Biochem. J. (1987) 248, 889–896 (Printed in Great Britain)

Increased sulphation improves the anticoagulant activities of heparan sulphate and dermatan sulphate

Frederick A. OFOSU,*†§ Gaman J. MODI,* Morris A. BLAJCHMAN,*† Michael R. BUCHANAN† and Edward A. JOHNSON‡

*The Canadian Red Cross Society Blood Transfusion Service, Hamilton, Ont. L8N 1H8, Canada, †Department of Pathology, McMaster University Medical Centre, Hamilton, Ont. L8N 3Z5, Canada, and ‡National Institute for Biological Standards and Control, London NW3 6RB, U.K.

Heparan sulphate and dermatan sulphate have both antithrombotic and anticoagulant properties. These are, however, significantly weaker than those of a comparable amount of standard pig mucosal heparin. Antithrombotic and anticoagulant effects of glycosaminoglycans depend on their ability to catalyse the inhibition of thrombin and/or to inhibit the activation of prothrombin. Since heparan sulphate and dermatan sulphate are less sulphated than unfractionated heparin, we investigated whether the decreased sulphation contributes to the lower antithrombotic and anticoagulant activities compared with standard heparin. To do this, we compared the anticoagulant activities of heparan sulphate and dermatan sulphate with those of their derivatives resulphated *in vitro*. The ratio of sulphate to carboxylate in these resulphated heparan sulphate and dermatan sulphate derivatives was approximately twice that of the parent compounds and similar to that of standard heparin. Anticoagulant effects were assessed by determining (*a*) the catalytic effects of each glycosaminoglycan on the inhibition of thrombin added to plasma, and (*b*) the ability of each glycosaminoglycan to inhibit the activation of ^{125}I-prothrombin in plasma. The least sulphated glycosaminoglycans were least able to catalyse the inhibition of thrombin added to plasma and to inhibit the activation of prothrombin. Furthermore, increasing the degree of sulphation improved the catalytic effects of glycosaminoglycans on the inhibition of thrombin by heparin cofactor II in plasma. The degree of sulphation therefore appears to be an important functional property that contributes significantly to the anticoagulant effects of the two glycosaminoglycans.

INTRODUCTION

The antithrombotic and anticoagulant activities of dermatan sulphate (DS) and heparan sulphate (HS) have been demonstrated previously (Grossman & Dorfmann, 1957; Kirk, 1959; Morrison *et al.*, 1968; Izuka & Murata, 1972; Murata *et al.*, 1975; Björnsson *et al.*, 1982; Buchanan *et al.*, 1985; Ofosu *et al.*, 1985, 1986). These activities are significantly weaker on a weight basis than those of unfractionated heparin. Anticoagulant and antithrombotic activities of glycosaminoglycans (GAGs) depend on their ability to catalyse thrombin inhibition and/or to inhibit prothrombin activation (Buchanan *et al.*, 1985; Ofosu *et al.*, 1985, 1987). Approximately one-third of heparin molecules have a unique pentasaccharide sequence that results in the binding of these molecules of heparin to antithrombin III with high affinity (Hopwood *et al.*, 1976; Rosenberg *et al.* 1978; Choay *et al.*, 1983). This high-affinity binding, which activates antithrombin III, is primarily responsible for the antithrombotic and anticoagulant effects of heparin. However, the fraction of heparin with low affinity to antithrombin III inhibits the activation of prothrombin in normal human plasma but much less effectively than does unfractionated heparin (Ofosu *et al.*, 1982). The low-affinity fraction also augments the antithrombotic effects of high-affinity oligosaccharides of heparin (Thomas *et al.*, 1982). Although HS possesses this pentasaccharide sequence, the proportion of HS chains with this sequence is significantly lower than that of heparin. DS lacks this pentasaccharide sequence (Lindahl & Höök, 1978; Casu, 1985).

A second major difference between unfractionated heparin, HS and DS is that heparin is more sulphated than the others (Lindahl & Höök, 1978; Casu *et al.*, 1983; Casu, 1985). We therefore investigated whether the lower anticoagulant effects of DS and HS relative to that of pig mucosal heparin could be improved by chemical modification to increase their degree of sulphation.

MATERIALS AND METHODS

Materials

Some properties of the seven GAGs used in the present study are summarized in Table 1. The source and methods of preparation of these materials have been described previously (Johnson, 1982; Casu *et al.*, 1983; Cerskus *et al.*, 1984; Johnson, 1984; Johnson & Paterson, 1986). HS type IIA (Johnson, 1984) and DS (sample 6) were sulphated *in vitro* by the following procedure. Resulphation of HS type IIA *in vitro* yielded HS-S listed in Table 1. The GAG (0.5 g) was freeze-dried, covered with dry pyridine (0.8 ml), and SO_3 solution (1 M in dimethylformamide, 5 ml) was then added. The mixture was heated at 60 °C for 5 h with occasional shaking.

Abbreviations used: GAG, glycosaminoglycan; HS, heparan sulphate; DS, dermatan sulphate; Pip, pipecolic acid; Cbz-, benzyloxycarbonyl-; -NH-Np, *p*-nitroanilide.

§ To whom correspondence should be addressed.

