Br. J. Pharmac. (1980), **68**, 645–649

EVIDENCE FOR ANTITHROMBIN III INVOLVEMENT IN THE ANTICOAGULANT ACTIVITY OF CELLULOSE SULPHATE

G. KINDNESS, W.F. LONG & F.B. WILLIAMSON

Department of Biochemistry, Marischal College, University of Aberdeen, Aberdeen, AB9 1AS

1 Cellulose sulphate, like heparin, prolonged the clotting time in partial thromboplastin time (PTT) assays, inhibited the amidolytic activity of thrombin, was without effect on amidolysis catalysed by activated coagulation factor X(Xa), and potentiated the inhibition of both thrombin and Xa by antithrombin III (AT).

2 The anticoagulant activity of cellulose sulphate in PTT assays was, like that of heparin and heparan sulphate, but unlike that of dermatan sulphate, reduced by prior incubation of plasma with antiserum specific for AT.

3 These results, which suggest that the anticoagulant activity of cellulose sulphate is at least partially mediated through AT, are discussed in terms of the structural features of polysaccharides required for AT activation.

Introduction

Cellulose sulphate is a semi-synthetic product initially prepared and pharmacologically examined because of its general chemical resemblance to heparin, the mammalian anticoagulant glycosaminoglycan (Astrup, Galsmar & Volkert, 1944). Its potential value to the food, drug and cosmetic industries has been recognised for some time (Morrow, Hodge, Neuman, Maynard, Blanchet, Fassett, Birk & Manrodt, 1952). However, preparations of the compound exert a wide variety of effects when administered to experimental animals, or when examined in appropriate *ex vivo* assays. These effects include activation of coagulation factor XII (Kellermeyer & Kellermeyer, 1969), induction of plasma kinin (Rothschild & Gascon, 1966), depletion of plasma complement (Eisen & Loveday, 1971), inhibition of induced lipaemia (Constantinides, Cairns & Werner, 1954), activation of fibrinolysis (Rosa, Rothschild & Rothschild, 1972), and, under certain conditions, induction of widespread haemorrhages (Eisen & Loveday, 1971).

Recently, pharmacological interest in cellulose sulphate has centred on its capacity to deplete plasma kininogen (Rothschild, 1968), a property exploited in order to investigate the involvement of kinins in a variety of effects (Eisen & Loveday, 1971; Saeki, 1972; Seidel & Wendel, 1972; Wendel & Seidel, 1972; Antonio, Fernades, Gonçalves & Rocha e Silva, 1973; McCormick, Senior & Whalley, 1974).

Because of the current interest in the molecular mechanisms underlying polysaccharide-activated anticoagulation, and particularly the involvement of antithrombin III(AT) (Barrowcliffe, Johnson & Thomas, 1978; Rosenberg, 1978), we have used *ex vivo* assay systems to re-examine the anticoagulant effect of cellulose sulphate, and to compare it to that of heparin. In this paper we describe the effects of cellulose sulphate and heparin on the activities of thrombin and activated coagulation factor X(Xa), and on the reaction between AT and these clotting enzymes.

Methods

Cellulose sulphate (Lot No. 185) was obtained from Kelco, San Diego, California, U.S.A. The sample had a degree of substitution of 2.2 sulphate ester residues per glucose residue. Heparin (from porcine intestinal mucosa, Grade 1, 170 USP units/mg) and dermatan sulphate (from porcine skin) were obtained from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey. Heparan sulphate (from bovine lung) was a gift from Professor J. A. Cifonelli, Department of Pediatrics, University of Chicago, U.S.A. Polysaccharide samples, which are all sodium salts, were dissolved immediately before use at 4 mg/ml in veronal buffer (0.015 M 5,5-diethylbarbituric acid, 0.01 M sodium 5,5-diethylbarbiturate, 0.125 M sodium chloride, pH 7.4), and were subsequently diluted in buffer as appropriate. Antiserum specific for human antithrombin III (AT) was obtained from Behringwerke AG, Marburg, West Germany. Platelin was obtained from General Diagnostics, Eastleigh, Hants.

The sources and methods of preparation of thrombin, activated bovine coagulation factor X(Xa),

0007-1188/80/040645-05 $01.00

human antithrombin III (AT), polybrene, and substrates N-benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide (S-2160) and N-benzoyl-L-isoleucyl-L-glutamyl-L-glycyl-L-arginine-*p*-nitroanilide (S-2222) were as previously described (Kindness, Long & Williamson, 1979).

