ELSEVIER

PII: S0144-8617(96)00173-7

Carbohydrate Polymers 33 (1997) 63–71

0144-8617/97/$17.00 + .00

Anticoagulant activity of functionalized dextrans. Structure analyses of carboxymethylated dextran and first Monte Carlo simulations

L. Krentsel[a], F. Chaubet[b]*, A. Rebrov[a], J. Champion[b], I. Ermakov[a], P. Bittoun[c], S. Fermandjian[c], A. Litmanovich[a], N. Platé[a] and J. Jozefonvicz[b]

[a]*Topchiev Institute of Petrochemical Synthesis, Russian Academy of Sciences, Leninsky prosp. 29, 117912, Moscow B-71, Russia*
[b]*Laboratoire de Recherches sur les Macromolecules, CNRS-URA 502, Université Paris-Nord, Av. J.B. Clement 93430, Villetaneuse, France*
[c]*Laboratoire de Biologie Structurale, CNRS-URA 147, I.G.R., Villejuif, France*

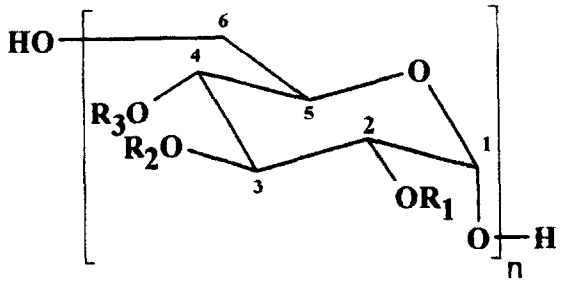
(Received 25 March 1996; accepted 26 July 1996)

During the last decade the synthesis and heparin-like properties of Carboxymethyl Dextran Benzylamide Sulfonate (CMDBS) have been investigated extensively. The carboxymethylation of the sugar hydroxyl groups of dextrans is the first and determining step during the CMDBS synthesis. The kinetics of this reaction as well as the structure of the resulting carboxymethylated compounds (CMD) have been investigated by joint 1D and 2D $^{1}H/^{13}C$ NMR spectroscopies. Using the corresponding data within kinetic equations provided 9 rate coefficients of carboxymethylation. At the same time Monte Carlo simulations of the CMDBS structures taking the NMR data and making some simplified assumptions allowed the analysis of the subsequent benzylamidification and sulfonation steps. Structure–function relationships, based on the preliminarly Monte Carlo results and anticoagulant properties of CMDBS, indicated that the anticoagulant activity of these compounds can be explained in terms of units distribution along the polymer chain. © 1997 Elsevier Science Ltd

INTRODUCTION

It is now well established that synthetic or modified natural polymers (these being copolymers with statistical distribution of the various functional groups along the chain) are capable of specific interactions with a biological species such as proteins, proteoglycans and nucleic acids (Jozefonvicz and Jozefowicz, 1990, 1992). The type and the level of biological activity vary with the composition and modification of the polymer. Glycosaminoglycan heparin is a highly charged anionic polysaccharide with a heterogenous structure that exerts a variety of biological effects (Bourrin and Lindahl, 1993) including the anticoagulant activity which arises from a complex process. Thus the structure of heparin corresponds to repeating uronic acid-*N*-acetyl-D-glucosamine disaccharide units which have undergone a variety of biochemical modifications including the sulfation and epimerization of the D-glucuronic acid resulting in L-iduronic acid (Casu, 1985). A heparin derived pentasaccharide is the minimal fragment required for binding to antithrombin and for eliciting the anticoagulant properties of the whole polysaccharide (Choay *et al.*, 1983; Lindahl *et al.*, 1980). The derivatized dextrans called CarboxyMethyl Dextran Benzylamide Sulfonate (CMDBS) (Fig. 1) present some of the biological activities of heparin including anticoagulant capacity (Fischer *et al.*, 1985), anticomplementary activity (Mauzac *et al.*, 1985; Crépon *et al.*, 1987) and modulation of cell proliferation (Logeart *et al.*, 1994; Letourneur *et al.*, 1993a, b). CMDBS delay the plasma coagulation by catalyzing the inactivation of the procoagulant enzyme thrombin by its natural inhibitors antithrombin and heparin co-factor II (Fischer *et al.*, 1985). It is thus postulated that CMDBS exhibits specific binding sites for both antithrombin and thrombin, similarly to heparin (Fischer *et al.*, 1985; Mauzac and Jozefonvicz, 1984). Evidence has been accumulated that the anticoagulant properties of CMDBS polymers are due to their high contents in carboxylic and sulfonate groups distributed along the polysaccharide backbone (Mauzac and Jozefonvicz, 1984). For instance, the

*To whom correspondence should be addressed.