Table 1. Summary of some of the physicochemical properties and U.S.P. specific activity of GAGs

Abbreviations: SH, standard unfractionated heparin from pig mucosa; LAH, heparin with low affinity to antithrombin III; HS, heparan sulphate from pig mucosa; HS-S, heparan sulphate type IIA, mean molecular mass 8000 Da, with a sulphate/carboxylate ratio of 1.6 (Johnson, 1984) resulphated *in vitro*; DS, bovine lung dermatan sulphate; DS-S, dermatan sulphate resulphated *in vitro*. GAG 1, is identical with PM III in the reference Casu *et al.* (1983). GAG 2 was obtained by affinity chromatography of heparin on antithrombin III–Sepharose (Cerskus *et al.*, 1984). The remainder are preparations isolated from heparin by-product materials (Johnson, 1982). HS type I (sample 3) and HS type IIB (sample 5) are identical with HS I (also Hes A) and HS IIB (also Hes G) respectively, as previously described (Casu *et al.*, 1983; Johnson, 1984). In all heparan sulphates, glucosamine accounted for 97% or more of the total hexosamine (Johnson, 1984). DS (sample 6), for which over 99% of the total hexosamine was galactosamine and which was free of other GAGs by cellulose acetate electrophoresis, was isolated by the procedures of Johnson & Paterson (1986). The sulphate/carboxylate ratio refers to the ratio of sulphate to carboxylate per disaccharide unit. This ratio was determined by conductimetric titration (Casu & Gennaro, 1975; Johnson, 1982).

GAG	Mean molecular mass (Da)	Sulphate (mequiv./g)	Sulphate/carboxylate ratio	U.S.P. specific activity (units/mg)
1. SH	12000	3.65	2.2	188
2. LAH	12000	3.40	2.0	35
3. HS type I	20000	1.94	1.0	< 2
4. HS type IIB	9000	3.26	1.9	66
5. HS-S	9000	3.34	2.2	< 2
6. DS	25000	1.90	1.0	< 2
7. DS-S	25000	4.20	2.4	16

After decantation, the solid product was washed with dimethylformamide (2×) and propan-2-ol (2×), then dissolved in water, neutralized with NaOH and dialysed exhaustively against water. The molecular mass [by gel-permeation chromatography, by using the procedure of Johnson (1982)] appeared to be essentially unaltered even though the degree of sulphation was increased substantially. The sulphate/carboxylate ratios were determined by the procedures of Casu & Gennaro (1975) and Johnson (1982). It may be noted in Table 1 that resulphation of HS type IIA (Hes E; Johnson, 1984) to yield HS-S, or resulphation of DS to yield DS-S, did not increase their U.S.P. specific activities to values comparable with that of heparin even though the sulphate/carboxylate ratio achieved was similar to that of unfractionated heparin.

Factor IX concentrate (human) was obtained from the Canadian Red Cross Society Blood Transfusion Service, Hamilton, Ont., Canada. APTT (activated partial thromboplastin time) reagent was obtained from Organon Teknika, Toronto, Ont., Canada. The chromogenic substrates D-Phe-Pip-Arg-NH-Np (S-2238) and Cbz-Ile-Glu-Gly-Arg-NH-Np (S-2222) were obtained from Maynard Scientific, Toronto, Ont., Canada. Fatty acid-free bovine serum albumin, the Factor X activator from Russell's-viper venom and other reagent-grade chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The reagents for polyacrylamide-gel electrophoresis in buffers containing SDS and the Protean II slab-gel electrophoresis system were obtained from Bio-Rad Laboratories, Mississauga, Ont., Canada. Human α-thrombin was generously provided by Dr. John Fenton, II, New York Department of Health, Albany, NY, U.S.A. The chloromethyl ketone inhibitors of thrombin (Phe-Pro-Arg-CH_2Cl) and of Factor Xa (dansyl-Glu-Gly-Arg-CH_2Cl) were obtained from Behring–Calbiochem, San Diego, CA, U.S.A.

Purification of proteins

Human prothrombin and Factor X were isolated from Factor IX concentrate (human) by using previously described procedures (Modi *et al.*, 1984). The prothrombin was labelled with $Na^{125}I$ to an initial specific radioactivity of 5×10^6 c.p.m./μg (Tollefsen & Blank, 1981). Factor X was activated with Factor X activator from Russell's-viper venom and freed of the activator by chromatography on DEAE-Sephadex (Ofosu *et al.*, 1981).

Effect of the GAGs on the inactivation of thrombin or Factor Xa by human plasma

Previously described procedures were used to assess the effect of each of the seven GAGs listed in Table 1 on the inhibition of Factor Xa or thrombin by human plasma (Ofosu *et al.*, 1984*a*,*b*, 1985). Each inhibition reaction was carried out in defibrinated plasma that contained 10 mM-$CaCl_2$ and human brain kephalin (6 μg of organic phospholipid/ml of plasma) (Ofosu *et al.*, 1981, 1984*a*,*b*). These additions facilitated direct comparison of the catalysis of the inhibition of the two proteinases and the inhibition of the generation of their activities under the same experimental conditions. Ca^{2+} and coagulant phospholipids are known to influence the catalytic effect of heparin on the inhibition of Factor Xa and thrombin by antithrombin III (Marciniak, 1973; Walsh *et al.*, 1974; Ofosu *et al.*, 1984*b*). The final dilution of plasma [after the addition of $CaCl_2$, kephalin, Factor Xa (20 nM) or thrombin (100 nM)], with or without an added GAG, was 1 in 2. The final concentration of the added GAG was 0.66, 6.6 or 66 μg/ml of incubation mixture. The defibrinated plasma and the suspension of Factor Xa (or thrombin), $CaCl_2$, kephalin and the GAG under study were prewarmed separately at 37 °C for 3 min before mixing. The inhibition reaction (the second incubation) was performed for 1 min at 37 °C, after which a sample was added to 9 vol. of the chromogenic substrate and the residual enzymic activity measured. Residual Factor Xa and thrombin were then determined (Ofosu *et al.*, 1981, 1984*a*).

