be considered as a candidate for the role of zona lysin. The mechanism of sperm penetration of the zona is discussed in the light of these observations.

INTRODUCTION

There are at least two possible reasons why an acrosome reaction is essential for sperm penetration of the zona. One is that sperm need to change their shape. The other is that an acrosome reaction releases or exposes a zona lysin which enables sperm to digest a path (Bedford, 1970). The shape of the penetration slit suggests that any lysin is attached to the sperm surface.

The zona lysin has been identified with acrosin, a trypsin-like protease which is stored in the acrosome as an inactive zymogen precursor proacrosin (Meizel, 1972; Mukerji & Meizel, 1975; Schleuning, Hell & Fritz, 1976). Proacrosin is activated to acrosin during the acrosome reaction (Green, 1978*b*). The evidence that acrosin is important for zona penetration is firstly, that acrosin extracts can remove the zona *in vitro* (Polakoski & McRorie, 1973; Meizel & Mukerji, 1976) and secondly, that fertilization is inhibited by acrosin inhibitors (Stambaugh, Brackett & Mastroianni, 1969; Zanefeld, Robertson, Kessler & Williams, 1971; Gaur, Goswami & Talwar, 1972; Yang, Zanefeld & Schumacher, 1976). However, the site at which fertilization is blocked is unknown and the experimental observations have themselves been disputed (Miyamoto & Chang, 1973).

If acrosin is to be a candidate for the zona lysin, it must be shown that it remains attached to the sperm surface after an acrosome reaction. This has not so far been done, although acrosin has been localized by fluorescence and light microscopy in sperm which had not previously undergone an acrosome reaction (Stambaugh &

Buckley, 1972; Garner, Easton, Munson & Doane, 1975; Morton, 1975; Schill *et al.* 1975; Garner & Easton, 1977).

Histochemically, acrosin has been identified in one of two ways: by Kunitz soybean trypsin inhibitor (Stambaugh & Buckley, 1972) and by anti-acrosin antibodies (Garner *et al.* 1975; Morton, 1975; Schill *et al.* 1975; Garner & Easton, 1977). This article describes the formation of a conjugate between the Kunitz soybean trypsin inhibitor and the electron-dense marker ferritin and its use in the localization of acrosin in guinea-pig sperm after an acrosome reaction induced by the divalent cation ionophore A23187 (Green, 1976; Summers *et al.* 1976; Talbot *et al.* 1976; Green, 1978*a*).

METHODS

Ferritin, soybean trypsin inhibitor (STI) Type I-S, bovine serum albumin (BSA), benzamidine and benzoyl arginine ethyl ester (BAEE) were purchased from Sigma, 25 % glutaraldehyde from Koch-Light, Sepharose 4B, Sepharose CL-6B, DEAE Sephadex A-50, Sephadex G-50 (fine) and Ficoll 400 from Pharmacia and carrier-free Na [^{125}I] from the Radiochemical Centre Ltd., Amersham. All other reagents were A.R. grade. A23187 was a generous gift from Dr R. L. Hamill of Eli Lilly Laboratories, Indianapolis. Schleicher & Schüll UH100 Ultrathimbles were used to concentrate protein. Trypsin was assayed by following the hydrolysis of BAEE at 256 nm in a Zeiss PMQ II spectrophotometer. The assay solution contained 1 mM BAEE, 50 mM $CaCl_2$ and 100 mM Tris chloride buffer, pH 8·0. Trypsin was coupled to Sepharose 4B with cyanogen bromide (Cuatrecasas, 1970) using 1 mM benzamidine throughout to inhibit reversibly trypsin autodigestion: the final derivative contained about 2 mg trypsin/ml Sepharose 4B. STI was purified by the method of Rackis, Sasame, Anderson & Smith (1959). Siliconized glassware was used throughout.