Preparation of human plasma and measurement of partial thromboplastin times (PTT)

Blood donors were male non-smokers in good health, had no known bleeding disorders, had taken no medication for at least 14 days, were fasted for 12 h before bleeding, and were aged 18 to 35 years. Blood was withdrawn from the median cubital vein into a polypropylene syringe, and transferred to a plastic tube containing anticoagulant (9 volumes of blood: 1 volume of 3.8% (w/v) trisodium citrate). Cell-free plasma was prepared by centrifuging blood at 750 *g* for 10 min. The supernatant was added to a pool of plasma derived ultimately from 20 donors, which was stored at −70°C in 1 ml portions.

Plasma (0.1 ml) was added to platelin (0.1 ml of a 1 in 5 dilution with veronal buffer of a solution of the material reconstituted as directed by the manufacturer) in a glass tube. After mixing, the contents were incubated at 37°C for 60 s. Polysaccharides (0.1 ml; 0 to 1 mg/ml final concentrations) were then added, the contents again mixed, and incubated at 37°C for 2 min. Following incubation, calcium chloride (0.1 ml of an 0.025 M solution) was added, mixed, and the time taken for clot formation noted. Throughout the period following the addition of calcium chloride, the contents of the tube were gently agitated manually by continuously tilting the tubes through an angle of 180°. The clotting time was taken as the mean of quadruplicate results. Standard deviations were computed for all PTT values; coefficients of variations varied within the range 5.6 to 11.0.

Results are expressed as clotting ratios (PTT in presence of polysaccharide/PTT in absence of polysaccharide). Control clotting times (in absence of polysaccharide) were in the range 50 to 60 s. If no clot formed in polysaccharide-containing tubes after 300 s, the clotting ratio was recorded as infinity. In some cases, plasma (100 µl) was incubated with antiserum specific for AT (30 µl) for 10 min at 37°C before addition of polysaccharide, or, in controls, addition of veronal buffer (100 µl).

Colorimetric experiments

Methods were modifications of those used by Teien, Abildgaard & Höök (1976).

(*i*) *Effect of polysaccharides on thrombin activity:* 200 µl polysaccharide (0 to 1 mg/ml) was incubated with 100 µl thrombin (2.5 units /ml) for 2 min at 37°C; 100 µl S-2160 (0.15 mg/ml) was then added. The reaction was stopped after 20 min incubation at 37°C.

(*ii*) *Effect of polysaccharides on Xa activity:* 100 µl polysaccharide (0 to 1 mg/ml) was incubated with 100 µl Xa (1 unit/ml) for 2 min at 37°C; 200 µl S-2222 (0.3 mg/ml) was then added. The reaction was stopped after 5 min incubation at 37°C.

(*iii*) *Effect of polysaccharides on inactivation of thrombin by AT:* 100 µl polysaccharide (0 to 1 mg/ml) was incubated with 100 µl AT (0.075 units/ml) for 2 min at 37°C; 100 µl thrombin (2.5 units/ml) were then added. After a further 60 s, 100 µl of a mixture containing S-2160 (0.15 mg/ml) and polybrene (0.1 mg/ml) was added. The reaction was stopped after a further 2 min incubation at 37°C.

(*iv*) *Effect of polysaccharides on inactivation of Xa by AT:* 50 µl polysaccharide (0 to 1 mg/ml) was incubated with 50 µl AT (0.075 units/ml for 2 min at 37°C; 100 µl Xa (0.032 units/ml was then added. After a further 60 s, 200 µl of a mixture containing S-2222 (0.3 mg/ml) and polybrene (0.1 mg/ml) was added. The reaction was stopped after a further 10 min incubation at 37°C.

In all assays, the reaction was stopped by addition of 300 µl 50% (v/v) acetic acid per tube. Reagent concentrations quoted are final concentrations in the reaction mixture before acetic acid addition. Appropriate control tubes containing separately no polysaccharide, no enzyme or no substrate, and in assays (iii) and (iv), no AT, were included. Polybrene, the polycation included in assays (iii) and (iv), electrostatically interacts with the polyanionic polysaccharides and prevents the compounds directly inactivating the enzyme (Teien *et al.*, 1976). Paranitroaniline (pNA) released from substrates in quadruplicate was determined at 405 nm, with a Cecil spectrophotometer model 272. Standard deviations were computed for all values; coefficients of variation varied within the range 3.2 to 10.1. pNA released was expressed as a fraction of that released from substrates in the absence of polysaccharides. Optical densities of pNA released in absence of polysaccharide were in the range 0.5–0.6.

