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The Blood Anticoagulant Activity of *N*-Carboxymethylchitosan Trisulfate

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SUMMARY

N-Carboxymethylchitosan trisulfate, prepared by submitting partially depolymerized N-carboxymethylchitosan to sulfation in pyridine and chlorosulfonic acid mixture, was characterized as a β(1–4) glucan carrying N-carboxymethyl, sulphamido, acetamido and sulfate ester groups on C3 and C6 (sulfur content 11–15·2%). Assays for its blood anticoagulant activity showed that this compound acts on the factors participating in the intrinsic pathways, while it is not active on the factors of the extrinsic and common pathways of the coagulation process. It therefore possesses antithrombogenic activity while preserving an alternative route to coagulation.

INTRODUCTION

Recent papers on blood anticoagulants obtained from chitosan demonstrate that these compounds should possess O-sulfate, acetamido and carboxyl groups. Sulphamido groups may also contribute to anticoagulant activity although the sulphamido/acetamido group ratio is important. It appears that the carbohydrate chain length is also important and this can be tailored to impart optimum blood anticoagulant properties (Muzzarelli *et al.*, 1984, 1986; Hirano *et al.*, 1985, 1986).

In the course of evaluating the blood anticoagulant activity of the modified chitosans, various authors have observed that the biological activity of one particular product can be classified differently compared with heparin, depending on which test is performed, and, more specifically, depending on which blood coagulation factor is involved in the test. Moreover, it would seem that certain well-recognized tests for

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heparin fail or lead to unreliable results when performed with sulfated chitosan derivatives.

We have undertaken the present work with the intention of evaluating the blood anticoagulant activity of *N*-carboxymethylchitosan 3,6,*N*-trisulfate (NCMC trisulfate) with respect to all of the blood coagulation factors. Further objectives of our work are to provide compounds chemically related to heparin which might find use in defining the regulatory mechanism of hyaluronic acid synthesis in synovial membrane treated with sulfated glycosaminoglycans (Nishikawa *et al.*, 1985), and to obtain an exogenous glycosaminoglycan with nonspecific antiadherence properties for pathogens which may prove clinically useful in the treatment of patients subject to bladder infection (Hanno *et al.*, 1981, 1983). Heparin or fragments of heparin deprived of anticoagulant activity were found to inhibit angiogenesis in the presence of steroids that lack glucocorticoid activity (Crum *et al.*, 1985). The possible use of chitosan derivatives in this field also requires their chemical and biological characterization.

EXPERIMENTAL

Chitosans

Chitosan from antarctic krill (*Euphausia superba*) with a degree of acetylation of 2%, as determined by first derivative ultraviolet spectrophotometry (Muzzarelli & Rocchetti, 1985), was used.

N-Carboxymethylchitosans (NCMC)

Chitosan previously swollen with water was treated with glyoxylic acid as previously described (Muzzarelli *et al.*, 1982). The amount of the latter was one half of the stoichiometric quantity referred to the free amine, thus, one half of the amino groups were left for subsequent sulfation. The resulting NCMC degrees of substitution were: acetamido, 2%; free amine, 49%; and *N*-carboxymethyl, 49%.

Partially depolymerized *N*-carboxymethylchitosans

NCMC (1 g) was dissolved in water (*ca* 30 ml) and then HCl was added (20 ml, 1 M). Upon warming under gentle stirring, a clear solution was obtained to which concentrated HCl was added until reaching 6 M. The

solution was then boiled for the desired length of time (10 or 20 min). After rapid cooling, the polysaccharide was precipitated by the addition of acetone.

N-Carboxymethylchitosan 3,6,*N*-trisulfate

NCMC (1 g) was stirred at −10°C in anhydrous pyridine (30 ml) while chlorosulfonic acid (2·5 ml) was added dropwise. The temperature was then raised to 60°C and stirring was continued overnight. The brown suspension thus obtained was cooled to −10°C and a saturated solution of $NaHCO_3$ was added to neutralize the acidity. After dialysis, filtration and lyophilization, NCMC trisulfate was obtained as a pale yellow powder. Four separate preparations gave sulfur contents in the range of 11–15·2%, corresponding to sulfate groups per monosaccharide (d.s.) of 1·72–2·38, the maximum theoretical sulfur content being 16%, giving a d.s. of 2·5. In comparison, the products obtained from non-depolymerized NCMC had sulfur contents in the range of 4·5–6% and a corresponding d.s. of 0·75–0·93. The samples used in this study were: **(1)**, NCMC trisulfate containing 11·8% S from NCMC hydrolyzed for 10 min; **(2)**, NCMC trisulfate containing 15·2% S from NCMC hydrolyzed for 20 min; and **(3)**, NCMC trisulfate containing 11% S from NCMC hydrolyzed for 20 min.