Ferritin was coupled to STI using a modification of the procedure of Kishida, Olsen, Berg & Prockop (1975). Glutaraldehyde (2 ml) was added to 0·1 M sodium phosphate buffer, pH 7·4 (2 ml) and the pH readjusted to 7·4 with 0·1 N NaOH. Ferritin (100 mg) was added to the glutaraldehyde solution which was then stood for 0·5 h before the glutaraldehyde was removed by gel filtration on Sephadex G-50 (fine) (2·5 cm × 90 cm). The ferritin fractions were amalgamated but left unconcentrated; they were mixed with STI (20 mg) dissolved in a small volume of 0·1 M sodium phosphate buffer, pH 7·4 to which a trace of ^{125}I-STI had been added. This solution was stood at room temperature for 24 h and then dialysed against 0·1 M Tris buffer, pH 7·4, concentrated to about 4 ml, and fractionated on Sepharose CL-6B (2 × 1 cm × 95 cm). Fractions containing monomeric ferritin were amalgamated and dialysed against calcium medium. The conjugate was used immediately after preparation at a ferritin concentration of 10 μM. Each mol. of ferritin had about 1·4 mol. of STI attached on average, estimated from incorporation of ^{125}I-STI.

STI was labelled with ^{125}I according to the method of Helmkamp, Contreras & Bale (1967). STI (20 μg, 8 nmol) in 20 μl 0·4 M NH_4Cl, pH 8·0, was added to 2 mCi of carrier-free Na[^{125}I] (1·27 nmol) and to this was added 20 μl of 500 μM stock ICl solution. After 1 min, 10 μl of 0·1 M sodium thiosulphate solution (made up in slightly alkaline water to avoid precipitation of sulphur) and 10 μl of 0·1 M sodium iodide solution were added. The reaction mixture was purified on a trypsin-agarose column (1 ml) which had been equilibrated with 0·1 M phosphate buffer, pH 7·4. The column was washed with 40 ml of the same buffer. ^{125}I-STI was eluted almost quantitatively with 0·1 N HCl. The eluted material was neutralized by addition of an equal volume of 0·25 M sodium phosphate buffer, pH 7·4, followed by titration with 0·1 N NaOH; the solution was immediately adjusted with albumin to a concentration of 0·1 % (w/v). The specific activity was about 100 Ci/mmol when STI was estimated by its inhibition of trypsin.

The acrosome reaction was induced by the divalent cation ionophore A23187. 10 μl of a stock solution of A23187 in dimethyl sulphoxide (2 mg/ml) were pipetted into 1 ml of sperm suspension in ferritin conjugate. Sperm were then allowed to stand for 30 min at 37 °C. At

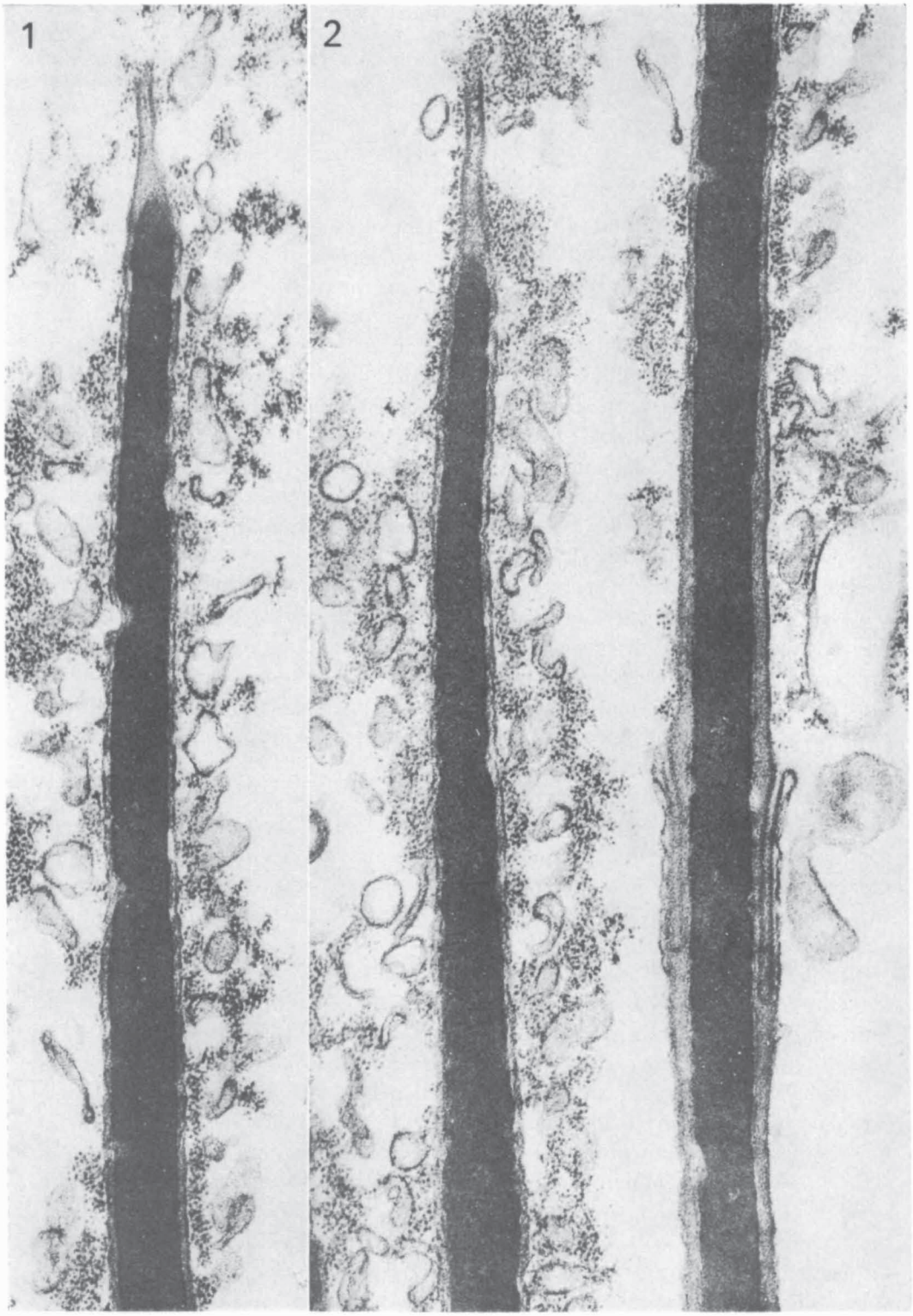
the end of the incubation, the medium was diluted to 10 ml with magnesium medium at room temperature and the sperm centrifuged at 1500 g_{av} for 5 min. The pellet was resuspended in 1 ml magnesium medium and fixed by addition of a further 1 ml of 0·15 M sodium cacodylate buffer, pH 7·2 containing 2 % glutaraldehyde. It was then prepared for electron microscopy as before (Green, 1978*a*).