Results

Figure 1 shows that both cellulose sulphate and heparin prolonged the clotting time of plasma. Cellulose sulphate and heparin directly inhibited amidolysis of the chromogenic substrate S-2160 by thrombin and potentiated the inactivation of thrombin by AT (Figure 2), heparin being, on a weight basis, the

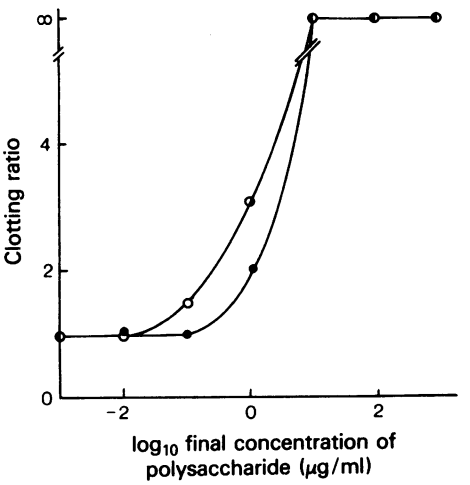

Figure 1 Effect of cellulose sulphate (●) and heparin (○) on partial thromboplastin time (PTT). Results are expressed as clotting ratios (see Methods).

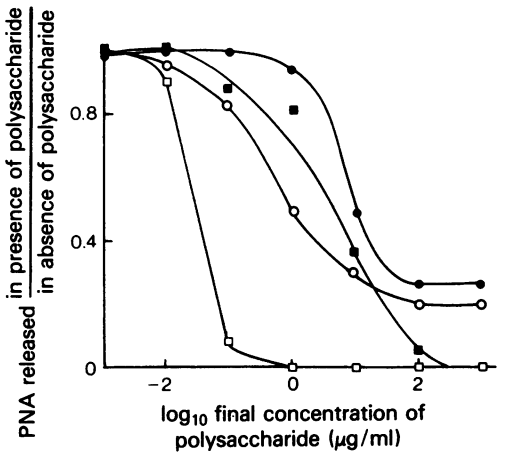

Figure 2 Effect of cellulose sulphate (closed symbols) and heparin (open symbols) on amidolytic activity of thrombin (circles) and on inactivation of thrombin by antithrombin III (squares).

more effective. In contrast, the polysaccharides did not inhibit Xa directly, although they did potentiate the inactivation of Xa by AT (Figure 3).

A concentration of cellulose sulphate (1 μg/ml) and of heparin (1 μg/ml) was selected which produced suitably elevated clotting ratios when the compounds were incubated with plasma (see Figure 1). Clotting ratios were then determined in an experiment in which plasma was preincubated with antiserum specific for AT before addition of polysaccharide or veronal buffer (see Methods section). The glycosaminoglycans heparan sulphate and dermatan sulphate were similarly examined (at concentrations producing elevated clotting ratios), as their relative effectiveness in AT potentiation has already been documented (Teien *et al.*, 1976; Kindness *et al.*, 1979). Preincubation of plasma with antiserum reduced the recorded clotting ratio in the case of cellulose sulphate, heparin and heparan sulphate, but not in the case of dermatan sulphate (Table 1).

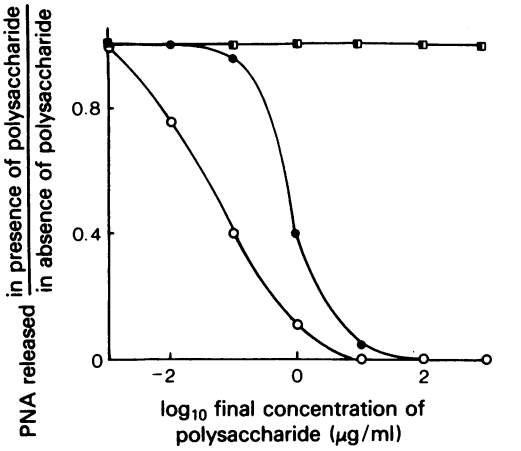

Figure 3 Effect of cellulose sulphate (closed symbols) and heparin (open symbols) on amidolytic activity of activated coagulation factor X (Xa, squares) and on inactivation of Xa by antithrombin III (circles).

Discussion

The results obtained show that cellulose sulphate, like heparin, prolongs the clotting time in PTT assays, inhibits the amidolytic activity of thrombin, is without direct effect on Xa-catalysed amidolysis, and potentiates the inhibition of both thrombin and Xa by AT.