General methods

Infrared spectra were recorded with a Perkin Elmer spectrometer Mod. 299-B. Circular dichroism spectra were recorded with a Jasco spectropolarimeter Mod. J-500-A. Ultraviolet spectra were recorded with a Kontron spectrophotometer Mod. Uvikon 810. The sulfur content was obtained using a sulfur analyzer manufactured by Leco, in which sulfur is oxidized to sulfate and titrated.

Biological activities

The anticoagulant activity was determined for human plasma with respect to partial thromboplastin time (PTT), prothrombin time (PT) and thrombin time (TT) tests.

Fresh human plasma, treated with citrate and combined with heparin (0·0, 0·2, 0·4, 0·6 and 0·8 IU ml^{-1}, 1 IU of heparin corresponding to 5·766 μg), or with the same weight of NCMC trisulfate, was used to carry out the tests with reagents supplied by Kabi Diagnostica, Stockholm, Sweden.

The partial thromboplastin time test (PTT) was carried out with Thrombofax®, a buffered reagent which contains phospholipids for optimal platelet-like activity and a plasma activator. To a test tube, plasma (0·1 ml) and Thrombofax® (0·1 ml) were added, mixed and incubated at 37°C for 4 min. Prewarmed calcium chloride (0·1 ml) was then added, the stop-watch started and the time necessary to form a fibrin clot in the gently tilted tube was read.

The prothrombin time test (PT) was carried out by adding Ortho Brain Thromboplastin (0·2 ml) at 37°C to human plasma with citrate (0·1 ml). The time from the addition until clot formation was taken.

The thrombin time test (TT) was carried out by adding Ortho Fibrindex® (human thrombin with calcium) (0·2 ml) to human plasma (0·2 ml). Time was measured until clot formation.

Factor assays were performed with factor deficient plasmas supplied by Mezz & Dade AG, Dudingen, Switzerland. The clotting times were measured on a mixture of normal plasma (0·1 ml) and factor deficient plasma (0·1 ml) against specific calibration curves for each factor. Clotting times were determined in terms of PTT for factors VII, IX, XI and XII; and in terms of PT for factors II, V, VII and X. All measurements were made in triplicate.

RESULTS

Optical properties of *N*-carboxymethylchitosan trisulfate

Evidence for the introduction of sulfate groups in NCMC trisulfate was obtained by i.r. spectrometry and spectropolarimetry. The i.r. spectra of NCMC sulfate showed evident absorption bands at 1230 and 800 cm^{-1} due to the presence of the sulfate group in all of the products obtained.

The u.v. spectrum of NCMC trisulfate showed absorption peaks at 190 and 260 nm, the latter being due to very small amounts of pyridine (Cushing *et al.*, 1954). The u.v. spectrum was consistent with the c.d. spectra which show Cotton bands at *ca* 190, 220 and 270, as indicated in Fig. 1. All of the c.d. spectra for the trisulfates were pH-dependent. Fig. 2 shows the $|\theta|$ values for (**1**) and (**2**), calculated at 210 and 230 nm respectively. The latter showed a large increase in $|\theta|$ over a narrow pH interval centered at pH 4, probably due to conformational changes that take place more readily at lower molecular weights.

Moreover, the more degraded NCMC trisulfate (**2**) showed a positive Cotton band at *ca* 190 nm whilst the 10 min hydrolyzed product (**1**) had a negative Cotton band at the same wavelength (compare Figs 1 and 3).