RESULTS AND DISCUSSION

Soybean trypsin inhibitor (STI) is a protein of molecular weight about 22 500 Daltons which forms 1 : 1 inhibitory complexes with serine proteases. The dissociation constants of these complexes are often small and the half-life for dissociation correspondingly long (for example, K_D for the STI-trypsin complex is about 10^{-10} mol^{-1} (Green, 1953; Lebowitz & Laskowski, 1962)). It is known empirically that acrosin is inhibited by STI but although the dissociation constant and rate of dissociation are both small, they have never been accurately determined (Polakoski & McRorie, 1973). Fluorescein-labelled STI has been used to localize acrosin by fluorescence microscopy in air-dried mounts of whole sperm in which an acrosome reaction had not taken place (Stambaugh & Buckley, 1972), but this was criticized as a method (Morton, 1975) on the ground that STI is a non-specific inhibitor. It is true that STI will not readily distinguish between serine proteases, but acrosin is the only one known to be present in sperm. If there were other serine proteases present it is not clear that the alternative method of localizing them, that of anti-acrosin antibodies, would distinguish between them. It is of course, always possible that STI binds non-specifically to sperm; *a priori* there is no way of knowing this and a similar objection can be raised against antibody labelling. It will be assumed throughout this discussion that STI binds specifically to acrosin and that its properties in this respect are not altered by formation of the conjugate.

Ferritin has a diameter of 12·0 nm but several molecules of STI attached round one ferritin molecule would increase this to 18·0 nm. If acrosin-binding sites on sperm were closer than 18·0 nm they could prove mutually exclusive and this would certainly be true if they were closer than 12·0 nm. It remains a possibility, therefore, that the potential density of binding sites is much greater than that shown by the ferritin labelling.