This was shown by examination of thrombin- and Xa-catalysed amidolysis of synthetic chromogenic substrates. It should be noted that effects exerted in such systems by glycosaminoglycans (Teien *et al.*, 1976; Kindness *et al.*, 1979), or, in this report by cellulose sulphate, do not definitively establish whether or not the *in vivo* or *ex vivo* anticoagulant activity of these compounds is mediated through AT.

Nevertheless, pre-incubation of plasma with antiserum specific for AT reduced the anticoagulant activity of cellulose sulphate subsequently added to the plasma. The anticoagulant activity of heparin (as expected) and of heparan sulphate was also reduced following pre-incubation of plasma with antiserum, while that of dermatan sulphate was not reduced. The results suggest that in the *ex vivo* clotting assay, the anticoagulant activity of cellulose sulphate (and of heparin and heparan sulphate) is at least partially mediated through potentiation of AT activity.

Table 1 Effect of antiserum specific for antithrombin III (AT) on anticoagulant activity of cellulose sulphate, heparin, heparan sulphate and dermatan sulphate

Polysaccharide	Concentration (μg/ml)	PTT(s) (means ± s.d.) without antiserum	PTT(s) (means ± s.d.) with antiserum	Clotting ratio without antiserum	Clotting ratio with antiserum
Cellulose sulphate	1	118 ± 9	104 ± 8	2.19	1.77
Heparin	1	178 ± 10	122 ± 12	3.24	2.06
Heparan sulphate	10	140 ± 8	116 ± 6	2.54	1.96
Dermatan sulphate	100	134 ± 9	147 ± 11	2.44	2.49
Control	—	55 ± 6	59 ± 7	—	—

PTT: partial thromboplastin time.

Relatively few compounds, other than naturally-occurring glycosaminoglycans, have been shown to potentiate AT activity (Yin & Tangen, 1976; Machovich & Horváth, 1977; Thomas, Lane, Michalski, Johnson & Kakkar, 1977; Kindness *et al.*, 1979,) and it has been suggested that the ability of compounds to activate AT may be related to the molecular rigidity of the polymer (Kindness *et al.*, 1979). Consistent with this suggestion is the observation of a specific, co-operative, binding of calcium ions by heparin (association constant 10^4 M^{-1}) accompanied by a major conformational change and increased rigidity (Williamson *et al.*, unpublished results). In brief, these studies suggest that calcium binding locks heparin tetrasaccharide units into a particular, rigid, conformation. In this form, the 2-0 sulphates of the iduronate residues, and the 2-N-sulphates of the glucosamine residues may be hydrogen-bonded in pairs. These anionic pairs would be ideal candidates for electrostatic interaction with the cationic lysyl ε-amino groups of AT which appear to be essential for its activation (Rosenberg, 1977). Heparan and dermatan sulphates, with lower calcium ion binding capacity (Vannucchi, del Rosso, Cella, Urbano & Chiarugi, 1978) are expected to be less capable of conversion into a rigid conformation, and should therefore be less effective in AT activation.

Cellulose sulphate is a polyanion. In addition, the criteria formulated by Morris, Rees, Welsh, Dunfield & Whittington (1978) for predicting the molecular flexibility of sulphated polysaccharides, suggest that the freedom of rotation between adjacent glucose residues in cellulose sulphate is restricted: 1 → 4 linked diequatorial glycoside bonds in the glucan are likely to impose an overall extended molecular rigidity to the compound (Rees & Skerrett, 1968). It is therefore not surprising that cellulose sulphate can, at least under some conditions, potentiate AT activity. Further information about the structural prerequisites for AT activation may be gained by an examination of other acidic polymers of defined stereochemistry.

We thank Professor H.M. Keir for his continued interest and support. We gratefully acknowledge grants from the Medical Research Council, the Humanitarian Trust and the Humane Research Trust, which financed part of the work reported here. G.K. was in receipt of a Science Research Council Studentship.

References

ANTONIO, M.P.O., FERNADES, F., GONÇALVES, J.A. & ROCHA e SILVA, M. (1973). Pharmacological analysis of the mediators involved in the acute pulmonary edema produced in rats by adrenaline. *Agents and Actions*, **3/5**, 383.

ASTRUP, R., GALSMAR, I. & VOLKERT, M. (1944). Polysaccharide sulfuric acids as anticoagulants. *Acta physiol. scand.*, **8**, 215–226.

BARROWCLIFFE, T.W., JOHNSON, E.A. & THOMAS, D. (1978). Antithrombin III and heparin. *Br. med. Bull.*, **34**, 143–150.

CONSTANTINIDES, P., CAIRNS, A. & WERNER, A. (1954). Antilipemic activity of sulfated polysaccharides. *Archs int. Pharmacodyn. Thér.*, **99**, 334–345.