$R_1, R_2, R_3 = H$ (D)

$= {}^{-}CH_2COONa$ (CM)

$= {}^{-}CH_2CONHCH_2C_6H_5$ (B)

$= {}^{-}CH_2CONHCH_2C_6H_4SO_3Na$ (S)

Fig. 1. Schematic structure of CMDBS.

anticoagulant capacity of CMDBS varies with the overall ratio of the chemical groups and the molecular weight of the polymer. Similarly to heparin, short sequences spanning different units may be responsible for the specific interactions of CMDBS with thrombin and antithrombin (Jozefonvicz and Jozefowicz, 1990, 1992; Fischer *et al.*, 1985; Mauzac and Jozefonvicz, 1984). We hereby postulate that the probability of finding such sequences in CMDBS depends on both the chain structure and the units distribution, the latter property being governed by the kinetic peculiarities of the reactions used for modification. The first and most determining step for the CMDBS synthesis is the dextran carboxymethylation (Logeart *et al.*, 1994; Mauzac and Jozefonvicz, 1984). Each dextran unit bears three hydroxyl groups; both the mono- and disubstitutions are possible during the reaction. However, the first substitution may affect the reactivity of the remaining OH groups, so that carboxymethylation is not a purely random process (unlike the subsequent benzylamidification and sulfonation are assumed to proceed randomly).

The present report concerns the study of carboxymethylated dextran structures using NMR measurements to estimate individual rate coefficients of carboxymethylation. The derived data were used to run computer simulations on short selective CMDBS fragments. Thus, the probabilities of formation of such sequences were estimated and results were used to interpret the experimental data upon anticoagulant activities in terms of units distribution of previously described CMDBS (Mauzac and Jozfonvicz, 1984).

MATERIALS AND METHODS

Dextran T-10 (Dx) (batch 06671, $M\bar{w} = 10200$, $M\bar{n} = 5400$) was purchased from Pharmacia Fine Chemicals, St Quentin en Yvelines, France. Monochloroacetic acid was purchased from Janssen Chimica, Noisy le Grand, France.

Derivatized dextrans

Carboxymethylation of dextran was carried out as in the following (Mauzac and Jozefonvicz, 1984; Chaubet *et al.*, 1995). Briefly: an aqueous solution of dextran and NaOH cooled to 4°C was heated quickly up to 20°C under vigorous stirring. An aqueous solution of monochloroacetic acid cooled to 4°C was then added at once, allowing the temperature of the reaction mixture to reach 50°C, due to the heat of neutralization. The reaction vessel was immersed immediately into a temperature-controlled bath at 50°C which timed the beginning of the reaction. The total volume of the mixture did not exceed 8–10 ml, initial dextran concentration being 0.75 mol/l. Kinetics of the aliquots at low conversions (d.s. < 0.40) were monitored by UV spectroscopy (at 287 nm). At higher conversions, UV spectra became more complex, thus chemical analysis was used. The samples of carboxymethylated dextran (CMD) were transformed into H-form and CM groups contents have been estimated by titration with NaOH. The total decay of monochloroacetic acid was monitored by titration of Cl^- ions with $Hg_2(NO_3)_2$. Eight samples were prepared with d.s. of carboxymethyl groups from 0.10 to 0.80. CMDB and CMDBS samples were previously prepared and characterized by Mauzac and Jozefonvicz (Mauzac and Jozefonvicz, 1984). In summary, dextran units were successively substituted with carboxymethyl groups, benzylamide groups and sulfonate groups. Thus, native dextran (T10), one sample of CMDB and 12 samples of CMDBS were tested in the anticoagulant assay. All these samples were washed with water and equilibrated at pH 7.35 with Michaelis buffer using a 'Diaflo' ultrafilter (Amicon; membrane YM5: 5000) and finally lyophilized. Chemical characterizations were performed by acidimetric titrations in acetone–water 1:1 (v/v) as the solvent. The benzylamine content and the sulfonate content were calculated from elemental analysis.

Anticoagulant activity

The anticoagulant activity has been determined by measuring thrombin clotting time (ThNIH units) of poor platelets human plasma in the presence of various amounts of polymer. Clotting system: 0.1 mL of polymer solution (various contents) or 0.1 mL of Michaelis buffer (control time) were incubated with 0.2 mL of PPP at 37°C for 5 min. Then 0.1 mL of thrombin (20 NIH u./mL) was added and the thrombin clotting time measured. As previously described (Fischer *et al.*, 1985), the anticoagulant activity is expressed as the number of thrombin NIH units inactivated by one milligram of polymer. Thrombin clotting times were compared with data obtained using H108 heparin from Choay Sanofi France (173 IU/mg). The anticoagulant activity of

heparin was found to express 3500 ThNIHu/mg in this assay.

NMR measurements

1D ^{13}C NMR spectra were recorded using a Bruker MSL-300 spectrometer at 75.47 MHz set on the inverse gated heteronuclear decoupled mode without NOE, operating in quadrature detection, using 10%(w/v) solutions in D_2O at 20°C and TMS (tetramethylsilane) as internal reference standard. About 30,000–60,000 scans were accumulated for each spectrum with a 6 s repetition time, 0.62 s acquisition time and a 90° ^{13}C pulse of 6.7 s. 1D ^{1}H and ^{2}D homonuclear and heteronuclear spectra were recorded using a Bruker AMX-400 instrument set at 50°C.