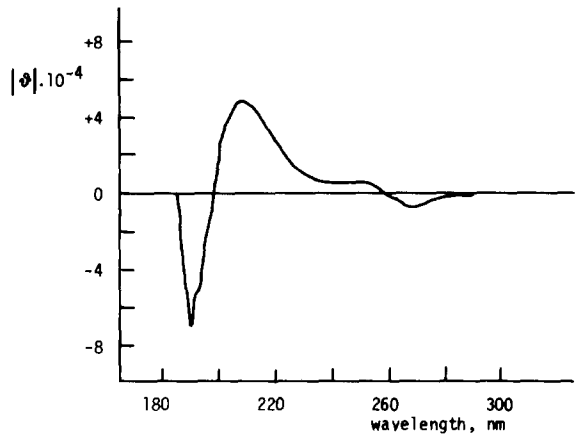

Fig. 1. Circular dichroism spectrum of NCMC trisulfate (**1**) (sulfur content 11·8%) at pH 6·7 obtained with pyridine and chlorosulfonic acid mixture. The NCMC was previously hydrolyzed for 10 min.

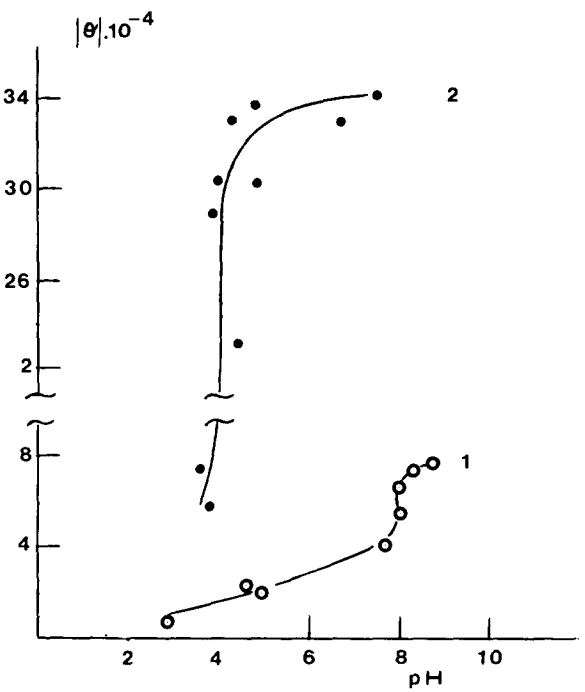

Fig. 2. pH-Dependence of the molecular ellipticity for NCMC trisulfate (samples (**1**) and (**2**)).

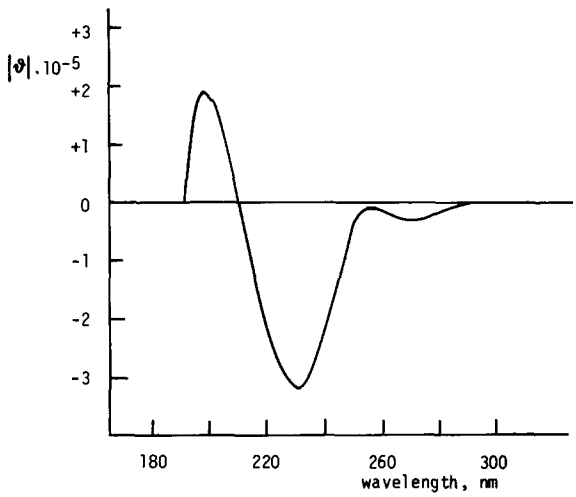

Fig. 3. Circular dichroism spectrum of NCMC trisulfate (**2**) (sulfur content 15·2%) at pH 6·7 obtained with pyridine and chlorosulfonic acid mixture. The NCMC was previously hydrolyzed for 20 min.

When the size of the macromolecule was reduced below a certain value by the hydrolysis, the sulfation reaction gave rise not only to products with higher sulfur content, but also probably with a different conformation.

As previously indicated, the c.d. spectra gave preliminary information about the molecular size of the product obtained after hydrolysis (compare Figs 1 and 3). The remarkable increase in $|\theta|$ at pH 4 was also related to the molecular size and degree of sulfation of the product; small molecules with a high degree of sulfation could in fact assume more regular conformations at certain pH values, thus contributing to the $|\theta|$ increase.

