Glycosaminoglycan Stimulation of the *In Vitro* Conversion of Boar Proacrosin into Acrosin

RICHARD F. PARRISH,* THOMAS J. WINCEK,† AND KENNETH L. POLAKOSKI†

*From the Departments of *Urology and †Obstetrics/Gynecology, Washington University School of Medicine, St. Louis, Missouri*

Glycosaminoglycans obtained from shark cartilage, whale cartilage, porcine intestinal mucosa, porcine skin, and human umbilical cord were found to accelerate the *in vitro* conversion of highly purified boar sperm proacrosin into m_α-acrosin. Since none of the glycosaminoglycan preparations demonstrated esterase activity (BzArgOEt), general proteinase activity (Azocoll), or stimulation of acrosin activity, the glycosaminoglycan stimulation of proacrosin conversion into acrosin results from a direct interaction between proacrosin and the glycosaminoglycans. These results demonstrate that the glycosaminoglycan stimulation of proacrosin conversion into acrosin is a general phenomenon that is not species- or organ-specific and indicate that glycosaminoglycans could function in the regulation of the *in vivo* conversion of proacrosin into acrosin.

Key words: acrosomal enzymes, acrosome reaction, proacrosin, acrosin, glycosaminoglycan.

The necessity of acrosin (EC 3.4.21.10) activity in both *in vitro* and *in vivo* fertilization (reviewed by McRorie and Williams, 1974, and Zaneveld et al, 1975) and the fact that enzymatically inactive proacrosin accounted for more than 95% of the potential acrosin activity in ejaculated sperm indicated that the conversion of proacrosin into acrosin could serve an important function in mammalian reproduction (reviewed by Parrish and Polakoski, 1979). In order to understand the possible intracellular regulation of this important enzyme system, it is necessary to investigate the factors that affect the rate of proacrosin conversion into acrosin. Compounds that inhibit the conversion have been investigated in more detail than those that accelerate the conversion because there is an apparent contraceptive potential for such inhibitors and because there was a need for the prevention of premature proacrosin conversion during purification procedures. The synthetic materials benzamidine (Zahler and Polakoski, 1977; Tobias and Schumacher, 1977), *p*-aminobenzamidine (Schleuning et al, 1976), *p*-nitrophenyl-*p'*guanidino benzoate (Borhan et al, 1976), and tosyllysine chloromethyl ketone (Polakoski and Parrish, 1977) blocked conversion of proacrosin into acrosin. The naturally occurring materials calcium (Polakoski and Zaneveld, 1976), spermine (Parrish and Polakoski, 1977), and zinc (Mukerji and Meizel, 1975) retarded the conversion.

It has recently been established that porcine uterine flushings contained a factor that stimulated the *in vitro* conversion of boar proacrosin into acrosin. Since the factor was sensitive to digestion by hyaluronidase and condroitinase ABC, it was identified as a glycosaminoglycan (Wincek et al, 1979). In this article we establish that the glycosaminoglycan stimulation of proacrosin conversion into acrosin is not a unique property of the porcine uterine glycosaminoglycans but a general effect that is independent of the tissue or species from which the glycosaminoglycans were purified.

Materials and Methods

Materials

BzArgOEt (N-α-benzoyl-L-arginine ethyl ester HCl), BzArgNNab (N-α-benzoyl-DL-arginine-β-naphthyl-

Reprint requests: Richard F. Parrish, Ph.D., Department of Urology, Washington University School of Medicine, St. Louis, Missouri 63110.

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amide HCl), chondroitin-4-sulfate (whale cartilage), chondroitin-6-sulfate (shark cartilage), dermatan sulfate (porcine skin), heparin (porcine intestinal mucosa), hyaluronic acid (human umbilical cord), and HEPES (N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) were Sigma products. Black K salt was a product of ICN K & K Laboratories, Inc. Azocoll was purchased from Cal-Biochem. The electrophoretic supplies were purchased from Bio-Rad Laboratories. All other chemicals were reagent grade. Solutions were prepared with deionized, glass-distilled water.

