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THE ANTICOAGULANT ACTIVITY OF DERMATAN SULPHATES: EVIDENCE AGAINST THE INVOLVEMENT OF ANTITHROMBIN III

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1 Anticoagulant activity of dermatan sulphates is unaffected by antiserum specific for antithrombin III (AT III) unless the glycosaminoglycan preparation contains demonstrable heparin.

2 Only dermatan sulphate preparations of considerable heparin content potentiate AT III inhibition of thrombin, factor X_a and plasmin.

3 These data suggest that dermatan sulphates exert anticoagulant activity which, unlike that of heparin, is largely or totally independent of AT III.

Introduction

Dermatan sulphates are glycosaminoglycans that characteristically contain extensive amounts of repeating disaccharide units consisting of α-L-iduronosyl-(1 → 3)-β-D-*N*-acetylgalactosamine 4-sulphate-(1 → 4) (Lindahl & Höök, 1978). Like other glycosaminoglycans, dermatan sulphates show considerable intra- and inter-molecular structural heterogeneity, the idealised structure frequently being masked by extra sulphations (Suzuki, Suzuki, Nakamura & Koizumi, 1976), and by the occurrence of β-D-glucuronate in place of its C-5 epimer, α-L-iduronate (Malmström, Carlstedt, Aberg & Fransson, 1975). The precise conformations of component residues of the polymer are still under investigation (Gatti, Casu, Torri & Vercellotti, 1979).

Such compounds have been isolated from several tissues, including blood vessel walls (Murata, Nakazawar & Hamai, 1975; Wagner & Salisbury, 1978). Like heparins and heparan sulphates, which are the only other iduronate-containing glycosaminoglycans, dermatan sulphates exhibit anticoagulant activity in conventional clotting assays *in vitro* (Teien, Abildgaard & Höök, 1976; Cofranesco, Radaelli, Pogliani, Amici, Torri & Casu, 1979; Kindness Long & Williamson, 1979a, b, c: Kindness, Williamson & Long, 1980a; Kindness, Long, Williamson, Edward, Winter, & Bennett, 1980b; Kindness, Long & Williamson, 1980c; Long, Williamson, Kindness, Edward & Winter, 1980). Vessel wall proteoglycans containing dermatan sulphates may therefore play a role in haemostasis as immobilised anticoagulants (Teien *et al.*, 1976). Glycosaminoglycans displaced from vessel walls into the circulation may also exert anticoagulant activity following the administration of exogenous glycosaminoglycans *in vivo* (Thomas, Merton, Barrowcliffe, Mulloy & Johnson, 1979; Thomas, Barrowcliffe, Johnson, Stocks, Dawes & Pepper, 1979).

Anticoagulant activity of heparins, the clinically-used sulphated glycosaminoglycans, appears to involve potentiation of the inhibition by antithrombin III (AT III) of thrombin (EC 3.4.21.5), factor X_a (EC 3.4.21.6) and other proteinases which catalyse particular steps of the coagulation casade. *In vitro* clotting assays, and assays involving thrombin- and factor X_a-catalysed cleavage of chromogenic substrates, suggest that AT III may not be involved in the activity of dermatan sulphates (Teien *et al.*, 1976; Kindness *et al.*, 1979a, b, c; 1980a, b, c; Long *et al.*, 1980). In contrast, Hatton, Berry & Regoeczi (1978) recently reported that a preparation of dermatan sulphate potentiated AT III inhibition of thrombin-catalysed hydrolysis of a chromogenic ester, and suggested that dermatan sulphates could play a role, similar to that of heparins, in the inactivation of thrombin by AT III.

In the present paper, the possible role of AT III in the anticoagulant activity of dermatan sulphates was investigated using plasma treated with antiserum specific for AT III, and using assays designed to detect potentiation of the AT III inhibition of proteinases *in vitro*.

Methods

Glycosaminoglycans

Four preparations of dermatan sulphate were used.