Biological activities of *N*-carboxymethylchitosan 3,6,*N*-trisulfate

Coagulation tests carried out on human venous blood showed that (**1**), (**2**) and (**3**) had to be used at concentrations respectively 8·5, 10 and 6 times higher than heparin to prevent coagulation after 24 h at room temperature.

The PTT test gave the results shown in Fig. 4. In comparison to heparin, (**1**), (**2**) and (**3**) were respectively 6, 7·3 and 4·9 times less effective. The results of the PT test are shown in Fig. 5: (**3**) did not show any activity, while (**1**) and (**2**) showed activities respectively 3·9 and 2·1 times lower than heparin. The TT data showed absence of activity towards fibrinogen (factor I) for all samples studied. This set of data demon-

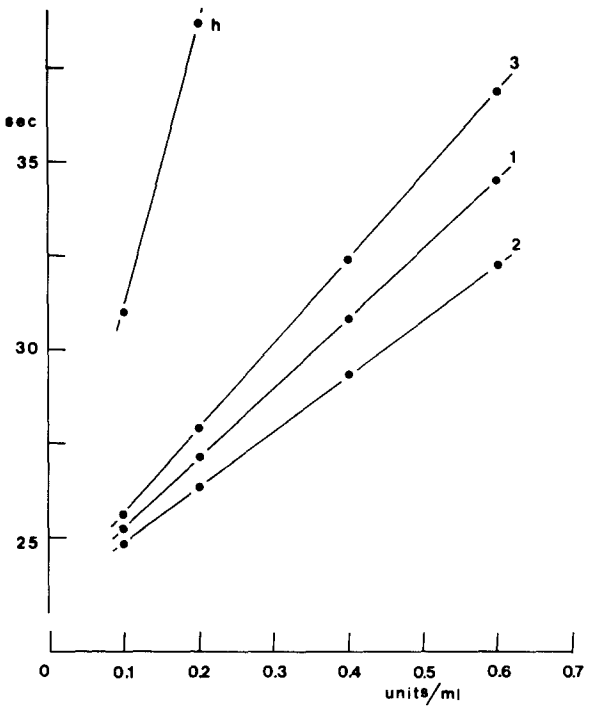

Fig. 4. Partial thromboplastin time (PTT) as a function of the amount of anticoagulant added to human plasma, for heparin (h) and NCMC trisulfate (**1**), (**2**) and (**3**).

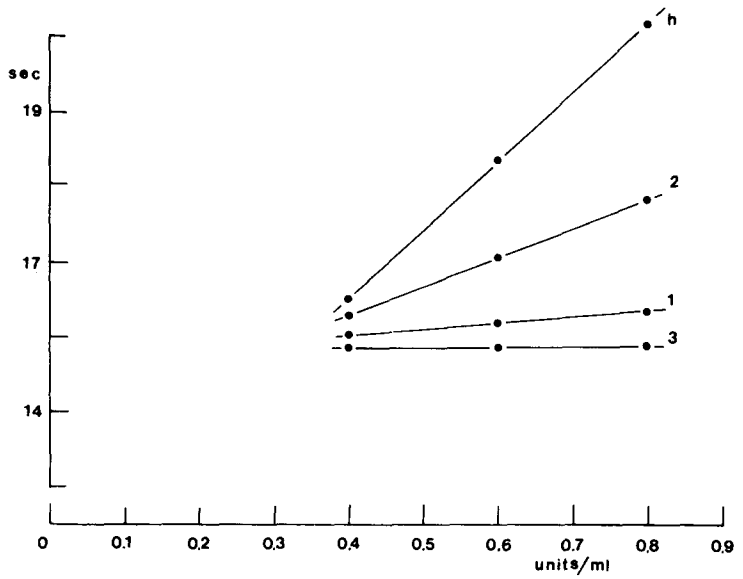

Fig. 5. Prothrombin time (PT) as a function of the amount of anticoagulant added to human plasma, for heparin (h) and NCMC trisulfate, (**1**), (**2**) and (**3**).

strates that a single test is inadequate to evaluate a blood anticoagulant, because there was no uniform trend in a group of tests for a given anticoagulant.