Table 2. Effects of GAGs on the inhibition of thrombin or Factor Xa added to pooled normal human plasma

The coagulant proteinase, $CaCl_2$, kephalin and GAG (all in 1 vol. of 30 mM-sodium barbiturate buffer, pH 7.4, containing 0.1 mg of fatty acid-free bovine serum albumin/ml) were added to 1 vol. of defibrinated human plasma. After incubation for 1 min at 37 °C, residual thrombin activity was measured with S-2238, and residual Factor Xa was measured with S-2222. The values reported are the means for six to eight determinations. The standard deviation for each value reported was ±1%. See Table 1 for explanation of the abbreviations used.

		Inactivation of added proteinase (%)					
		Thrombin			Factor Xa		
GAG	Concn. of GAG ...	0.66 μg/ml	6.6 μg/ml	66 μg/ml	0.66 μg/ml	6.6 μg/ml	66 μg/ml
1. SH		91	92	98	52	80	86
2. LAH		45	86	98	8	27	40
3. HS type I		41	85	90	19	80	89
4. HS type IIB		72	91	94	27	90	94
5. HS-S		55	86	95	10	72	70
6. DS		40	60	81	5	8	24
7. DS-S		65	80	83	5	23	55
8. None		35	35	35	5	5	5

Effect of GAGs on the appearance of thrombin and Factor Xa activities in plasma

APTT reagent (1 vol.) was added to the plasma (2 vol.). After an incubation of 3 min at 37 °C, 1 vol. of 40 mM-$CaCl_2$ that had been prewarmed to 37 °C was added. The enzymically active thrombin and Factor Xa generated subsequently were determined quantitatively with S-2238 and S-2222 respectively (Ofosu *et al.*, 1981, 1984*a*). To do this, a sample (1 vol.) was added to 4 vol. of 5 mM-EDTA kept at 4 °C to stop further prothrombin activation. Then 1 vol. of this diluted plasma was added to 39 vol. of 0.25 mM-S-2238 (for thrombin determination) or 9 vol. of 0.4 mM-S-2222 (for Factor Xa determination) prewarmed to 37 °C and the ΔA/min determined. The effects of each of the seven GAGs on the generation of thrombin and Factor Xa activities were also determined. The GAG was added to the contact-activated plasma simultaneously with the $CaCl_2$. Three final concentrations of each GAG were used: 0.66 μg, 6.6 μg or 66 μg of GAG/ml of contact-activated plasma with re-added Ca^{2+}. The appearance of thrombin activity, and its inhibition by each GAG, were determined 45 s, 60 s and 120 s after the re-addition of Ca^{2+}. The appearance of Factor Xa activity, or its inhibition by a GAG, was determined 2 min after the re-addition of Ca^{2+}.

Pathways for the inhibition of the generation of thrombin activity by glycosaminoglycans

In order to determine the pathways by which each GAG inhibits the appearance of thrombin activity in plasma, ^{125}I-labelled human prothrombin was added to the plasma before contact activation and subsequent addition of $CaCl_2$ to initiate the activation of prothrombin (Ofosu *et al.*, 1986, 1987). After the $CaCl_2$ addition, samples of the contact-activated plasma were withdrawn 45 s and 60 s later and added to 4 vol. of 30 mM-sodium barbiturate buffer, pH 7.4, which also contained 5 mM-EDTA, 0.1% bovine serum albumin, 1 μM-Phe-Pro-Arg-CH_2Cl and dansyl-Glu-Gly-Arg-CH_2Cl for 2 min at 37 °C. This buffer served to stop further activation of prothrombin and further inhibition of the thrombin formed by its plasma inhibitors. Prothrombin activation was assessed by the appearance of ^{125}I-prothrombin fragment 1+2 (F_{1+2}) and ^{125}I-thrombin after SDS/polyacrylamide-gel electrophoresis and autoradiography (Ofosu *et al.*, 1987). Catalysis of the inhibition of the thrombin activity generated *in situ* was assessed by the formation of ^{125}I-thrombin–inhibitor complexes in plasma containing the GAG under study.

RESULTS

Catalytic effects of GAGs on the inhibition of thrombin and Factor Xa by plasma

The effects of the seven GAGs on the inhibition of the activities of thrombin and Factor Xa are summarized in Table 2. Unfractionated heparin (sample 1) is the most active catalyst on the inhibition of the two proteinases at the lowest concentration of GAGs (0.66 μg/ml). Heparin with low affinity to antithrombin III (sample 2) is only as effective as DS and HS type I in catalysing thrombin inhibition. It is even less effective than HS type I in catalysing the inhibition of Factor Xa. Although the other GAGs are less active than unfractionated heparin in catalysing the inhibition of thrombin, further sulphation of an HS *in vivo* (i.e. the naturally occurring HS IIB) and resulphation of DS (*in vitro*) markedly increase their ability to enhance the inhibition of thrombin at the lowest concentration. It is evident that there are no great differences in performance, at 66 μg/ml, between any of unfractionated heparin, low-affinity heparin or the HS preparations (samples 1 to 5), whereas DS is a moderately effective catalyst for thrombin inhibition but has little effect on Factor Xa inhibition. Against Factor Xa, resulphation of DS *in vitro* increases its activity whereas sulphation *in vitro* of HS decreases its activity, in each case to a moderate extent.