The distribution of STI-ferritin complex on the surface of guinea-pig sperm after an acrosome reaction is shown in Figs. 1–3. The right-hand sperm in Fig. 2 is the lower part of that shown in Fig. 1. The principle conclusions to be drawn from Figs. 1–3 are as follows. (1) Acrosin is attached to the outer surface of the inner acrosomal membrane after an acrosome reaction but not to any membrane which was part of the plasma membrane of the cell prior to the acrosome reaction (in other words it does not bind to the equatorial segment). (2) The distribution of acrosin immediately round the sperm head is extremely uneven (Figs. 1, 2): in some places it exists as amorphous arrays and in others it is completely absent from the membrane. No two sperm have the same distribution. (3) Acrosin is present throughout what remains of the acrosomal material (Fig. 3). And (4) a web of adhesive material lies between the vesicles of acrosomal and plasma membrane formed during



Figs. 1, 2. Cross-section through 2 guinea-pig sperm after an A23187-induced acrosome reaction and incubation with Kunitz soybean trypsin inhibitor-ferritin conjugate. The sperm in Fig. 1 is the anterior part of the right-hand sperm in Fig. 2. Ferritin is distributed unevenly in blebs over the inner acrosomal membrane but there is no evidence of any binding to the equatorial segment, the area of original plasma membrane immediately posterior to the acrosome. ×40000.

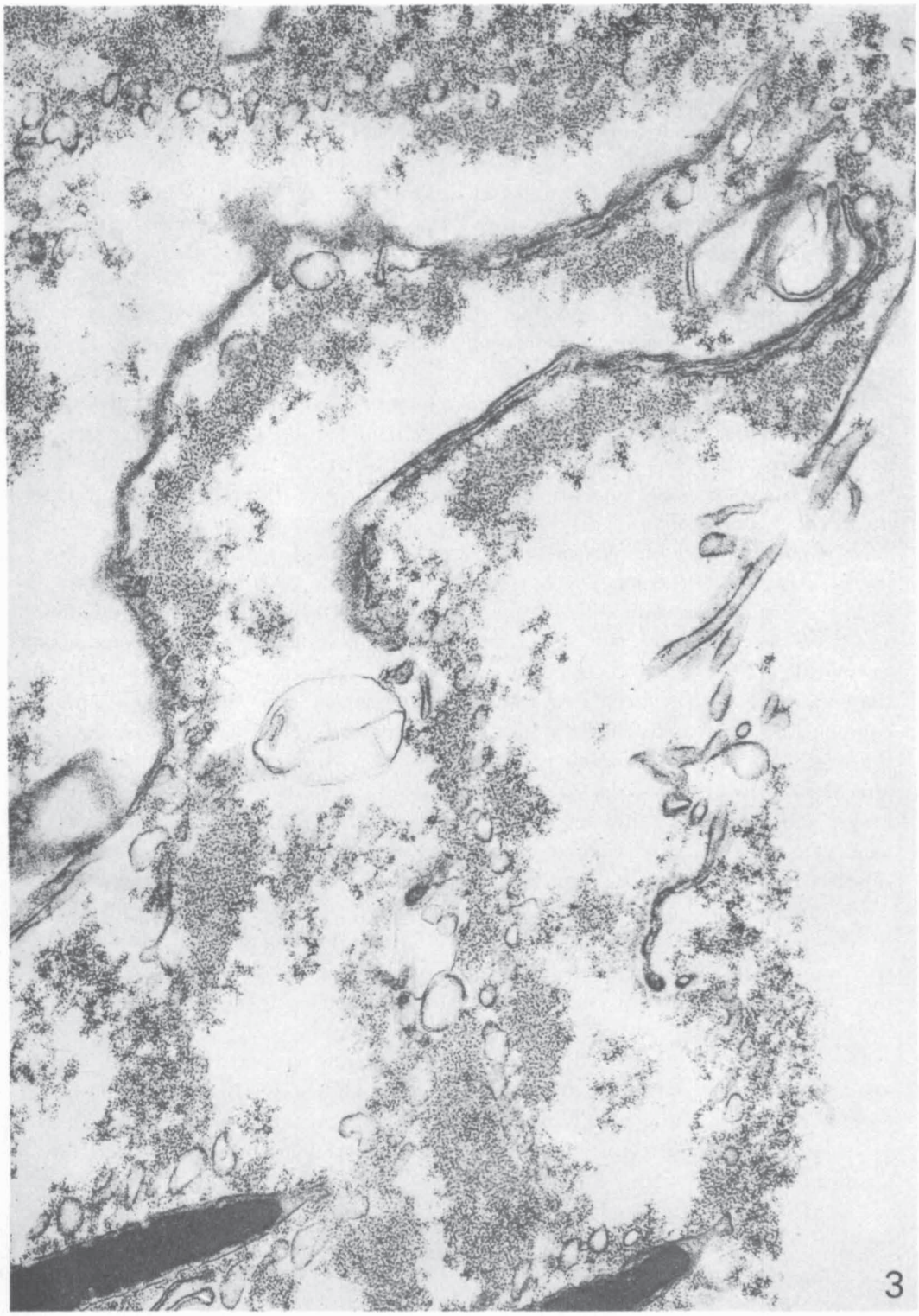


Fig. 3. The remains of the acrosome anterior to the nucleus after an A23187-induced acrosome reaction and incubation with Kunitz soybean trypsin inhibitor-ferritin conjugate. The remains of the acrosome are considerably broken down. It is nevertheless possible to see the vestigial remains of the acrosomal matrix and to see that ferritin is distributed evenly throughout it. × 50 100.