RESULTS AND DISCUSSION

Six carboxymethylated dextran samples (CMD) were prepared according to published procedure (Chaubet *et al.*, 1995; Mauzac and Jozefonvicz, 1984). The degree of substitution of carboxymethyl residues (CM) is varying from 0.10 to 0.80. Carboxymethylbenzylamide dextran samples (CMDB) and subsequent CMDBS were previously prepared by Mauzac and Jozefonvicz. The composition of all compounds is reported in Table 1.

NMR and kinetics studies

The ^{13}C NMR chemical shifts of the carbons of the glucosyl rings in dextran and in derivatives are gathered in Table 2.

Dextran

^{1}H and ^{13}C resonances of dextran samples were assigned from ^{1}H COSY spectra and 2D HETEROCOSY spectra, respectively, and when needed through comparison with the results from the literature on linear dextrans (Ramirez *et al.*, 1993, 1994; Arranz *et al.*, 1992; Cheethman *et al.*, 1991; Davis *et al.*, 1986, 1987; Seymour *et al.*, 1976, 1979a, 1979b, 1980; Meyer *et al.*, 1979).

In the case of ^{13}C NMR of dextran compounds the peak found at the lowest field i.e. at 98.9 ppm from TMS was assigned to the hemiacetal carbon C1 (Fig. 1) and that at the highest field (66.7 ppm) to the methylene carbon C6. The complex resonance pattern in the 69–76 ppm range (data not shown) arises from the four

Table 1. Composition and anticoagulant activity of the dextran derivatives

Samples	Sample composition (d.s.)			Specific anticoagulant activity (ThNIHu./mg)
	CM±0.01	B±0.01	S±0.01	±5
Dextrane	0	0	0	0
CMD	0.10	0	0	0
	0.17	0	0	0
	0.23	0	0	0
	0.44	0	0	0
	0.65	0	0	0
	0.80	0	0	0
CMDB[a]	0.37	0.21	0	0
CMDBS[a]	0.37	0.15	0.06	1
	0.45	0.00	0.05	20
	0.48	0.00	0.05	25
	0.58	0.05	0.06	18
	0.64	0.00	0.05	18
	0.60	0.14	0.05	16
	0.41	0.04	0.10	6
	0.43	0.04	0.11	23
	0.49	0.01	0.12	29
	0.50	0.03	0.10	22
	0.68	0.04	0.12	31
	0.72	0.00	0.10	40

a: see Fischer *et al.*, 1985; Mauzac and Jozefonvicz, 1984.

Table 2. ^{13}C NMR chemical shifts of unsubstituted dextran and mono- and disubstituted CMD

Unsubstituted dextran		Monosubstituted CMD		Disubstituted CMD	
Attributed carbon	Chemical shift (ppm)	Attributed carbon[a]	Chemical shift (ppm)	Attributed carbon[b]	Chemical shift (ppm)
C1	98.9	C1(2)	96.7	C1(2,3)	99.6
		C1(3)	99.6	C1(3,4)	99.2
C2	72.5	C2(2)	80.9	C2(2,4)	80.2
				C2(2,3)	80.0–80.9
C3	74.5	C3(2)	73.5	C3(2,3)	83.9
		C3(3)	84.8	C3(3,4)	83.9
C4	70.7	C4(3)	69.9	C4(2,3)	69.6
		C4(4)	80.5	C4(2,4)	80.0–80.9
				C4(3,4)	80.0–80.9
C5	71.3	—	—	—	—
C6	66.7	—	—	—	—

a: C*n*(*i*) = C*n* in a cycle bearing a carboxymethyl substituant on C*i*.
b: C*n*(*i*,*j*) = C*n* in a cycle bearing two carboxymethyl substituants on C*i* and C*j*.

remaining carbons of the sugar ring: C3 (74.5 ppm), C2 (72.5 ppm), C5(71.3 ppm) and C4(70.7 ppm). The chemical shifts of the differently substituted glucosyl ring are gathered in the Table 2. Spectra performed on unsubstituted dextran gave expected results in accordance with the literature mentioned above. Briefly: small peaks observed at 100.4 (one-unit side chain) and 100.7 ppm (two-unit side chain) reflect the existence of a α-1,3-linked-D-glucosyl residues attached to the α-1,6-D-glucosyl residues in the main chain. While those at 82.3 and 81.6 ppm were assigned to the C3 branching point (Ito and Schuerch, 1979). Resonances from the end groups under the α- and β-conformation were also detectable at 93.3 ppm and 97.2 ppm, respectively.

Carboxymethylated dextran

Figure 1 shows expanded regions of the inverse gated proton-decoupled ^{13}C NMR spectra recorded at 75.47 MHz of the CMD samples obtained at four different conversion degrees d.s. = 0.10, 0.23, 0.44 and 0.80.