Table 3. Inhibition of the appearance of enzymically active Factor Xa activity by various GAGs

Maximum Factor Xa activity is developed 2 min after $CaCl_2$ is added to contact-activated plasma. Results are expressed as percentage inhibition of this development in the presence of a given concentration of GAG. These results are the means for four to six determinations and the standard deviation of each value was less than 10% of the mean value. See Table 1 for explanation of the abbreviation used.

GAG	Concn. of GAG ...	Inhibition of the appearance of Factor Xa activity (%)		
		0.66 μg/ml	6.6 μg/ml	66.0 μg/ml
1. SH		52	80	80
2. LAH		5	25	35
3. HS type I		20	60	87
4. HS type IIB		27	77	87
5. HS-S		18	59	70
6. DS		2	2	18
7. DS-S		1	24	22

Table 4. Inhibition of the appearance of enzymically active thrombin in normal plasma containing a variety of GAGs

The data are reported as percentage inhibition of the generation of thrombin activity in the plasma containing the given concentration of GAG and are obtained 1 min after the re-addition of Ca^{2+} to contact-activated plasma. See Table 1 for explanation of the abbreviations used.

GAG	Concn. of GAG ...	Inhibition of the appearance of enzymically active thrombin (%)		
		0.66 μg/ml	6.6 μg/ml	66.0 μg/ml
1. SH		96	97	95
2. LAH		10	90	95
3. HS type I		7	45	78
4. HS type IIB		20	81	95
5. HS-S		14	44	90
6. DS		8	42	61
7. DS-S		7	98	98

Effects of the degree of sulphation of GAGs on the formation of enzymically active Factor Xa in contact-activated plasma

Table 3 lists the effectiveness of the seven GAGs in inhibiting the appearance of enzymically active Factor Xa. None of the seven can completely inhibit the appearance of Factor Xa activity even at 66 μg/ml, a concentration that for heparin *in vivo* would be excessive. Heparin with low affinity to antithrombin III is less effective than any of the HS preparations in inhibiting the appearance of Factor Xa activity, suggesting that most of the inhibition seen with HS preparations is due to the catalysis of the inhibition of the Factor Xa formed. The HS preparations are less effective than heparin at the lower concentrations, and resulphation *in vitro* has no additional effect. DS shows a weak action at the highest concentration only, an effect that is somewhat enhanced by further sulphation.

Table 5. Concentrations of GAG that inhibited the generation of thrombin activity by 50% 60 s after $CaCl_2$ had been added to contact-activated plasma containing the GAG under study

GAG	Concn. of GAG (μg/ml of plasma)
1. SH	0.4
2. LAH	3.3
3. HS type I	16.0
4. HS type IIB	1.0
5. HS-S	6.6
6. DS	10.0
7. DS-S	2.0

Effects of the degree of sulphation on the appearance of enzymically active thrombin in contact-activated plasma

The appearance of enzymically active thrombin can be almost completely inhibited, at least at the highest concentration, by five of the seven GAGs, the remaining two being HS type I and DS, the least sulphated GAGs (Table 4). Heparin is most active; the activity of heparin is probably attributable to the fraction with high affinity to antithrombin III, since the lowest concentration of heparin with low affinity to antithrombin III has little inhibitory effect. Also, the sulphation *in vitro* of HS and DS to heparin-like extents does not make these derivatives as active as unfractionated heparin, though for DS the increase in activity over that of the parent GAG is noteworthy. None of the HS preparations inhibited the generation of enzymically active thrombin at the lowest concentration as well as unfractionated heparin. HS IIB was the most effective of the three HS preparations. The low-sulphate preparation HS I was the least active; the resulphated preparation HS-S, with the highest sulphate content of the three preparations, had only marginally increased activity over that of HS I. Neither HS I nor HS-S achieved complete inhibition (i.e. 95% or greater) even at the highest concentration used.

Another approach to comparing the effectiveness of the seven GAGs as anticoagulants is summarized in Table 5, which lists the concentration of each GAG required to inhibit the generation of enyzmically active thrombin by 50%. Heparin with low affinity to antithrombin III was approx. 10% as effective as unfractionated heparin. Resulphation of DS *in vitro* yields a product better able to inhibit the generation of thrombin activity in plasma. The effect achieved with increased sulphation *in vivo* for HS (i.e. HS II) markedly exceeded that associated with resulphation *in vitro*.

Pathways for the inhibition of the appearance of enzymically active thrombin in plasmas containing the various GAGs

The activation of ^{125}I-prothrombin could readily be demonstrated by SDS/polyacrylamide-gel electrophor-

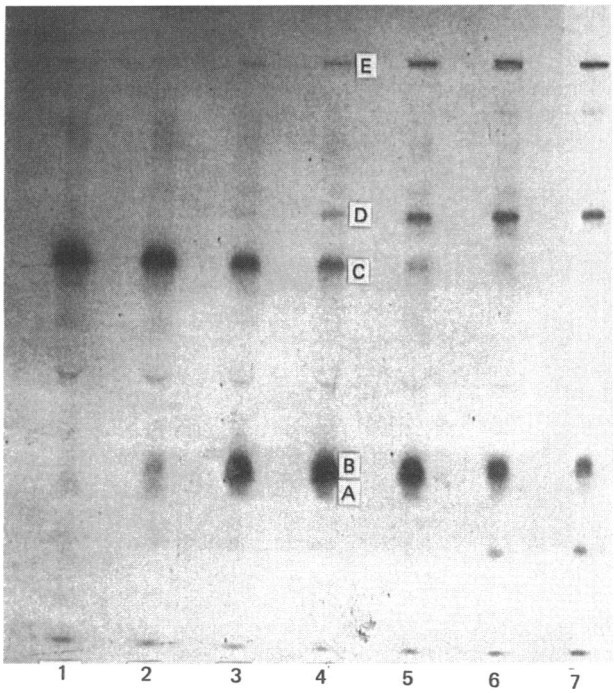


Fig. 1. Intrinsic-pathway activation in normal human plasma containing ^{125}I-prothrombin, and the subsequent formation of ^{125}I-thrombin–inhibitor complexes