the acrosome reaction, not just round the sperm head but throughout the more distant parts of what was the acrosome.

These results established that acrosin remains attached to the sperm surface after an acrosome reaction and they confirm that the acrosomal contents consist, in part, of a matrix of material which disperses only slowly after the acrosome reaction. In a previous paper (Green, 1978*b*) it was shown that dispersal of the acrosomal material was dependent on acrosin activity and that 40 μM STI failed to inhibit the dispersal. Fig. 3 shows the remnants of 2 acrosomes in sperm which have undergone an acrosome reaction at an actual STI concentration of about 14 μM (but whose effective concentration may be much lower than 14 μM because of the masking of the active site of STI, which may have taken place as a result of conjugation). It is therefore not surprising that suppression of the loss of the acrosomal contents by the conjugate is not wholly effective.

It is essential, if acrosin is to be considered as the agent responsible for digesting the penetration slit, that it can be shown to be bound to the sperm surface after an acrosome reaction. The reason for this is simply that the penetration slit has the width of the sperm head and it is formed as sperm move through the zona: this is inconsistent with release of a soluble digesting enzyme at the zona surface as the cause of the slit (it does not eliminate a role for soluble acrosomal enzymes in weakening the structure of the zona).

There is one important difference in the history of sperm in these experiments and those that have undergone an acrosome reaction and then penetrated the zona during the course of normal fertilization. This is that sperm which have penetrated the zona have been subjected to shearing forces which may remove their surface components, particularly those at the leading edge (since this establishes most of the width of the penetration slit). It is therefore one thing to show that acrosin is attached to the sperm surface after an acrosome reaction in the absence of these forces, and another to show that acrosin is still attached to the sperm surface in the zona. The random attachment of the acrosin with its total absence in some areas suggests that it may not be strongly attached to the sperm head. The question of whether acrosin remains attached to the sperm head in the zona could, in principle, be resolved by the technically more difficult experiment of localizing acrosin on sperm in the act of penetration. But even if acrosin were shown to be absent under these circumstances, this would not eliminate the possible existence of a zona lysin; it would merely eliminate acrosin as a possible candidate.

In this paper, the ultrastructural localization of acrosin in sperm after an acrosome reaction has been established for the first time. It shows clearly why, if acrosin is an important part of the zona lysin, inhibiting it after an acrosome reaction is likely to be an extremely effective way of preventing zona penetration and, therefore, fertilization.

REFERENCES

BEDFORD, J. M. (1970). Sperm capacitation and fertilisation in mammals. *Biol. Reprod.*, Suppl. 2, 128–158.

CUATRECASAS, P. (1970). Protein purification by affinity chromatography. *J. biol. Chem.* **245**, 3059–3065.

GARNER, D. L. & EASTON, M. P. (1977). Immunofluorescent localisation 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 localisation of bovine acrosin. *J. exp. Zool.* **191**, 127–131.

GAUR, R. D., GOSWAMI, A. K. & TALWAR, G. P. (1972). Studies on sperm-ovum interaction. I. Effect of an inhibitor of proteolytic enzyme on fertilisation. *Indian J. med. Res.* **60**, 932–937.

GREEN, D. P. L. (1976). Induction of the acrosome reaction in guinea pig spermatozoa *in vitro* by the Ca ionophore A23187. *J. Physiol., Lond.* **260**, 18P–19P.

GREEN, D. P. L. (1978*a*). The induction of the acrosome reaction in guinea-pig sperm by the divalent metal cation ionophore A23187. *J. Cell Sci.* **32**, 137–151.

GREEN, D. P. L. (1978*b*). The activation of proteolysis in the acrosome reaction of guinea-pig sperm. *J. Cell Sci.* **32**, 153–164.