Proacrosin was purified from ejaculated boar spermatozoa as previously described (Polakoski and Parrish, 1977). m_α-acrosin and m_β-acrosin were prepared by selective conversion of highly purified proacrosin (Parrish and Polakoski, 1977; 1978).

Assay of Acrosin and Proacrosin

Acrosin esterolytic activity was measured spectrophotometrically by following the hydrolysis of BzArgOEt (Schwert and Takenaka, 1955). The reaction was monitored at 253 nm at 10-second intervals using a Gilford Model 240 spectrophotometer equipped with a Gilford 410 digital absorbance meter, a Gilford 4009 data lister, and a constant temperature bath set at 30 C ± 0.1. A molar absorbance difference of 1150 $M^{-1}cm^{-1}$ was used to convert the change in absorbance into micromoles of BzArgOEt hydrolyzed (Whitaker and Bender, 1965). The proteolytic activity of acrosin was measured against Azocoll, a general proteinase substrate, as previously described (Parrish and Polakoski, 1977). Proacrosin conversion was monitored by measuring the acrosin activity generated. Detailed experimental procedures are presented in the appropriate figure or table legend. Protein concentration was measured spectrophotometrically at 280 nm (Polakoski et al, 1973).

Electrophoresis

Electrophoresis in the presence of sodium dodecyl sulfate was performed essentially by the method of Laemmli (1970). The following procedure was used for sample preparation prior to electrophoresis: proacrosin and the appropriate glycosaminoglycan, in a total reaction volume of 0.20 ml, were incubated on ice for 30 seconds in a 7.0 pH solution consisting of 0.05 M HEPES, 0.05 M calcium chloride. The reaction was terminated by the addition of 1.0 ml of a 2.8 pH solution consisting of 0.2 M sodium formate, 0.05 M benzamidine. A proacrosin control was prepared by adding proacrosin to 1.0 ml of a 2.8 pH solution consisting of 0.2 M sodium formate, 0.05 M benzamidine, and then adding 0.10 ml of a 7.0 pH solution of 0.1 M HEPES, 0.1 M calcium chloride. The salts were removed by dialysis against 0.001 M HCl, 3.0 pH, and the samples were lyophilized. The lyophilized samples were dissolved in 0.25 ml of 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and then subjected to electrophoresis at a constant current of 4 mA/gel. The gels were stained for protein with Coomassie brilliant blue and destained in 7% acetic acid as previously described (Polakoski and Parrish, 1977). The standard proteins used for molecular weight determinations were bovine serum albumin (68,000); ovalbumin (45,000); trypsin (23,300); and lysozyme (14,300).

Analytical disc gel electrophoresis at 4.3 pH was performed by the method of Brewer and Ashworth (1969). Proacrosin was incubated on ice with the appropriate glycosaminoglycan in 0.4 ml of a 7.0 pH solution consisting of 0.05 M HEPES, 0.05 M calcium chloride. After a 30-second incubation period, 0.10 ml was removed and the reaction was terminated by the addition of 0.2 ml of a solution consisting of 0.01 M benzamidine, 0.025 M HCl (final pH was 3.2). After electrophoresis, BzArgNNab hydrolyzing activity was measured essentially by the method of Garner (1975) with the previously noted modifications (Polakoski and Parrish, 1977).

Results

The Effect of Glycosaminoglycans on the Conversion of Proacrosin into Acrosin

Initial experiments with heparin (Fig. 1) indicated that the conversion of proacrosin into acrosin was complete within 30 seconds. In order to obtain a more accurate half-time for the proacrosin conversion, a series of short-time incubations was used and, as shown in Fig. 1B, 70% of the conversion occurred after only 5 seconds. The half-time for proacrosin conversion in the absence of heparin (Fig. 1A) was 60 minutes. A series of glycosaminoglycans was then examined in order to determine whether there was any organ or tissue specificity (Table 1). Although some slight concentration dependence was observed, all of the glycosaminoglycans dramatically stimulated the appearance of enzymatic activity from the enzymatically inactive zymogen.