This and the other Figures are autoradiographs after SDS/polyacrylamide-gel electrophoresis. Lane 1, ^{125}I-prothrombin in contact-activated plasma to which Ca^{2+} was not re-added. Lanes 2–7, ^{125}I-prothrombin in contact-activated plasma to which Ca^{2+} had been re-added at the following times previously: lane 2, 30 s; lane 3, 45 s; lane 4, 60 s; lane 5, 120 s; lane 6, 300 s; lane 7, 600 s. Key: A, thrombin; B, prothrombin fragment 1+2 (F_{1+2}); C, prothrombin; D, thrombin–antithrombin III complex; E, thrombin-α_2-macroglobulin complex; F, thrombin–heparin cofactor II complex.

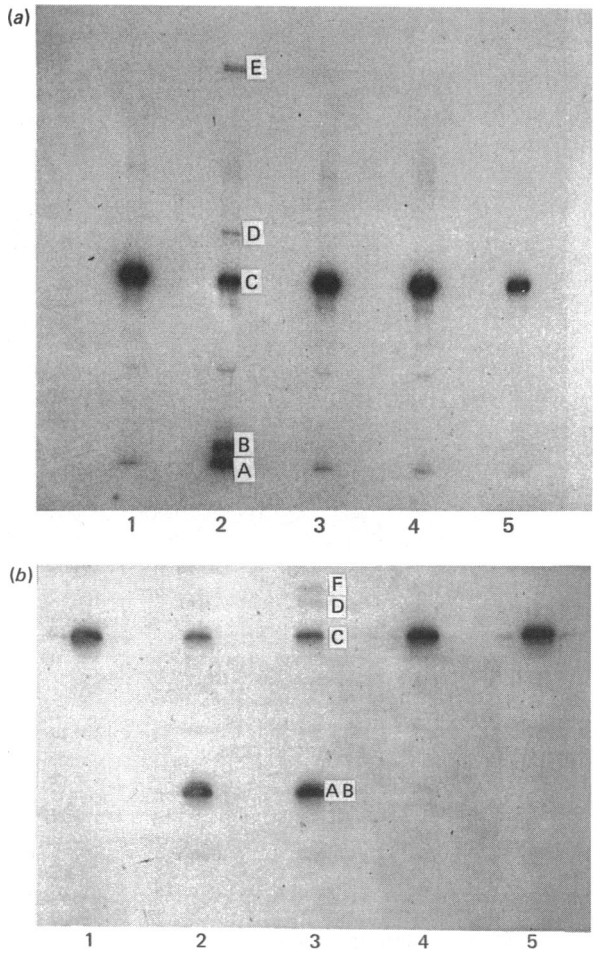


Fig. 2. Effects of (*a*) unfractionated pig mucosal heparin and (*b*) heparin with low affinity to antithrombin III on the activation of ^{125}I-prothrombin in contact-activated plasma

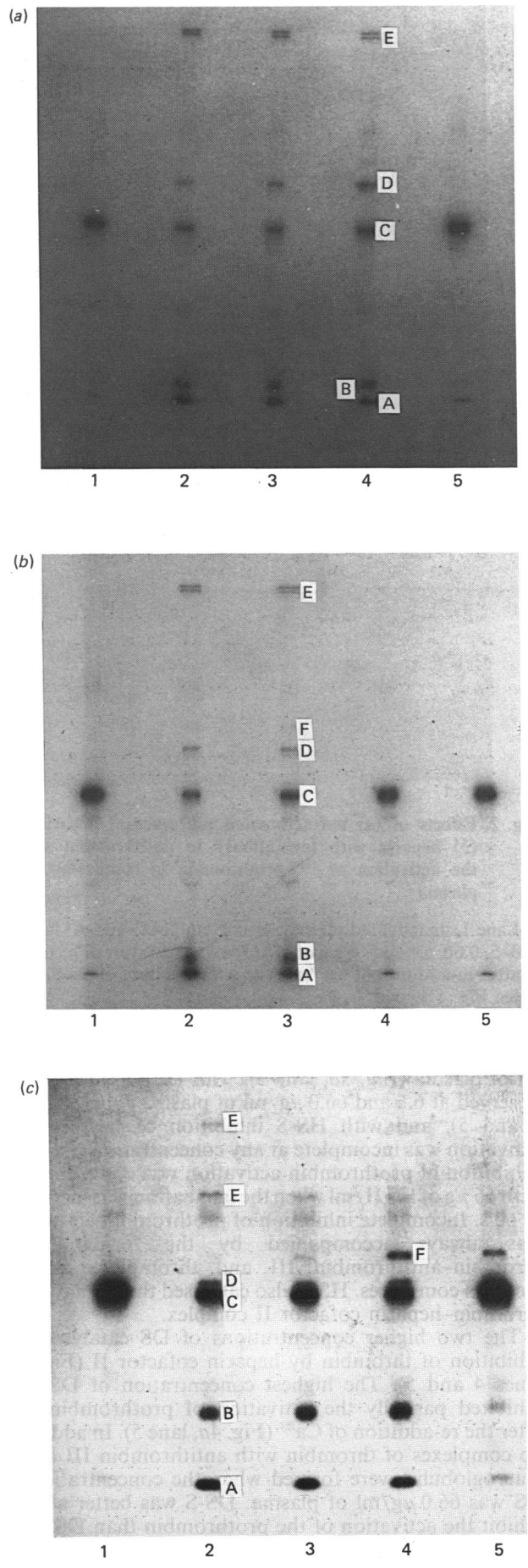
Lane 1, unactivated plasma: lane 2, no GAG added; lanes 3–5, 0.66, 6.6 and 66 μg of GAG/ml respectively. The time after re-addition of Ca^{2+} was 45 s. For coding of products see Fig. 1 legend.