GREEN, N. M. (1953). Competition among trypsin inhibitors. *J. biol. Chem.* **205**, 535–551.

HELMKAMP, R. W., CONTRERAS, M. A. & BALE, W. F. (1967). ^{131}I-labelling of proteins by the iodine monochloride method. *Int. J. appl. Radiat. Isotopes* **18**, 737–746.

KISHIDA, Y., OLSEN, B. R., BERG, R. A. & PROCKOP, D. J. (1975). Two improved methods for preparing ferritin-protein conjugates for electron microscopy. *J. Cell Biol.* **64**, 331–339.

LEBOWITZ, J. & LASKOWSKI, M. JR. (1962). Potentiometric measurement of protein-protein association constants. Soybean trypsin inhibitor-trypsin association. *Biochemistry, N.Y.* **1**, 1044–1055.

MEIZEL, S. (1972). Biochemical detection and activation of an inactive form of a trypsin-like enzyme in rabbit testes. *J. Reprod. Fert.* **31**, 459–462.

MEIZEL, S. & MUKERJI, S. K. (1976). Biochemical studies of proacrosin and acrosin from hamster cauda epididymal spermatozoa. *Biol. Reprod.* **14**, 444–450.

MIYAMOTO, H. & CHANG, M. C. (1973). Effects of protease inhibitors on the fertilising capacity of hamster sperm. *Biol. Reprod.* **9**, 533–537.

MORTON, D. B. (1975). Acrosomal enzymes: immunochemical localisation of acrosin and hyaluronidase in ram spermatozoa. *J. Reprod. Fert.* **45**, 375–378.

MUKERJI, S. K. & MEIZEL, S. (1975). The molecular transformation of rabbit testis proacrosin into acrosin. *Archs Biochem. Biophys.* **168**, 720–721.

POLAKOSKI, K. L. & MCRORIE, R. A. (1973). Boar acrosin. II. Classification, inhibition and specificity studies of a proteinase from sperm acrosomes. *J. biol. Chem.* **248**, 8183–8188.

RACKIS, J. J., SASAME, H. A., ANDERSON, R. L. & SMITH, A. K. (1959). Chromatography of soybean proteins. I. Fractionation of whey proteins on diethyl aminoethylcellulose. *J. Am. chem. Soc.* **81**, 6265–6270.

SCHILL, W. B., SCHLEUNING, W.-D., FRITZ, H., WENDT, V. & HEIMBURGER, N. (1975). Immunofluorescent localisation of acrosin in spermatozoa by boar acrosin antibodies. *Naturwissenschaften* **62**, 540–541.

SCHLEUNING, W.-D., HELL, R. & FRITZ, H. (1976). Multiple forms of boar acrosin and their relationship to proenzyme activation. *Hoppe-Seyler's Z. physiol. Chem.* **357**, 207–212.

STAMBAUGH, R., BRACKETT, B. G. & MASTROIANNI, L. (1969). Inhibition of *in vitro* fertilisation of rabbit ova by trypsin inhibitors. *Biol. Reprod.* **1**, 223–227.

STAMBAUGH, R. & BUCKLEY, J. (1972). Histochemical subcellular localisation of the acrosomal proteinase effecting dissolution of the zona pellucida using fluorescein-labelled inhibitors. *Fert. Steril.* **23**, 348–353.

SUMMERS, R. G., TALBOT, P., KEOUGH, E. M., HYLANDER, B. L. & FRANKLIN, L. E. (1976). Ionophore A23187 induces acrosome reactions in sea urchin and guinea pig spermatozoa. *J. exp. Zool.* **196**, 381–386.

TALBOT, P., SUMMERS, R. G., HYLANDER, B. L., KEOUGH, E. M. & FRANKLIN, L. E. (1976). The role of calcium in the acrosome reaction: an analysis using ionophore A23187. *J. exp. Zool.* **198**, 383–392.

YANG, S. L., ZANEFELD, L. J. D. & SCHUMACHER, G. F. B. (1976). Effect of serum proteinase inhibitors on the fertilising capacity of rabbit spermatozoa. *Fert. Steril.* **27**, 557–581.

ZANEFELD, L. J. D., ROBERTSON, R. T., KESSLER, M. & WILLIAMS, W. L. (1971). Inhibition of fertilisation *in vivo* by pancreatic and seminal plasma trypsin inhibitors. *J. Reprod. Fert.* **25**, 387–392.

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