Since several different molecular weight species of acrosin can be obtained from proacrosin (Polakoski and Parrish, 1977), it was necessary to determine which form(s) of acrosin resulted from the incubations in the presence of the glycosaminoglycans. Figure 2 shows the results of the sodium dodecyl sulfate disc gel electrophoresis of the products resulting from 30-second incubations of proacrosin (gel A) or proacrosin plus heparin (gel B). A comparison to standard reference proteins established the molecular weights of the two bands in gel A to be 55,000 and 53,000, which corresponded to the two proacrosin bands seen in the highly purified extracts (Polakoski and Parrish, 1977). An examination of gel B shows that in the presence of heparin these two proacrosin bands disappeared and a new band of protein with a molecular weight of 49,000 appeared. This is the molecular weight previously established for

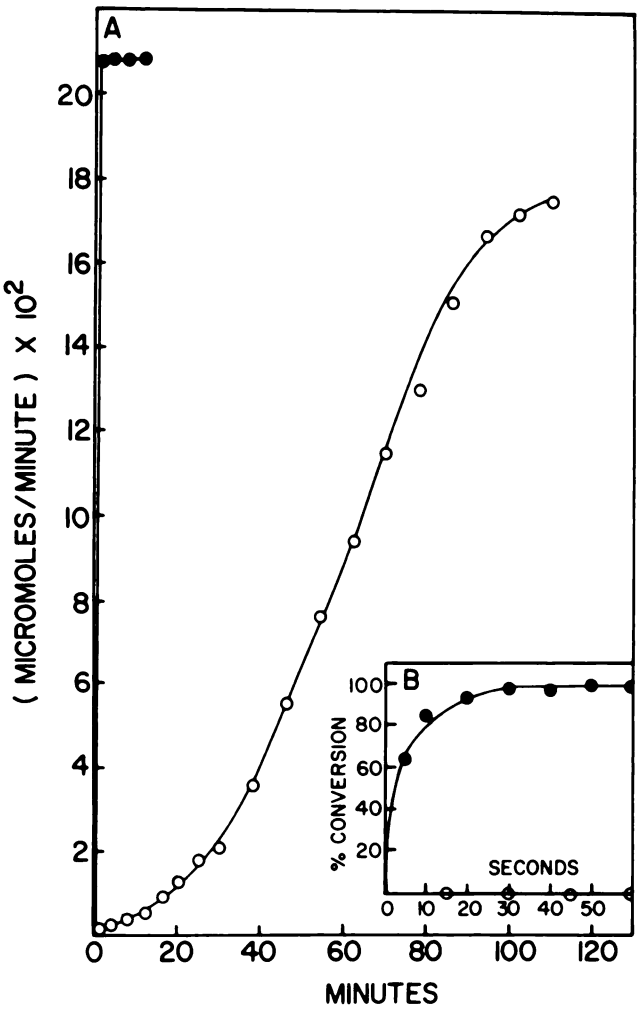

Fig. 1.A. The effect of heparin on the time course for the appearance of BzArgOEt hydrolyzing activity from proacrosin. The reaction solution on ice at 7.0 pH in a total volume of 0.20 ml consisted of 0.05 M HEPES, 0.05 M calcium chloride, proacrosin (26 μg), and 0.8 μg heparin (●) or no heparin (○). At the indicated times, 0.01 ml was removed and added to 3.0 ml of a 7.0 pH solution maintained at 30 C that consisted of 0.05 M HEPES, 0.05 M calcium chloride, 0.5 mM BzArgOEt, in order to measure the acrosin activity. **B.** (*inset*) The reaction solution on ice in a total volume of 0.2 ml at 7.0 pH consisted of 0.05 M HEPES, 0.05 M calcium chloride, proacrosin (26 μg), and 0.8 μg heparin (●) or no heparin (○). After the indicated incubation periods, 0.01 ml was removed and assayed for acrosin activity as in A.