esis and subsequent autoradiography 45 s after $CaCl_2$ had been added to contact-activated plasma (Fig. 1). The activation of prothrombin resulted in the formation of ^{125}I-prothrombin fragment 1+2 (F_{1+2}) and ^{125}I-thrombin, and the associated decrease in the band that corresponded to ^{125}I-prothrombin. Formation of ^{125}I-thrombin–antithrombin III and ^{125}I-thrombin–α_2-macroglobulin complexes was also evident.

The effects of the unfractionated heparin and heparin with low affinity to antithrombin III on the activation of prothrombin in contact-activated plasma are summarized in Figs. 2(*a*) and 2(*b*) respectively. Each of the three concentrations of unfractionated heparin completely inhibits the activation of prothrombin 45 s and 60 s after the re-addition of Ca^{2+}. Complete inhibition of prothrombin activation is obtained only with the two higher concentrations of heparin with low affinity to antithrombin III 45 s after $CaCl_2$ addition. The lowest concentration did not inhibit the activation of prothrombin but weakly catalysed the formation of thrombin–heparin cofactor II complex.

The effects of HS type I, HS type IIB and HS-S 45 s after $CaCl_2$ addition are shown in Figs. 3(*a*), 3(*b*) and 3(*c*) respectively. With HS type I complete inhibition of prothrombin activation was only observed with 66 μg/ml of plasma (Fig. 3*a*, lane 5), with HS type IIB it was observed at 6.6 and 66.0 μg/ml of plasma (Fig. 3*b*, lanes 4 and 5), and with HS-S inhibition of prothrombin activation was incomplete at any concentration (Fig. 3*c*). Inhibition of prothrombin activation was complete only with 66 μg of HS II/ml when the incubation was increased to 60 s. Incomplete inhibition of prothrombin activation was always accompanied by the formation of thrombin–antithrombin III and thrombin–α_2-macroglobulin complexes. HS-S also catalysed the formation of thrombin–heparin cofactor II complex.

The two higher concentrations of DS catalysed the inhibition of thrombin by heparin cofactor II (Fig. 4*a*, lanes 4 and 5). The highest concentration of DS also inhibited partially the activation of prothrombin 45 s after the re-addition of Ca^{2+} (Fig. 4*a*, lane 5). In addition, no complexes of thrombin with antithrombin III or α_2-macroglobulin were formed when the concentration of DS was 66.0 μg/ml of plasma. DS-S was better able to inhibit the activation of the prothrombin than DS since complete inhibition of prothrombin activation was



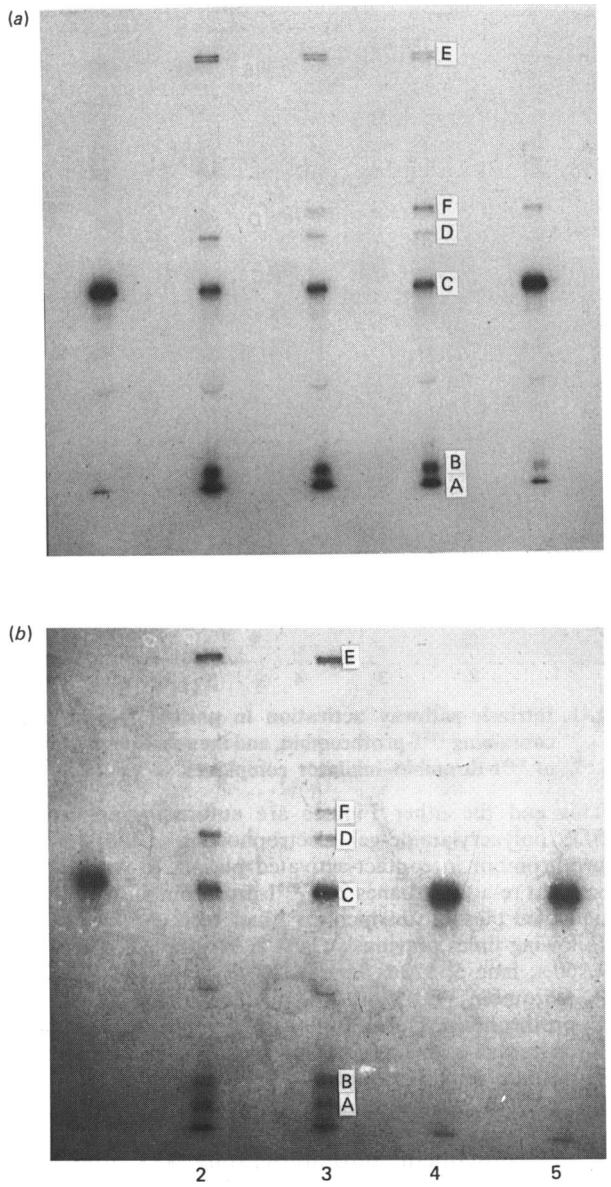


Fig. 4. Pathways for the inhibition of the appearance of enzymically active thrombin in plasma containing (*a*) pig mucosal DS and (*b*) DS resulphated *in vitro* (DS-S)

Lane 1, unactivated plasma; lane 2, no GAG; lanes 3–5; 0.66, 6.6 and 66 μg of the GAG/ml of activated plasma. For coding of products see Fig. 1 legend.