Preparation A was isolated from by-products remaining after isolation of heparin from porcine intestinal

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mucosa. Its average molecular weight was 45,000. This material, produced under contract from the U.S.A. National Institute of Health, was a generous gift from Professors M.B. Mathews and J. A. Cifonelli, Dept. of Pediatrics, University of Chicago, Illinois, U.S.A., from whom further analytical information is available in the form of a data sheet. This was the dermatan sulphate used by Hatton *et al.* (1978).

Preparation B, also isolated from porcine intestinal mucosa after heparin preparation, was a generous gift from Dr U. Lindahl, Dept. of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Preparation C, a dermatan sulphate from porcine skin, was supplied by Sigma Chemical Co. Ltd., Poole, Dorset, (Lot. No. 66C-3875), who reported that its molecular weight was 12,000 to 17,000. It was used in previous dermatan sulphate-AT III experiments (Kindness *et al.*, 1979a, b, c; 1980a, b, c; Long *et al.*, 1980).

Preparation D, also isolated from porcine skin, was supplied by Seikagaku Fine Biochemicals, Tokoyo, Japan (Lot. No. S9401).

Heparin, from porcine intestinal mucosa, was supplied by Sigma Chemical Co. Ltd. (Grade 1; Lot No. 46C-0035; activity stated by supplier as 170 U.S.P. units/mg).

Other materials

Bovine topical thrombin (Lot No. Ye 584) was from Parke, Davis & Co., Detroit, Michigan, U.S.A. Chondroitin AC lyase (EC 4.2.2.5), which specifically catalyses the degradation of chondroitin 4- and 6-sulphates; chondroitin ABC lyase (EC 4.2.2.4), which specifically catalyses the degradation of chondroitin 4- and 6-sulphates and dermatan sulphates. Polybrene; and porcine plasmin (EC 3.4.21.7) were from Sigma Chemical Co. Ltd., Poole, Dorset. The chromogenic substrates *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide hydrochloride (S-2160) for thrombin, *N*-benzoyl-L-isoleucyl-L-glutamyl-L-glycyl-L-arginine-*p*-nitroanilide hydrochloride and its methyl ester (S-2222) for factor X_a, and D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251) for plasmin, were from KabiVitrum Ltd., Ealing, U.K. Bovine factor X_a (Batch No. XA14) was from Diagnostic Reagents Ltd., Thame, Oxon. Antiserum specific for human AT III was from Behringwerke AG, Marburg, F.D.R.

Susceptibility of glycosaminoglycans to enzymatic and chemical degradation

Glycosaminoglycan (2 mg) was dissolved in Tris-acetate buffer (200 µl, pH 8.0, 0.05 M) containing NaCl (0.15 M), bovine serum albumin (0.1% w/v) and either chondroitin AC lyase or chondroitin ABC lyase (5 units/ml). Mixtures were then incubated at 37°C for 18 h.

Deamination with HNO_2 was by treatment of 2 mg glycosaminoglycan with a mixture of 200 µl 1.8 M acetic acid and 200 µl 0.24 M sodium nitrate for 9 h at 18°C. Such treatment specifically degrades heparin and heparan sulphate polymers (Lagunoff & Warren, 1962).

After enzymatic or chemical treatment, samples were freeze-dried, and either redissolved in distilled water and examined electrophoretically, or redissolved in 0.9% (w/v) NaCl solution and examined in the clotting or amidolytic assays.

Analyses of glycosaminoglycans

Electrophoresis was carried out using 0.1 M barium acetate (pH 8.0) on cellulose acetate strips (Sepraphore III from Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.) A loading corresponding to 80 to 100 µg glycosaminoglycan originally present before enzymatic or chemical treatment was used per strip. Following migration, glycosaminoglycans were detected by staining with Alcian Blue (Edward, Watson, Williamson & Long, 1979).