TABLE 1. The Effect of Glycosaminoglycans on the Conversion of Proacrosin into Acrosin*

Glycosaminoglycan	Constituents	Concentration	Activity†
None	—	—	0
Heparin	Glucosamine-6-sulfate, glucuronic acid-2-sulfate, iduronic acid	8 μg/ml	21.1 ± 0.8‡
Chondroitin-6-sulfate	N-acetyl-D-galactosamine-6-sulfate, glucuronic acid	8 μg/ml	21.1 ± 1.5
Chondroitin-4-sulfate	N-acetyl-D-galactosamine-4-sulfate, glucuronic acid	8 μg/ml	18.3 ± 0.8
Dermatan sulfate	N-acetyl-D-galactosamine-4-sulfate, iduronic acid	8 μg/ml	0
Dermatan sulfate	—	40 μg/ml	21.5 ± 1.1
Hyaluronic acid	N-acetyl-D-glucosamine, glucuronic acid	8 μg/ml	5.7

* The reaction mixtures on ice at 7.0 pH in a 0.05-ml reaction volume consisted of 0.05 M HEPES, 0.05 M calcium chloride, proacrosin (6.4 μg), and the glycosaminoglycans at the indicated concentrations. The reactions were initiated by the addition of proacrosin, and after a 30-second incubation period, 0.01 ml was removed and assayed for acrosin activity. The results are expressed as the mean ± mean deviation for duplicate determinations.

† (μmol BzArgOEt hydrolyzed/min) × 10^2.

‡ Full activity.