For samples of low conversion the ring carbon region displays an intense decrease for signals assigned to the C1, C2 and C3 carbons in standard dextran. This occurs together with the appearance of a series of new signals mainly ranging from 78 to 100 ppm. It is known that the modification of OH groups in glucopyranosyl compounds entails both a strong downfield shift of the carbon bearing the OH group itself (α-effect) and an upfield shift of the adjacent carbon (β-effect) with respect to carbons with unsubstituted OH group (Ramirez *et al.*, 1993, 1994; Arranz *et al.*, 1982, 1987). Thus, by comparison to published data on both dextran compounds modified with either ethyl chloroformate (Arranz *et al.*, 1987) or chloroacetyl (Ramirez *et al.*, 1994) and also on modified cellulose, we assign the above peaks as follow: at 80.9 ppm to the carbons C2(2) (bearing a CM group) and thus reflecting an α-effect; at 96.7 ppm to the carbons C1(2) and at 73.5 ppm to the carbons C3(2) adjacent to substituted C2 carbons (reflecting a β-effect); at 84.8 ppm to the C3(3) carbons (α-effect), at 99.6 ppm to the C1(3) carbons (γ-effect), at 69.9 ppm to the C4(3) carbons (β-effect), and at 80.5 ppm to the C4(4) carbons (α-effect). In increasing the conversion the following signals either emerge or grow in intensity: at 80.2 ppm that may be assigned to the carbons C2(2,4) (disubstituted glucosyl ring), at 83.9 ppm, to C3(2,3) + C3(3,4); and the slight signal at 69.6 ppm to C4(2,3) (data not shown). The integration of carbon signals from 78 to 86 ppm does not change with conversion, probably because the carbon of the substituent groups contribute in the same region. The region from 66 to 75 ppm is of no value for a quantitative analysis because of the very poor signal resolution of the modified and unchanged ring carbons. The signal intensity of the carbons C1 (Fig. 2A) decreases with degree of conversion while on the contrary that of the carbons C1(2) increases; at d.s. about 0.3 and above, two broad peaks are observed at 97.5 ppm that may be assigned to the carbons C1(2,3) and C1(3,4) respectively. The signal at 83.9 ppm (C3(2,3) + C3(3,4)) continues to grow (Fig. 2B) and simultaneously the peak within the 79–82 ppm region broadens and its overall shape is modified. The 2D inverse HETERO-COSY spectra were useful for the analysis of this latter peak; it was concluded that the peak arising beside the C2(2) signal consists of overlapping C4(4), C2(2,4), C2(2,3), C4(2,4) and C4(3,4) peaks. It remained several other ^{13}C signals which can not be assigned correctly because of the poor resolution of the CMD ^{1}H NMR spectra.

The quantitative analysis of the CMD structure was performed through the computer deconvolution of NMR spectra and using the results of carboxymethylation kinetics and the derived kinetic scheme.

Rate coefficients of carboxymethylation and CMD structure

The analysis of the NMR data reveals that at the very beginning of the reaction the three possible mono-substitutions are possible; beyond at d.s. about 0.30–0.35 disubstituted units are then observed. However, within the broad range of conversions investigated (d.s < 0.8) no ring trisubstitution is allowed. Figure 3 represents the course of the reaction where D is an unsubstituted unit, Ci (i = 2, 3, 4) and Ci,j (i,j = 2, 3, 4) designate the mono- and disubstituted rings respectively; k_i (i = 2, 3, 4) and $k_{i,j}$ (i,j = 2, 3, 4) are the rate coefficients. For example k_{24} designates the substitution rate coefficient at C4 for a ring already monosubstituted at C2. A concentration change of all these compounds can be described with the following set of equations:

$$\mathrm{d\,D}/\mathrm{d}\tau = -(k_2 + k_3 + k_4)\mathrm{D} \quad (1)$$

$$\mathrm{d\,C2}/\mathrm{d}\tau = k_2\mathrm{D} - (k_{23} + k_{24})\mathrm{C2} \quad (2)$$

$$\mathrm{d\,C3}/\mathrm{d}\tau = k_3\mathrm{D} - (k_{32} + k_{34})\mathrm{C3} \quad (3)$$

$$\mathrm{d\,C4}/\mathrm{d}\tau = k_4\mathrm{D} - (k_{43} + k_{42})\mathrm{C4} \quad (4)$$

$$\mathrm{d\,C23}/\mathrm{d}\tau = k_{23}\mathrm{C2} + k_{32}\mathrm{C3} \quad (5)$$

$$\mathrm{d\,C24}/\mathrm{d}\tau = k_{24}\mathrm{C2} + k_{42}\mathrm{C4} \quad (6)$$

$$\mathrm{d\,C34}/\mathrm{d}\tau = k_{34}\mathrm{C3} + k_{43}\mathrm{C4} \quad (7)$$

where

$$\tau = \int_0^t [\mathrm{A}][\mathrm{B}]\mathrm{d}t \quad (8)$$

is a transformed time, [A] and [B] are the current concentrations of chloroacetic acid and NaOH, respectively.