All dermatan sulphate preparations migrated as single major bands with identical mobilities. These bands did not appear when samples were incubated with chondroitin ABC lyase before electrophoresis; preincubation with chondroitin AC lyase or with nitrous acid did not lead to the disappearance of the bands. In preparations A and B, a minor band, migrating with a mobility corresponding to that of heparin, was detected. This band did not appear when A and B were subjected to nitrous acid treatment before electrophoresis; preincubation with chondroitin AC lyase or chondroitin ABC lyase did not lead to the disappearance of the band. Following electrophoresis of preparations C and D, a similar band, with the same mobility, and sensitive to nitrous acid but not chondroitin lyase treatments, was also just discernible under these analytical conditions. For preparation C, but for no other dermatan sulphate preparation, a minor band, migrating at a rate corresponding to that of chondroitin sulphates, and sensitive to chondroitin lyase treatments but not to nitrous acid treatment, was detected.

After electrophoresis of the heparin sample the single major band seen also appeared after chondroitin lyase treatments, but not after nitrous acid treatment. An additional minor band, migrating at a rate corresponding to that of dermatan sulphate, was removed from the pattern by chondroitin ABC lyase

pretreatment, but not by pretreatment with chondroitin AC lyase or with nitrous acid.

The extent of contamination of individual glycosaminoglycan preparations was estimated by elution and spectrophotometric determination of the Alcian Blue-glycosaminoglycan complexes (Edward *et al.*, 1979).

Analysis of dermatan sulphate hexosamine content was carried out by incubating 2 mg glycosaminoglycan in HCl (4 M; 1 ml) in a sealed tube under vacuum for 12 h at 100°C; the solution was then dried in a vacuum desiccator containing NaOH before being redissolved in HCl (0.1 M; 1 ml). Glucosamine and galactosamine contents were determined by the method of Spiro (1972) using a Locarte automatic amino acid analyser.

The results of the electrophoretic analyses and hexosamine determinations, and sulphate contents of the dermatan sulphate preparations as determined by the method of Dixon (1968), are summarised in Table 1.

Preparation of plasma and measurement of thrombin times

A pool of normal human citrated plasma was prepared as previously described (Kindness Long & Williamson 1980d). The effects of glycosaminoglycans on thrombin-initiated clotting of plasma were determined as previously described (Kindness *et al.*, 1980c); in some cases, plasma was preincubated with AT III antiserum (30 µl/100 µl plasma) for 2 min at 37°C before addition of glycosaminoglycan. Standard deviations were calculated for all thrombin time values; coefficients of variation were in the range 5.5 to 10.0. Control clotting times (in absence of glycosaminoglycan) were in the range 18 to 22 s. If no clot formed in glycosaminoglycan-containing tubes after 150 s, the clotting ratio was recorded as infinity.

Colorimetric experiments

Details of experiments in which the effects of glycosaminoglycans on inactivation of thrombin, factor X_a and plasmin by AT III were examined are described in the legend to Figure 3.

Optical densities of pNA released in control tubes lacking glycosaminoglycan were in the range 0.35–0.42. Reagents were dissolved in buffer (0.11 M NaCl; 0.05 M Tris, pH 7.7); reagent concentrations quoted are final concentrations in the reaction mix before addition of acid. In the absence of AT III, dermatan sulphates and several other sulphated polysaccharides affect proteinase activity (Kindness *et al.*, unpublished results), and the polycation Polybrene was included to interact electrostatically with the polyanionic polysaccharides and prevent them from directly interacting with the enzyme (Teien *et al.*, 1976). Coefficients of variation were in the range 4.2 to 10.2. pNA released was expressed as a fraction of that released from substrates in the absence of glycosaminoglycans.

Results

Effect of glycosaminoglycans on plasma thrombin times

Figure 1 (open circles) shows the effects of dermatan sulphate preparations A,B,C,D and of heparin on the thrombin time of normal pooled plasma. Preparations A and B were markedly more anticoagulant than preparations C and D.