Fig. 3. Effects of various HS preparations on the intrinsic-pathway activation of prothrombin in normal plasma containing (*a*) HS type I, (*b*) HS type IIB (HS-IIB) and (*c*) sulphated HS (HS-S)

Results shown were obtained 45 s after the re-addition of Ca^{2+} to contact-activated plasma. Lane 1, unactivated plasma; lane 2, no GAG; lanes 3–5, 0.66, 6.6 and 66.0 μg of the GAG/ml of plasma with Ca^{2+} re-added respectively. For coding of products see Fig. 1 legend. The samples in (*c*) were reduced with 2-mercaptomethanol before electrophoresis. Reduction facilitated the separation of thrombin (band A) and prothrombin fragment 1+2 (band B) and increased the migration of thrombin–α_2-macroglobulin complex (band E). Reduction, however, resulted in a poorer separation of prothrombin (band C) and thrombin–antithrombin III complex (band D).

observed at the two higher concentrations of DS-S (Fig. 4*b*, lanes 4 and 5).

DISCUSSION

The structures of the GAGs used in this work differ in a number of ways; the three major ones are (*a*) the constituent backbones, (*b*) the extent of sulphation and (*c*) molecular mass. The polysaccharide backbones differ, and among the seven samples there are three types of uronosylhexosamine backbones. The heparin–heparan class has hybrid iduronosyl/glucuronosyl-(1→4)-glucosamine units, with mostly iduronosyl in heparins and with less iduronosyl in heparans. The glucosamine units are *N*-acetylated or *N*-sulphated. The iduronosyl residues in heparins and heparans (the latter *in vivo*) increase in parallel with increased sulphation, and ultimately an increase in the proportion of chains able to activate antithrombin III. Dermatans have, primarily, iduronosyl-(1→3)-galactosamine (as *N*-acetylated) units (Lasker, 1977; Höök *et al.*, 1984; Casu, 1985). From Tables 2, 3, 4 and 5 it can be seen immediately that GAGs 1 and 4, which have the heparin/heparan backbone and are most sulphated *in vivo*, have the highest activity in all systems. The galactosaminoglycans, GAGs 6 and 7, show activities that are generally lower than those of unfractionated heparin (GAG 1) and HS type IIB (GAG 4). It is evident from Tables 2, 4 and 5 that in general the efficiency with which a GAG catalyses the inhibition of thrombin parallels its ability to inhibit the generation of enzymically active thrombin in plasma. Although at first glance this might indicate that the anticoagulant effects result strictly from the catalysis of the inhibition of the thrombin generated *in situ*, it is very likely that there is an additional contributory effect to anticoagulation. Specifically, some GAGS are better able to suppress the activation of prothrombin than others with similar catalytic effects on thrombin inhibition. The various effects of the seven GAGs support the concept that the ability to catalyse the inhibition of Factor Xa and/or its generation does not always translate directly into a similar effect on the generation of thrombin activity (Ofosu *et al.*, 1985). This is best demonstrated by the *N*-acetylgalactosamine-containing GAGS 6 and 7. They are the least able to inhibit the appearance of Factor Xa activity, yet they catalyse the inhibition of thrombin and inhibit the generation of enzymically active thrombin.

A second major difference among the GAGs is the degree of sulphation, increased over that of the natural product in HS-S and DS-S by further sulphation *in vitro*. Sulphation, as already noted, correlates better with inhibition of the appearance of thrombin activity in contact-activated plasma than with other activities. However, it should be borne in mind that in natural GAGs changes in the extent of sulphation are always accompanied by other structural variations. In the heparin/heparan class increased sulphation is always accompanied by decreased glucuronate and increased iduronate content, and also by other sequence changes including decrease in *N*-acetylation in the hexosamine and an increase in the fraction that can activate antithrombin III (Lasker, 1977; Höök *et al.*, 1984; Jacobson *et al.*, 1984; Johnson, 1984; Casu, 1985). The effects of resulphation *in vitro* of HS and DS on the appearance of thrombin activity, and the similarity of their effects to that of heparin with low affinity to antithrombin III, suggest that the sulphation of GAGs with predominantly iduronosyl residues yields products better able to inhibit the generation of thrombin activity than sulphation of GAGs with predominantly glycuronosyl residues. Heparin with low affinity to antithrombin III and DS-S, both likely to have iduronate as the predominant uronic acid, are better inhibitors of thrombin generation than HS-S, which probably has more glucuronate than the iduronate content.

The seven GAGs inhibit the generation of thrombin activity in plasma by a variety of ways. Heparin completely inhibits the activation of prothrombin. This is probably due to the fraction with high affinity to antithrombin III, as the activity of heparin with low affinity to antithrombin III is approx. 10% that of unfractionated heparin. Low concentrations of natural forms of HS inhibit the generation of thrombin activity by catalysing the inhibition of the thrombin generated *in situ* by antithrombin III (Fig. 3). Increased sulphation as found in HS type IIB, which *in vivo* makes the HS more heparin-like, also improves its ability to inhibit the activation of prothrombin. Increased sulphation as such, without an accompanying increase in the ability to activate antithrombin III (i.e. sulphation *in vitro*), does not improve the ability of HS to inhibit the activation of prothrombin (Tables 4 and 5 and Fig. 3).