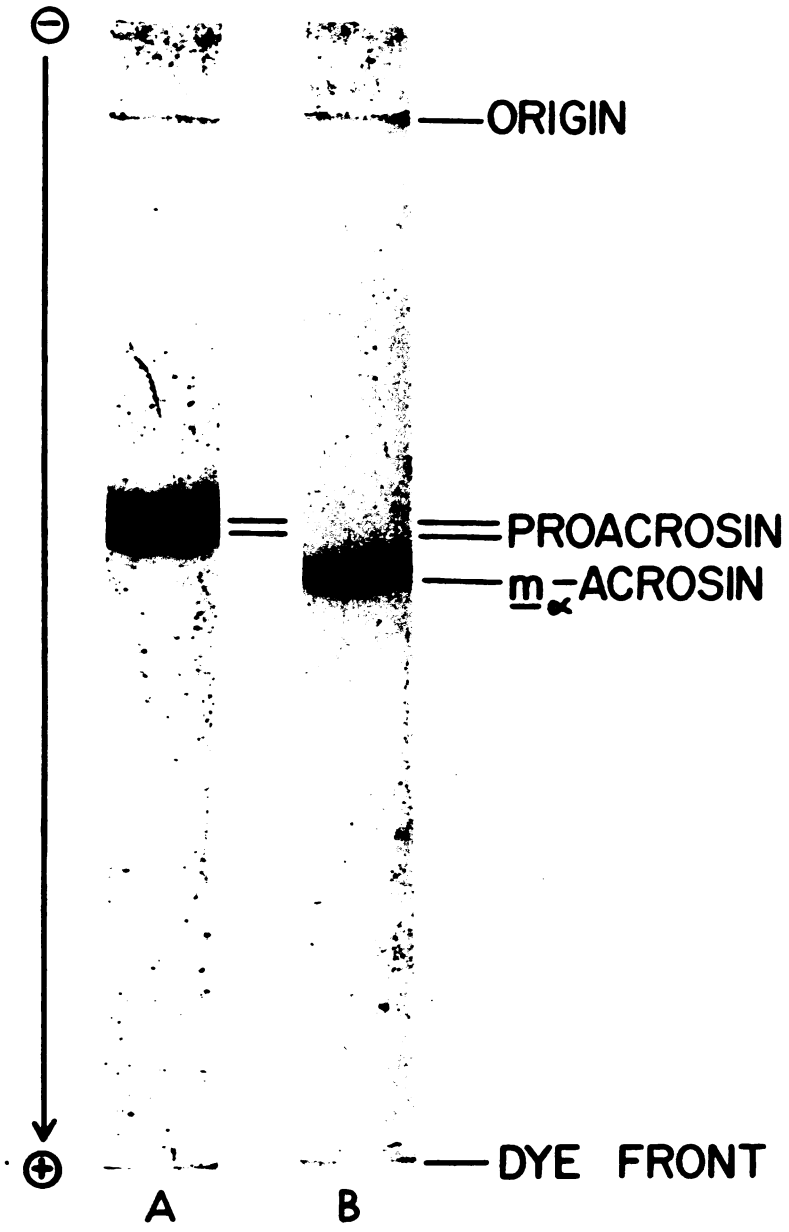

Fig. 2. Electrophoretic characterization of the product resulting from the incubation of proacrosin with heparin. Gels A and B show the protein-staining bands resulting from sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the products of 25-μg proacrosin not treated with heparin (gel A) or incubated with 0.8 μg heparin (gel B) as described in Materials and Methods.

m_α-acrosin (Polakoski and Parrish, 1977; Parrish and Polakoski, 1978). These results also establish that the observed enzymatic activity was not the result of a possible latent activity of the zymogen itself. Electrophoresis of the samples at 4.3 pH and staining for BzArgNNab hydrolyzing activity (results not shown) demonstrated that the product of the incubations containing proacrosin and heparin possessed a relative migration (r = 0.39) identical to that of homogeneous m_α-acrosin. m_α-acrosin was also the product resulting from the 30-second incubations of proacrosin and the other glycosaminoglycans listed in Table 1. If the incubations were extended to 30 or 60 minutes, m_β-

acrosin (molecular weight = 34,000, determined by sodium dodecyl sulfate disc gel electrophoresis) was produced. These results demonstrate that proacrosin is rapidly converted into acrosin in the presence of a wide range of structurally different glycosaminoglycans. The sequence of conversion is similar to that previously established for the *in vivo* (Polakoski et al, 1979) and the *in vitro* (Polakoski and Parrish, 1977) conversion of proacrosin into acrosin.

The Effect of Glycosaminoglycans on the Enzymatic Activity of Acrosin

The sigmoidal appearance of esterase activity from the zymogen proacrosin, in the absence of the glycosaminoglycans, indicates a reaction sequence in which the product of the reaction (acrosin) generates more product from the substrate (proacrosin) (Kassell and Kay, 1973). This has been substantiated by the observation that added acrosin shortens the time required for full conversion of proacrosin into acrosin (Mukerji and Meizel, 1975; Polakoski and Parrish, 1977). Therefore, the acceleration of the conversion of proacrosin into acrosin by the glycosaminoglycans could have resulted from stimulation of the enzymatic activity of acrosin generated from proacrosin during the reaction. To test this hypothesis, the effect of glycosaminoglycans on the esterolytic (Table 2) and the proteolytic (Table 3) activity of acrosin was measured. The data in Table 2 show that the glycosaminoglycans did not stimulate the esterolytic activity of acrosin. Table 3 shows that there was no meaningful effect of glycosaminoglycans on the proteolytic activity of acrosin. These results indicate that the stimulatory effect of glycosaminoglycans on the conversion of proacrosin into acrosin did not result from a glycosaminoglycan stimulation of the enzymatic activity of acrosin.