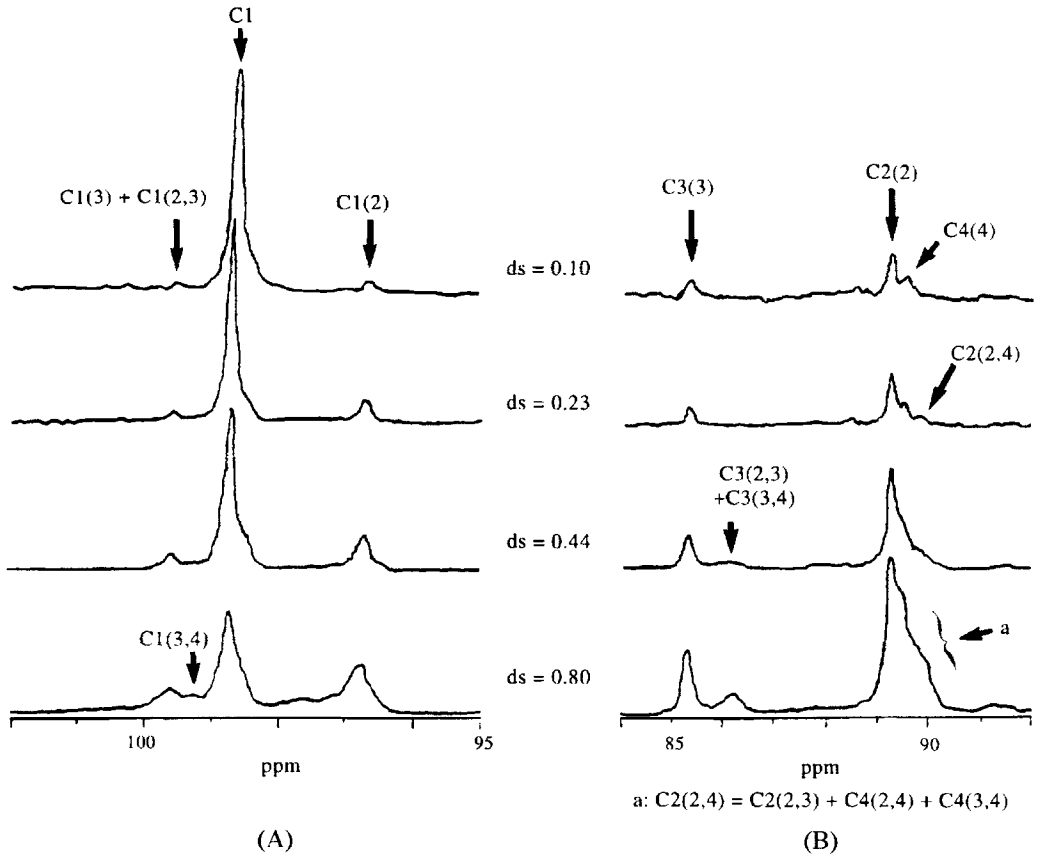


Fig. 2. ^{13}C 75.47 Mhz spectra of CMD of four conversions (d.s. + 0.10, 0.23, 0.44 and 0.80) in D_2O at 300 K in the two regions, A: 95 to 102 ppm and B: 78 to 86 ppm.

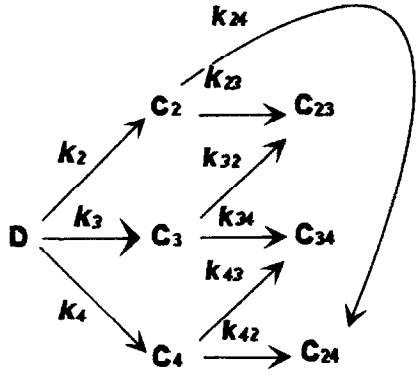


Fig. 3. Course of the reaction of carboxymethylation leading to mono and dicarboxymethylated derivatives from dextran, with corresponding rate coefficients (see text).

The NMR data used with Equations (1)–(8), allow both the rate coefficients and the CMD structure to be estimated. For NMR measurements eight samples of CMD were prepared under conditions of kinetic experiments. To estimate k_2, k_3 and k_4, we first considered the low conversion conditions (d.s < 0.30) where monosubstitution is prevailing. Secondly, an attempt to estimate the remaining eight rate coefficients was made using the obtained k_2, k_3 and k_4 values together with the NMR data for the CMD samples prepared at high conversions.

Kinetics of carboxymethylation at low conversions

The peaks at 96.7 ppm, C1(2), 84.8 ppm, C3(3), and 99.6 ppm, C1(3), were used to calculate the substitutions occurring at C2 and C3 positions. The poorly resolved signals at 80.9 ppm C2(2) and 80.5 ppm, C4(4), have been deconvolved using the computer program. The contents of the monosubstituted units in the five CMD samples (see Table 3) were estimated by dividing the corresponding peak areas by the integrated area of all the C1 peaks which remains unchanged during the conversion. These may be taken as an internal standard within the procedure. The quality of matching between the areas at C1(2) and C2(2) and also between the areas at C1(3) and C3(3) further indicated that the disubstitution was not proceeding significantly within the range of d.s. considered. The most probable values for the rate coefficients of monosubstitution were calculated using the data of Table 3 within Equations (1)–(4) as a result of the solution of the inverse kinetic task by a proper computer program (items containing rate coefficients of disubstitutions being omitted). Calculations were performed in terms of concentrations (mol/l) and τ (min.mol^{2}l^{-2}); initial D value being 0.75 mol/l. Calculations provided the following values ($\times 10^3$ mol^{-2} l^2 min^{-1}) $k_2 = 1.2$, $k_3 = 0.5$ and $k_4 = 0.8$.