Pretreatment of heparin with nitrous acid (closed triangles) led to a total loss of anticoagulant activity. The activities of preparations A and B were also greatly reduced following nitrous acid treatment; the anticoagulant activity resistant to nitrous acid treatment was comparable, on a weight basis, to that exhibited by untreated preparations C and D. Pretreatment of preparations C and D with nitrous acid did not detectably change their anticoagulant potency.

The anticoagulant activities of preparations A and B surviving after nitrous acid treatment were abol-

Table 1. Characteristics of glycosaminoglycans

Glycosaminoglycan	*Sulphur**	*Galactosamine**	*Glucosamine**	*Contaminant*†*
A	6.2	21.5	0.38	Heparin (1.8)
B	5.9	20.8	0.84	Heparin (2.9)
C	5.9	23.8	0.05	Chondroiton sulphates (2.6) Heparin (<0.2)
D	5.8	19.8	0.02	Heparin (<0.2)
Heparin	ND	ND	ND	Dermatan sulphate (3.8)

* % by weight; † electrophoretically-detected; ND = not determined.

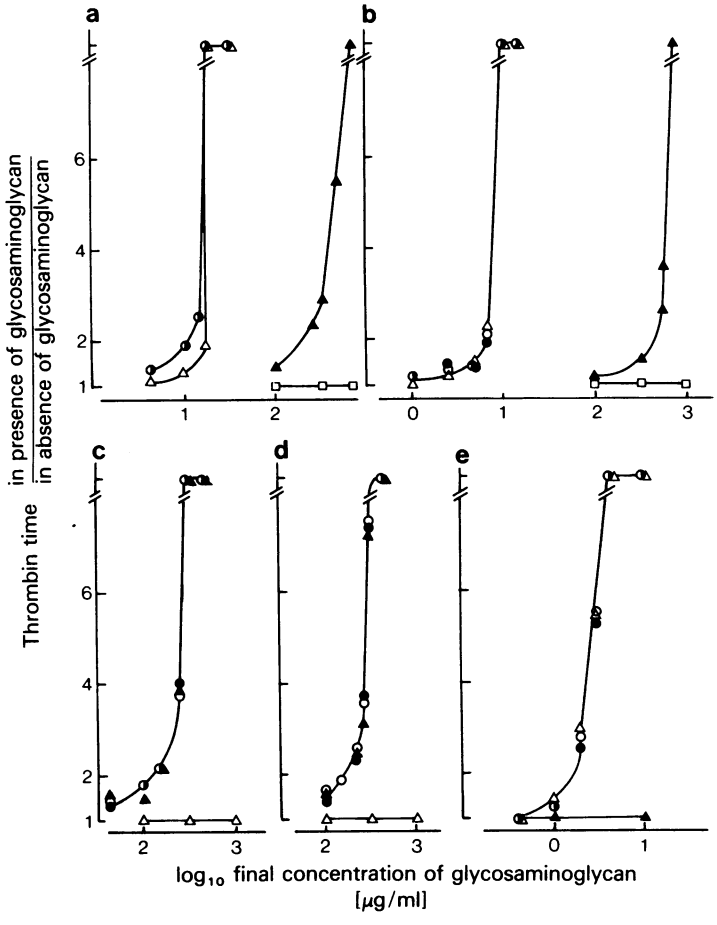

Figure 1. Effect of glycosaminoglycans on thrombin times: (a) dermatan sulphate A; (b) dermatan sulphate B; (c) dermatan sulphate C; (d) dermatan sulphate D; (e) heparin. Symbols: (○) untreated; (▲) nitrous acid treated; (□) nitrous acid treatment followed by chondroitin ABC lyase treatment; (△) chondroitin ABC lyase treated; (●) chondroitin AC lyase treated.

ished by incubation with chondroitin ABC lyase (open squares).