TABLE 2. The Effect of Glycosaminoglycans on the Esterolytic Activity of Acrosin*

Glycosaminoglycan Added	Enzymatic Activity†	
	m_α-acrosin	m_β-acrosin
None	25.7 ± 0.7	24.1 ± 0.1
Heparin	23.1 ± 0.4	21.5 ± 0.1
Chondroitin-6-sulfate	25.2 ± 0.7	24.0
Chondroitin-4-sulfate	25.1	24.4 ± 0.2
Dermatan sulfate	25.0 ± 0.5	24.1 ± 0.1
Hyaluronic acid	25.3 ± 0.3	24.5

* The 3.0-ml reaction mixture at 30 C and 7.0 pH consisted of 0.05 M HEPES, 0.05 M calcium chloride, 0.5 mM BzArgOEt, and either m_α-acrosin (1.6 μg) or m_β-acrosin (1.3 μg) and the indicated glycosaminoglycans (24 μg). Values are expressed as mean ± mean deviations for duplicate measurements.

† (μmol BzArgOEt hydrolyzed/min) × 10^2.

Discussion

The results obtained during this investigation demonstrate that the conversion of boar proacrosin into acrosin was stimulated by a number of structurally different glycosaminoglycans that were not derived from the porcine female reproductive tract. Indeed, as measured under the conditions of these experiments, neither the presence or position of the sulfate nor the configuration of the C-6 carboxyl group of the uronic acid moiety demonstrated any significant effect on the ability to stimulate proacrosin conversion. However, the dramatic glycosaminoglycan acceleration of the conversion of proacrosin into acrosin appears to be a unique property of proacrosin, relative to the known effects of glycosaminoglycans on other proteinase zymogens. Heparin has been reported to inhibit the enterokinase catalyzed conversion of trypsinogen into trypsin (Wolosowicz et al, 1977), while heparin and condroitin-4-sulfate accelerated the pepsinogen conversion into pepsin. This latter stimulation has been postulated to result from the binding of the glycosaminoglycans and the cationic inhibitor peptide released during pepsinogen conversion (Anderson, 1969). It seems unlikely that this is the mechanism for the stimu-

TABLE 3. The Effect of Glycosaminoglycans on the Proteolytic Activity of Acrosin*

Glycosaminoglycan Added	OD_{520}	
	+Acrosin	−Acrosin
None	0.487 ± 0.007	0.024 ± 0.002
Heparin	0.527 ± 0.025	0.020 ± 0.001
Chondroitin-6-sulfate	0.521 ± 0.012	0.021 ± 0.003
Chondroitin-4-sulfate	0.526 ± 0.029	0.022 ± 0.001
Dermatan sulfate	0.492 ± 0.024	0.023 ± 0.003
Hyaluronic acid	0.560 ± 0.007	0.021 ± 0.001

* The 1.0-ml reaction mixtures at 30 C and 7.0 pH consisted of 0.05 M HEPES, 0.05 M calcium chloride, Azocoll (10 mg), and the indicated glycosaminoglycans (8 μg). The reactions were initiated by the addition of m_β-acrosin (3.5 μg) or an equivalent amount of 0.001 M HCl. After a 40-minute incubation, the reactions were terminated by the addition of 0.2 ml of 0.3 M benzamidine. The mixtures were immediately filtered through glass wool-plugged Pasteur pipets and the absorbance at 520 nm was measured. Values are expressed as mean ± mean deviation for triplicate measurements.

lation of proacrosin conversion, for in our purified preparations we have been unable to detect acrosin inhibitors in the conversion incubations. The stimulation of proacrosin conversion was not the result of contaminating proteinases present in the glycosaminoglycan preparations, since none of the glycosaminoglycan preparations demonstrated: 1) protein-staining bands following sodium dodecyl sulfate disc gel electrophoresis; 2) BzArgOEt hydrolyzing ability; 3) general proteinase activity against Azocoll; 4) loss of stimulatory effect following boiling or pronase treatment. Finally, the proacrosin preparation was highly purified and there was no apparent effect of the glycosaminoglycans on acrosin esterase (Table 2) or proteinase (Table 3) activity. Therefore, the observed stimulation of proacrosin conversion (Fig. 1 and Table 1) was probably the result of a direct interaction between proacrosin and the glycosaminoglycans.