The availability of factor deficient plasmas makes it possible to measure the residual activity of each factor present in an anticoagulant-treated plasma. Table 1 shows the decrement of activity of the intrinsic pathway factors obtained with heparin and (**1**), (**2**) and (**3**). The different extent to which these factors were affected by the chitosan-derived blood anticoagulants is remarkable. In all cases (for (**1**), (**2**) and (**3**)) great differ-

TABLE 1
Decrements of Activity, %, for the Coagulation Factors of the Intrinsic Pathway, Measured as PTT.

Sample	*Concentration units ml^{-1}*	*Activity decrement (%)*			
		VIII	*IX*	*XI*	*XII*
Heparin	1·5	92	57	75	22
	3·0	97	97	92	78
(**1**)	13	97	94	17	0
	26	97	97	89	81
(**2**)	15	97	91	66	32
	30	97	97	91	82
(**3**)	9	74	57	0	0
	18	97	97	54	56

TABLE 2
Decrements of Activity, %, for the Coagulation Factors of the Common and Extrinsic Pathways, Measured as PT.

Sample	*Concentration units ml^{-1}*	*Activity decrement (%)*			
		Common pathway			*Extrinsic pathway*
		II	*V*	*X*	*VII*
Heparin	1·5	14	22	24	2
	3·0	30	25	52	5
(**1**)	13	0	0	0	0
	26	0	5	0	0
(**2**)	15	0	5	0	0
	30	5	22	0	0
(**3**)	9	0	0	0	0
	18	0	0	0	0

ences existed between factors XI and XII on one side and VIII and IX on the other side. This difference between heparin and the chitosan derivatives became more dramatic when the extrinsic pathway and the common pathway factors were considered (Table 2). It appears that samples (**1**), (**2**) and (**3**) did not produce any activity decrease for any of these factors of the extrinsic (VII) and common pathway (II, V and X).

DISCUSSION

The sulfation of NCMC in pyridine and chlorosulfonic acid mixtures leads to derivatives carrying sulfate groups on primary and secondary alcohol functions as well as on the residual amino groups in position 2. These products are effective blood anticoagulants, their efficacy depending on the coagulation factor examined. In general, the biological activities of NCMC trisulfates are lower than that of heparin. In comparison with *N*,O-sulfated chitosan and O-sulfated *N*-acetylchitosan studied by Hirano *et al.* (1985, 1986), it seems that the latter have anticoagulant activities higher than heparin, (**1**), (**2**) and (**3**), while sulfated O-CM-chitosan and O-sulfated chitosan have lower activities. It should be emphasized, however, that according to our results, the biological activity of these blood anticoagulants should be evaluated on the basis of a number of factors rather than one or two, because a particular product might exhibit high activity in one test and low activity in another. For example, the APTT value at zero concentration should be *ca* 30 s, (Kabi Diagnostica, 1985), as opposed to the reported value of 80 s (Fig. 2, Hirano *et al.*, 1985).

On the other hand, our results confirm, firstly, that sulfation on C-6, while essential for activity, should be preferably accompanied by sulfation on other positions; secondly, that the sulfamido group does not need to be present to a high degree of substitution; and thirdly, that the carboxymethyl group, which can be conveniently introduced on the amino group, adds to the biological activity. Carboxymethylation seems to impart activity if directed to the amino group rather than to the C-6.

While the results show that the molecular weight is an important parameter for the anticoagulant activity, enhanced activity could be obtained by defining the optimum molecular weight and by reducing polydispersity (Choay, 1986*). Data on the role of the molecular weight of NCMC trisulfate on blood coagulation will be reported in due course.

*Work by Choay (1986) on the anti-thrombogenic activity of the oligomer (4500 uma) of chitosan trisulfate (presently marketed in France under the tradename FRAXIPARINE) appeared after this paper was accepted for publication.

The most interesting aspect of our results is that the samples studied specifically interact with the factors of the intrinsic coagulation pathway, while they do not interact with those of the extrinsic and common pathway. Their behavior is distinctly different from that of heparin. The characteristic properties of NCMC trisulfate open the perspective of the use of blood anticoagulants which selectively act on only one branch of the coagulation cascade, thus leaving unaffected an alternative route to coagulation.

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