Pretreatment with chondroitin ABC lyase (open triangles) abolished the anticoagulant activity of preparations C and D, slightly diminished that of preparation A, and did not change the activities of heparin or of preparation B.

The anticoagulant activities of heparin and of preparations A,B,C,D were not affected by pretreatment with chondroitin AC lyase (closed circles).

Effect of AT III antiserum on glycosaminoglycan anticoagulant activity

Preincubation of plasma with antiserum specific for human AT III considerably diminished the anticoagulant activity of heparin, and of dermatan sulphate preparations A and B (Figure 2). The activity of preparations C and D was inhibited to a smaller extent.

Preincubation with antiserum also diminished the anticoagulant activity of preparations A and B which had been pretreated with chondroitin ABC lyase but did not alter the anticoagulant activity of any of the dermatan sulphate preparations which had been preincubated with nitrous acid.

The results described in this and the preceding section accord with data obtained from electrophoretic analyses and hexosamine determinations carried out on the dermatan sulphate preparations (Table 1). Preparations A and B were contaminated by

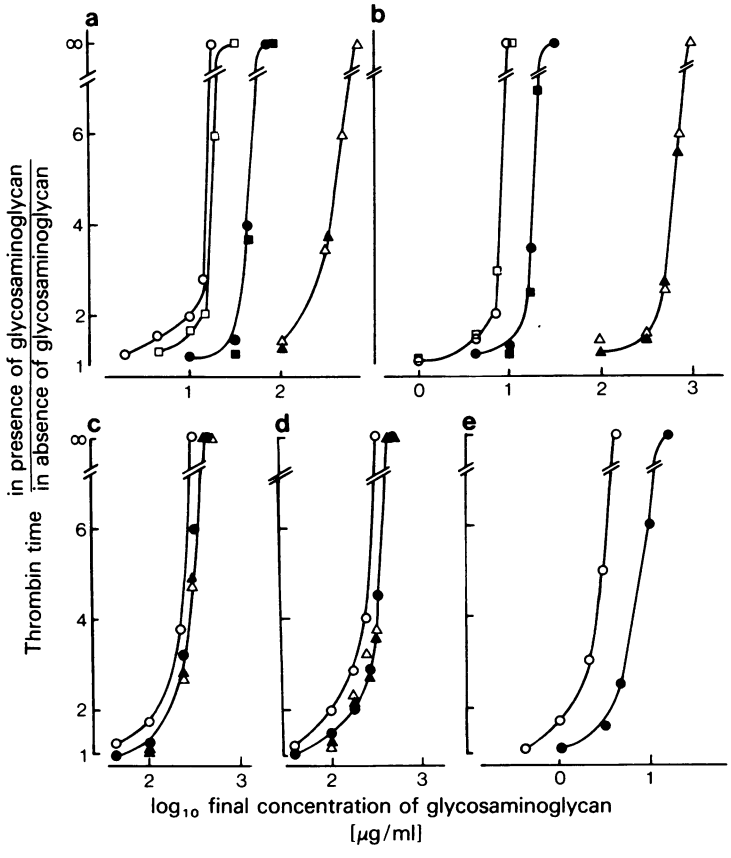

Figure 2 Effect of antiserum against AT III on glycosaminoglycan anticoagulant activity: (a) dermatan sulphate A; (b) dermatan sulphate B; (c) dermatan sulphate C; (d) dermatan sulphate D; (e) heparin. Open symbols: no antiserum; closed symbols: AT III antiserum-treated. Circles: untreated glycosaminoglycans; squares: chondroitin ABC lyase-treated glycosaminoglycans; triangles: nitrous acid-treated glycosaminoglycans.

small quantities of heparin. This was not surprising, as these preparations were made from a tissue source rich in heparin (Nader, Takahashi, Straus & Dietrich, 1980). Dermatan sulphate preparations C and D isolated from a tissue source of little heparin content (Nader *et al.*, 1980), contained less heparin. The small chondroitin sulphate content of preparation C was not important in the context of this study, since glycosaminoglycans not containing iduronate exhibit little or no anticoagulant activity *in vitro* (Teien *et al.*, 1976; Kindness *et al.*, 1980b).