EISEN, V. & LOVEDAY, C. (1971). *In vivo* effects of cellulose sulphate on plasma kininogen, complement and inflammation. *Br. J. Pharmac.*, **42**, 383–391.

KELLERMEYER, W.F. & KELLERMEYER, R.W. (1969). Hageman factor activation and kinin formation in human plasma induced by cellulose sulfate solutions. *Proc. Soc. exp. Biol. Med.*, **130**, 1310–1314.

KINDNESS, G., LONG, W.F. & WILLIAMSON, F.B. (1979). Enhancement of antithrombin III activity by carrageenans. *Thromb. Res.*, **15**, 49–60.

MACHOVICH, R. & HORVÁTH, I. (1977). Heparin-like effect of polymethacrylic acid on the reaction between thrombin and antithrombin-III. *Thromb. Res.* **11**, 765–772.

MCCORMICK, J.T., SENIOR, J. & WHALLEY, E.T. (1974).

Changes in plasma kininogen levels induced by cellulose sulphate during pregnancy in the rat. *Br. J. Pharmac.*, **52**, 533–537.

MORRIS, E.R., REES, D.A., WELSH, E.J., DUNFIELD, L.G. & WHITTINGTON, S.G. (1978). Relation between primary structure and chain flexibility of random coil polysaccharides: calculation and experiment for a range of model carrageenans. *J. chem. Soc. Perkin II*, 793–800.

MORROW, P.E., HODGE, H.C., NEUMAN, W.F., MAYNARD, E.A., BLANCHET, H.J., FASSETT, D.W., BIRK, R.E. & MANRODT, S. (1952). The gastrointestinal non-absorption of sodium cellulose sulfate labelled with S^{35}. *J. Pharmac. exp. Ther.*, **105**, 273–281.

REES, D.A. & SKERRETT, R.J. (1968). Conformational analysis of cellobiose, cellulose and xylan. *Carbohyd. Res.*, **7**, 334–348.

ROSA, A.T., ROTHSCHILD, A.M. & ROTHSCHILD, Z. (1972). Fibrinolytic activity evoked in the plasma of the normal and adrenalectomized rat by cellulose sulphate. *Br. J. Pharmac.*, **45**, 470–475.

ROSENBERG, R.D. (1977). Chemistry of the hemostatic mechanism and its relationship to the action of heparin. *Fedn. Proc.*, **36**, 10–18.

ROSENBERG, R.D. (1978). Heparin, antithrombin and abnormal clotting. *A. Rev. Med.*, **29**, 367–378.

ROTHSCHILD, A.M. (1968). Some pharmacodynamic properties of cellulose sulphate, a kininogen-depleting agent in the rat. *Br. J. Pharmac. Chemother.*, **33**, 501–512.

ROTHSCHILD, A.M. & GASCON, L.A. (1966). Sulphuric acid esters of polysaccharides as activators of a bradykinin-forming system in plasma. *Nature*, **212**, 1364.

SAEKI, K. (1972). Anti-inflammatory properties of sulfated polysaccharides and activation of the plasma kinin-forming systems. *Archs int. Pharmacodyn. Ther.*, **195**, 33–51.

SEIDEL, G. & WENDEL, U. (1972). Participation of kinins in the regulation of cerebral vasopermeability. *Adv. exp. Med. Biol.*, **21**, 197–208.

TEIEN, A.N., ABILDGAARD, U. & HÖÖK, M. (1976). The anticoagulant effect of heparan sulfate and dermatan sulfate. *Thromb. Res.*, **8**, 859–867.

THOMAS, D.P., LANE, D.A., MICHALSKI, R., JOHNSON, E.A. & KAKKAR, V.V. (1977). A heparin analogue with specific action on antithrombin III. *Lancet*, **i**, 120–122.

VANNUCCHI, S., del ROSSO, M., CELLA, C., URBANO, P. & CHIARUGI, V. (1978). Surface glycosaminoglycans and calcium distribution in 3T3 cells. *Biochem. J.*, **170**, 185–187.

WENDEL, U. & SEIDEL, G. (1972). Kinins—evidence for the involvement in lymphostatic encephalopathy in rats. *Pharmacology*, **7**, 17–28.

YIN, E.T. & TANGEN, P. (1976). Heparin. heparinoids and blood coagulation. In *Heparin Chemistry and Clinical Usage*. ed. Kakker, V.V. & Thomas, D.P. pp. 121–124. London, New York and San Francisco: Academic Press.

(Received February 2, 1979.
Revised July 2, 1979.)