Table 3. Composition of carboxymethylated Dextran at low conversions according to NMR data

t (min)	τ (min.mol^{2}l^{-2})	Numbers of monosubstituted units per 100 units					d.s.[a]
		C_2		C_3		C_4	
		C1(2) 96.7 ppm	C2(2) 80.9 ppm	C1(3) 99.6 ppm	C3(3) 84.8 ppm	C4(4) 80.5 ppm	
6	48	48	48	20	20	32	11
10	77	75	75	35	35	60	19
12	91	11	85	48	40	57	21
14	105	11	11	56	56	65	23
25	170	15	12	60	55	81	32

a: UV and chemical analysis data.

Table 4. Change vs time of areas of summative peaks in ^{13}C NMR spectra of carboxymethylated dextran

t (min)	τ (min.mol^{2}l^{-2})	Relative areas of peaks ($\times 10^2$)				
		C1(2) + C1(2,4) 96.7 ppm	C1(3) + C1(3,4) 99.6 ppm	C3(3) 84.8 ppm	C3(2,3) + C3(3,4) 83.9 ppm	C2(2) + C2(2,3) + C4(4) 80.0–81.5 ppm
6	48	48	20	20	0	8
10	77	75	35	35	0	15
12	91	11	48	40	0	17
14	105	11	56	56	0	19
25	170	15	60	55	0	28
40	239	19	91	80	20	35
100	445	28	12	11	47	53
200	659	33	15	12	60	57

Kinetics of carboxymethylation at high conversions

For these kinetics we used the following summative signals: C1(2) + C1(2,4) at 96.7 ppm; C1(3) + C1(3,4) at 99.6 ppm; C2(2) + C2(2,3) + C4(4) + 2C4(2,4) + C4(3,4) at 80–81.5 ppm; C3(2,3) + C3(3,4) at 83.9 ppm and also C3(3) at 84.8 ppm. The change in the relative areas of the above signals produced by conversion is presented in Table 4. Using Equations (1)–(7) and the k_2, k_3 and k_4 values calculated before, we searched for a set of six rate coefficients of disubstitution agreeing with the data of Table 4. This was done interactively with the computer. The initial values of all nine rate coefficients were estimated (see Table 5) from a detailed study of kinetics and NMR data analysis and their changes through conversion were then revealed and described quantitatively (Krentsel *et al.* 1997). Calculations using these results provided data fitting well NMR data presented on Fig. 4 in terms of concentrations and τ. The results were also used to perform Monte Carlo simulations (see next section).

Table 5. Initial values of the rate coefficients of dextran carboxymethylation ($\times 10^3$mol^{-2}.l^2.min^{-1})

k_2	k_3	k_4	k_{23}	k_{24}	k_{32}	k_{34}	k_{42}	k_{43}
1.2	0.50	0.80	0.25	0.56	0.60	0.40	0.84	0.25

Anticoagulant capacity and modelling

On Fig. 5 and Table 1 we show previous reported experimental results (Mauzac and Jozefonvicz, 1984) (except for CMD compositions) reflecting the specific anticoagulant activity of two series of CMDBS characterized by S content of 0.06 and 0.11 (d.s.), each series with different CM content ranging from d.s. = 0.37 to

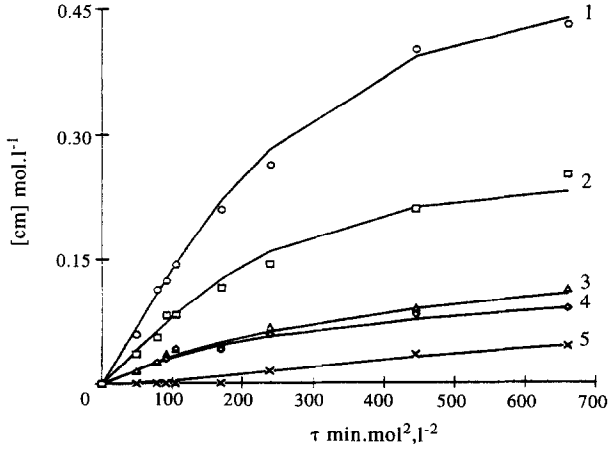


Fig. 4. Change of summative concentrations of various CM groups with transformed time; points—NMR data, curves—calculations for C2 + C4 + 2C2,4 + C2,3 + C3,4 (1); C2 + C2,4 (2); C3 + C3,4 (3); C3 (4) and C2,3 + C3,4 (5).