Effect of glycosaminoglycans on AT III-induced inhibition of serine proteinases

The ability of dermatan sulphate preparations to potentiate AT III inhibition of serine proteinases was examined in a system using purified proteinases, AT III and chromogenic substrates of the enzymes. In addition to thrombin and factor X_a, two proteinases of the coagulation cascade inhibited by heparin-modulated AT III (Teien *et al.*, 1976; Kindness *et al.*, 1979a), the fibrinolytic enzyme plasmin was examined, because it is also inhibited by AT III (Highsmith & Rosenberg, 1974; Crawford & Ogston, 1975) and this process is potentiated by heparin (Highsmith & Rosenberg, 1974; Kindness *et al.*, unpublished results).

Figure 3 shows the effects of dermatan sulphate preparations A–D and of heparin on the AT III-induced inhibition of thrombin, factor X_a and plasmin. Preparations A and B, like heparin, poten-

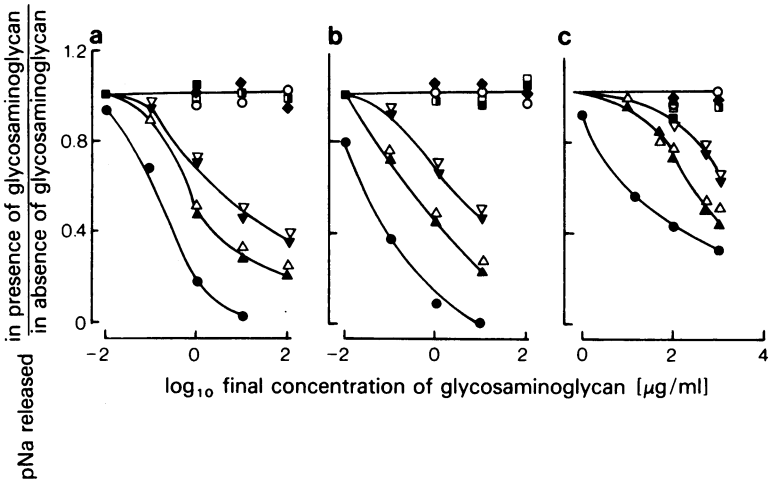

Figure 3 Effect of glycosaminoglycans on AT III-induced inhibition of serine proteinases: (a) thrombin; (b) factor X_a; (c) plasmin. Symbols: (●) heparin; (▼) dermatan sulphate A; (▽) dermatan sulphate A, chondroitin ABC lyase treated; (□) dermatan sulphate A, nitrous acid treated; (▲) dermatan sulphate B; (△) dermatan sulphate B, chondroitin ABC lyase treated; (○) dermatan sulphate B, nitrous acid treated; (■) dermatan sulphate C; (◆) dermatan sulphate D. One hundred μl glycosaminoglycan (0 to 1 mg/ml) was incubated with 100 μl AT III (0.075 units/ml) for 2 min at 37°C; 100 μl of thrombin (2.5 units/ml), factor X_a (0.0032 units/ml) or plasmin (0.01 units/ml) were then added. After a further 60 s, 100 μl of a mixture containing the appropriate substrate at 0.15 mg/ml (see Methods) and Polybrene (0.1 mg/ml) was added. The reaction in thrombin-, factor X_a- and plasmin-containing tubes was stopped after incubation at 37°C for 2, 10 and 5 min respectively, by the addition of 300 μl 50% (v/v) acetic acid. *p*-Nitroaniline (pNA) released from substrates in quadruplicate was estimated spectrophotometrically at 405 nm.

tiated the AT III inhibition of all three proteinases; preparations C and D did not affect the action of AT III.