The major anticoagulant effect of DS is the catalysis of the inhibition of thrombin generated *in situ* by heparin cofactor II (Fig. 4). Unlike HS, increased sulphation *in vitro* also increases the ability of DS to inhibit prothrombin activation directly. Thus sulphation of the DS *in vitro* confers on the new product properties similar to those of heparin with low affinity to antithrombin III (Table 5) and pentosan polysulphate (Ofosu *et al.*, 1986, 1987). Unlike HS, resulphated DS is also better able to accelerate the thrombin inhibition than is the parent DS (Table 2). These results suggest that the extent of sulphation of HS and DS *in vivo* may influence their ability to inhibit the activation of prothrombin and to catalyse the inhibition of thrombin. Alterations in the sulphation patterns of HS and DS may be an important contributory factor to the evolution of thrombosis *in vivo*. The extent to which sulphation of vessel-wall glycosaminoglycans varies within the vasculature may be an important factor in the pathogenesis of thromboembolic events.

This work was supported in part by Grants-in-Aid from the Ontario Heart and Stroke Foundation.

REFERENCES

Björnsson, T. D., Nash, P. V. & Schaten, R. (1982) Thromb. Res. **27**, 15–21

Buchanan, M. R., Boneu, B., Ofosu, F. & Hirsh, J. (1985) Blood **66**, 198–201

Casu, B. (1985) Adv. Carbohydr. Chem. Biochem. **43**, 51–134

Casu, B. & Gennaro, U. (1975) Carbohydr. Res. **39**, 168–176

Casu, B., Johnson, E. A., Mantovani, M., Mulloy, B., Oreste, P., Pescador, R., Prino, G., Torri, G. & Zopetti, G. (1983) Arzneim.-Forsch. **33**, 135–142

Cerskus, A. L., Birchall, K. J., Ofosu, F. A., Hirsh, J. & Blajchman, M. A. (1984) Can. J. Biochem. Cell. Physiol. **62**, 975–983

Choay, J., Petitou, M., Lormeau, J. C., Sihay, P., Casu, B. & Gatti, G. (1983) Biochem. Biophys. Res. Commun. **116**, 492–499
Grossman, B. J. & Dorfmann, A. (1957) Pediatrics **20**, 506–514
Höök, M., Kjéllen, L. & Johansson, S. (1984) Biochemistry **53**, 847–869
Hopwood, J., Höök, M., Linkder, A. & Lindahl, U. (1976) FEBS Lett. **69**, 51–54
Izuka, K. & Murata, K. (1972) Atherosclerosis **16**, 217–224
Jacobson, I., Lindahl, U., Jensen, J. W., Rodén, L., Prihar, H. & Feingold, D. S. (1984) J. Biol. Chem. **259**, 1056–1063
Johnson, E. A. (1982) Pharmacol. Res. Commun. **14**, 289–311
Johnson, E. A. (1984) Thromb. Res. **35**, 583–588
Johnson, E. A. & Paterson, M. S. (1986) Anal. Biochem. **158**, 111–116
Kirk, J. E. (1959) Nature (London) **184**, 369–370
Lasker, S. E. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. **36**, 92–97
Lindahl, U. & Höök, M. (1978) Annu. Rev. Biochem. **47**, 385–417
Marciniak, E. (1973) Br. J. Haematol. **24**, 391–400
Modi, G. J., Blajchman, M. A. & Ofosu, F. A. (1984) Thromb. Res. **36**, 537–547
Morrison, L. M., Rucker, P. G. & Ershoff, B. H. (1968) J. Atheroscler. Res. **8**, 319–327
Murata, K., Nakazawa, K. & Hamai, A. (1975) Atherosclerosis **21**, 93–193
Ofosu, F. A., Modi, G. J., Blajchman, M. A. & Hirsh, J. (1981) Thromb. Res. **23**, 331–345
Ofosu, F. A., Modi, G., Cerskus, A. L., Hirsh, J. & Blajchman, M. A. (1982) Thromb. Res. **28**, 487–497
Ofosu, F. A., Modi, G. J., Smith, L. M., Cerskus, A. L., Hirsh, J. & Blajchman, M. A. (1984*a*) Blood **64**, 742–747
Ofosu, F. A., Cerskus, A. L., Hirsh, J., Smith, L. M., Modi, G. J. & Blajchman, M. A. (1984*b*) Br. J. Haematol. **57**, 229–238
Ofosu, F. A., Blajchman, M. A., Modi, G. J., Smith, L. M., Buchanan, M. R. & Hirsh, J. (1985) Br. J. Haematol. **60**, 695–704
Ofosu, F. A., Modi, G. J., Hirsh, J., Buchanan, M. A. & Blajchman, M. A. (1986) Ann. N.Y. Acad. Sci. **485**, 41–55
Ofosu, F. A., Sie, P., Modi, G. J., Fernandez, F., Buchanan, M. R., Blajchman, M. A., Boneau, B. & Hirsh, J. (1987) Biochem. J. **243**, 579–588
Rosenberg, R. D., Armand, G. & Lam, L. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 3065–3069
Thomas, D. P., Martin, R. E., Barrowcliffe, T. W., Thunberg, L. & Lindahl, U. (1982) Thromb. Haemostasis **47**, 244–248
Tollefsen, D. M. & Blank, M. K. (1981) J. Clin. Invest. **68**, 589–593
Walsh, P. N., Biggs, M. & Cagnatelli, G. (1974) Br. J. Haematol. **26**, 405–419

Received 9 February 1987/8 July 1987; accepted 27 August 1987