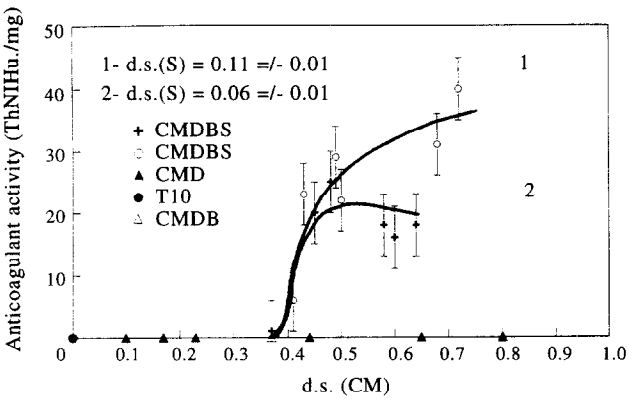


Fig. 5. Dependence of CMDBS anticoagulant activity on the content of CM and S groups.

0.72. Neither CMD, nor CMDB derivatives exhibit anticoagulant activity. Moreover the CMDBS do not reveal significant anticoagulant activity when their CM groups content is found below d.s. = 0.35–0.40; beyond this value the activity increases sharply to reach a maximum value for a CM content of d.s. = 0.72. At the same time, it is noted that the higher the S content, the higher the maximum of biological activity (Fig. 5). These peculiarities have to be taken into account when a search for possible active sites is undertaken. Using Monte Carlo simulations we modelled various sets of CMDBS chains in order to verify whether some short sequences of units of the functionalized polysaccharide might be active sites responsible for anticoagulant activity. The modelling approach involved the following: first, different OH groups are transformed into CM groups taking into account the results of the kinetics analysis mentioned above. Since benzylamdification proceeds randomly (see Section 1), any randomly chosen CM group may be transformed into B group with one and the same rate coefficient. It appeared also necessary to transform all B groups into S groups since some CMDBS samples not containing B groups exhibit anticoagulant activity (Mauzac and Jozefonvicz, 1984). Simulation was initiated by generating a set of dextran chains, each consisting of 50 units, as a two-dimensional array in the computer memory. The OH groups randomly chosen were transformed into CM ones with probabilities being proportional to the corresponding rate coefficients. The transformation of OH groups belonging to disubstituted units was considered as forbidden. Then a given number of CM groups chosen randomly was transformed directly into S groups with a probability equal to unity, independently of their type. Finally, we analyzed the distribution of the different units along the modified chains and calculated the number of chains containing at least one supposed active site. A good reproducibility of the results was achieved when at least 50 sets were tested, each consisting of 400 chains. Since during the dextran carboxymethylation the formation of disubstituted units started just at d.s. 0.30–0.35, it seemed natural to include such units in an assumed active site. A mono- or disubstituted unit bearing a S group appeared also a necessary constituent of active site. The existence of a maximum activity similarly requested the incorporation of D and/or monocarboxymethylated units in a supposed active site. We tested various triads, tetrads and pentads as possible active site, each consisting of D, monocarboxymethylated units CM1, dicarboxymethylated ones CM2, and sulfonated CM1 or CM2, respectively S1 or S2 (the latter containing one CM and one S groups) in all cases regardless of the position of the substituent. During the analysis of units distribution, a walk along the chain in both directions was used to account for both the sequence tested and the corresponding mirror image. Examples of Monte Carlo simulations are shown on Fig. 6, where F is a fraction of chains bearing at least one assumed active site. We note that the change of a single unit within the composition of an assumed active site already leads to a significant shift of calculated curves. From the totality of sequences tested, the pentad CM1DCM2CM1S1 (Fig. 6A) apparently deserves a preference. The calculated curves present similarities with the experimental ones, in their essential features: a threshold of CM value, a rise and a maximum belonging to almost the same CM region. Calculated and experimental curves, however, do not completely coincide. Several facts may explain these apparent discrepancies. In first, F and the anticoagulant activity are measured in different units. In second, parameters other than F must account for the complexity of the anticoagulant activity of polymers. Indeed, the presence of a thrombin binding oligosaccharide (i.e. as active site) able to catalyse interactions with its inhibitors is one of the prerequisites which account for CMDBS activity. As things stand now, a more adequate formulation of the requirements to qualify as active site can be reached by perfecting the studies on CMDBS composition vs anticoagulant activity, e.g. through an improvement of the synthetic aspects and structural determination.

The first simulations studies allow to conclude that anticoagulant properties of CMDBS, these being considered as statistical copolymers, might be, in principle, elucidated in terms of units distribution. The results of such an approach may be useful for experimental searches for the chain fragments contributing to the polymer bioactivity and consequently to protein interaction.

ACKNOWLEDGEMENTS

This work was supported by CNRS and INTAS (Project No 93-2276). We gratefully acknowledge Dr F. Pfluger for his help for the simulation work and Dr S. La Barre for his help during the writing of this work.