Pretreatment of preparations A and B with chondroitin ABC lyase did not prevent potentiation of AT III activity by the compounds; pretreated of preparations A and B with nitrous acid led to a total loss of ability to potentiate AT III activity.

Discussion

The results of the clotting assays and the assays using chromogenic substrates demonstrate that the anticoagulant activity of dermatan sulphate preparations is only affected by antiserum to AT III when the preparations contain heparin as a contaminant; similarly, only dermatan sulphate preparations contaminated with considerable quantities of heparin are able to potentiate AT III inhibition of proteinases *in vitro*. The presence of heparin in no way detracts from the use of material such as preparation A as an excellent reference standard in many situations in which the contaminant material is not important, or is undetectable (e.g. Edward *et al.*, 1979; Edward, Long, Watson & Williamson, 1980). These present data accord with the previous observations that the anticoagulant activity of heparin but not of dermatan sulphate, is decreased in plasma taken from a patient with familial AT III deficiency, and that the activity of heparin but not of dermatan sulphate, increased in plasma taken immediately after the intravenous administration of AT III to the patient (Kindness *et al.*, 1980b). Such data, and the results described here support the view that AT III may not be essential for the anticoagulant activity of dermatan sulphates (Teien *et al.*, 1976).

Dermatan sulphate preparations used in previous examinations of possible interactions between this glycosaminoglycan and AT III either contained little detectable heparin (Kindness *et al.*, 1979a, b, c; 1980a, b, c; Long *et al.*, 1980; preparation C in this communication), or were extracted from a heparin-rich tissue source and were then treated with nitrous acid before use (Teien *et al.*, 1976). However, Hatton *et al.* (1978) recently reported that a preparation of dermatan sulphate was capable of potentiating AT III inhibition of thrombin-catalysed hydrolysis of a chromogenic ester, and suggested that the dermatan sul-

phate was similar to that of heparin in the inactivation of thrombin by AT III. Tangen & Bygdeman (1972) emphasized that the clotting activity of thrombin need not parallel its esterolytic activity. The difficulties of comparing measurements of esterolytic and amidolytic activities (which were examined in the present study) are exemplified by the potentiating effects of glycosaminoglycans on the esterolytic activity of thrombin (Hatton *et al.*, 1978) and the inhibitory effects of the same compounds on the amidolytic activity of the proteinase (Teien *et al.*, 1976; Kindness *et al.*, 1979a). In addition, the dermatan sulphate preparation used by Hatton *et al.*, (1978) (preparation A in this paper) is known to contain heparin as a contaminant, and experiments involving it must therefore be assessed with caution. Finally, even this dermatan sulphate preparation, unlike heparin, did not bind to AT III (Hatton *et al.*, 1978).

The molecular mechanisms underlying the anticoagulant activity of dermatan sulphates are not yet known. As previously mentioned (Teien *et al.*, 1976) the low potency of dermatan sulphate (compared to heparin) as an anticoagulant *in vitro* does not necessarily imply that it is of less physiological importance. Plasma concentrations of circulating endogenous glycosaminoglycans have yet to be defined (e.g. Jacobsson & Lindahl, 1979). Moreover, glycosaminoglycans of blood-vessel-wall proteoglycans may exert considerable activity within their local microenvironments. There are also examples of chemically-related compounds which exhibit markedly different anticoagulant effects *in vivo* from those exerted *in vitro* (Thomas, Lane, Michalski, Johnson & Kakkar, 1977).

In summary, these data emphasize the caution required in interpreting results of experiments on the anticoagulant properties of glycosaminoglycan preparations which contain small amounts of potent anticoagulants as contaminants and reassert that, at least in the particular experimental circumstances described here, dermatan sulphates appear capable of exerting anticoagulant activity which differs from that of heparin in being largely or totally independent of AT III.

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