A working hypothesis that explains the glycosaminoglycan stimulation of proacrosin conversion into acrosin is shown in Fig. 3. In this schematic representation, proacrosin and the glycosaminoglycans bind in a complex resulting in the labilization of the peptide bond(s) that is(are) cleaved during proacrosin conversion into acrosin. This labilization results in an increase in the rate of conversion of proacrosin into m_α-acrosin relative to the rate of conversion in the absence of glycosaminoglycans. The cleavage site is represented as the small outward point in the schematic representation of proacrosin in the complex and as the small inward point in the free proacrosin. Examination of the approximate molar ratios of even the smallest glycosaminoglycan tested (1:7.5 heparin : proacrosin, based on an average molecular weight of 11,000 for heparin (Li et al, 1974), indicates that a catalytic amount of glycosaminoglycan is all that is required for complete proacrosin conversion into m_α-acrosin. This complete and rapid conversion could be used in the preparation and purification of m_α-acrosin, a form of acrosin which recent evidence suggests is physiologically significant (Polakoski et al, 1979). Purification of m_α-acrosin has previously been plagued by the necessity of long conversion incubations to ensure complete conversion, but this has resulted in the presence of significant amounts of m_β-acrosin (Parrish and Polakoski, 1978).

The exact physiologic significance of glycosaminoglycan stimulation of proacrosin conversion into acrosin is presently difficult to evaluate. It has been suggested that the interaction between proacrosin and the uterine fluid glycosaminoglycans may be one reason why sperm must reside in the female reproductive tract prior to acquiring fertilization potential (Wincek et al, 1979). Several other interesting corollary facts should be pointed out: 1) the outer investments of the ovum are composed, at least in part, of sulfated polysaccharides (Piko, 1969); 2) the normal sperm acrosome reaction occurs in the presence of the ovum and is essential for fertilization (reviewed by Bedford, 1970); 3) acrosin has been implicated in the acrosome reaction (Lui and Meizel, 1979). From these observations, the following hypothesis can be proposed. The uterine tract fluids or the ovum contain glycosaminoglycans that are

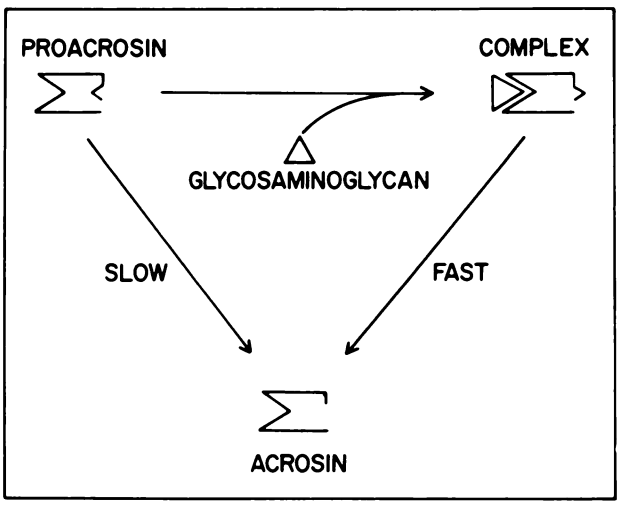

Fig. 3. Schematic representation of the mechanism of glycosaminoglycan stimulation of the conversion of proacrosin into acrosin.

taken up by the sperm and the conversion of proacrosin into acrosin is stimulated. The active acrosin is then used in the acrosome reaction and digests a path for the sperm through the zona pellucida of the ovum. Although this hypothesis remains to be tested, the results presented in this article indicate that glycosaminoglycans have the potential to serve as positive effectors of proacrosin conversion and thus could have a regulatory role in reproduction.

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