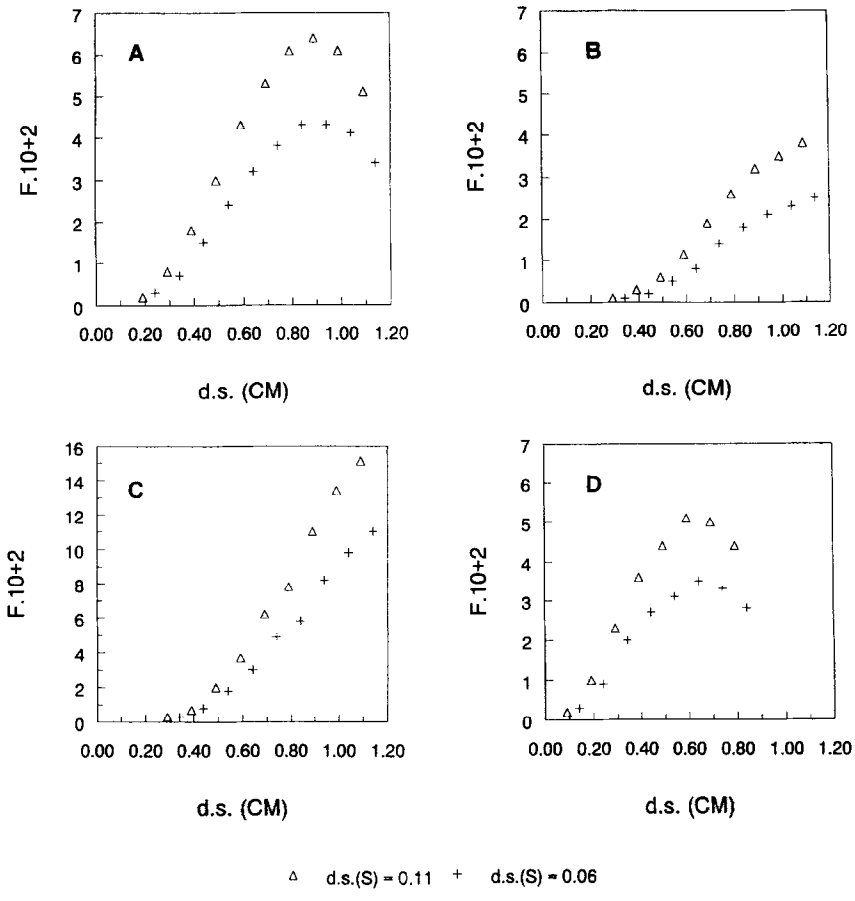


Fig. 6. Results of the Monte Carlo simulations. Dependence of fraction F of CMDBS chains containing at least one pentad on the contect of CM and S groups. A: CM1DCM2CM1S1; B: CM1DCM2CM1S2; C: CM1CM1CM2CM1S1; D: CM1DCM2DS1.

REFERENCES

Arranz, F., Roman, J.S. and Sanchez-Chaves, M. (1987) 13C NMR study of the selectivity in the modification of dextran with ethyl chloroformate. *Macromolecules* **20**, 801–806.

Arranz, F., Sanchez-Chaves, M. and Ramirez, J.C. (1992) Functionalization of dextran: incorporation of carboxy groups by O-succinoylation. *Angew. Makromol. Chem.* **194**, 79–89.

Bourrin, M.C. and Lindahl, U. (1993) Glycosaminoglycans and the regulation of blood coagulation. *Biochem. J.* **289**, 313–330.

Casu, B. (1985) Structure and biological activity of heparin. *Adv. Carbohydr. Chem. Biochem.* **43**, 51–132.

Chaubet, F., Champion, J., Maïga, O., Mauray, S. and Jozefonvicz, J. (1995) Synthesis and structure anticoagulant property relationships of functionalized dextrans: CMDBS. *Carbohydrate Polymers* **28**, 145–152.

Cheethman, N.W.H., Fiala-Beer, E. and Walker, G.J. (1991) Dextran structural details from high-field proton NMR spectroscopy. *Carbohydrate Polymers* **14**, 149–158.

Choay, J., Petitou, M., Lormeau, J.C., Sinaÿ, P., Casu, B., Gatti, G. (1983) Structure–activity relationship in heparin: a synthetic pentasaccharide with high affinity fo antithrombin III and eliciting high anti-factor Xa activity. *Biochem. Biophys. Res. Commun.* **116**, 492–499.

Crépon, B., Maillet, F., Kazatchkine, M.D. and Jozefonvicz, J. (1987) Molecular weight dependency of the acquired anticomplementary and anticoagulant activities of specifically substituted dextrans. *Biomaterials* **8**, 248–253.

Davis, H.M., Boyko, W.J. and Edwards, J.R. (1986) Molecular weight dependency of the acquired anticomplementary and anticoagulant activities of specifically substituted dextrans. *Carbohydr. Res.* **152**, 69–76.

Fischer, A.M., Mauzac, M., Tapon-Bretaudière, J. and Jozefonvicz, J. (1985) Anticoagulant activity of dextran derivatives. Part II: Mechanism of thrombin inactivation. *Biomaterials* **6**, 198–202.

Ito, H. and Schuerch, C. (1979) Anticoagulant activity of dextran derivatives. Part II: Mechanism of thrombin inactivation. *J. Am. Chem. Soc.* **101**, 5797–5806.

Jozefonvicz, J. and Jozefowicz, M. (1992) Bioactive specific biomaterials: present and future. *Pure and Appl. Chem.* **64**, 1783–1788.

Krentsel, L.B., Ermakov, I.V., Yashin, V.V., Rebrov, A.I., Litmanovich, A.D., Plate, N.A., Chaubet, F., Champion, J. and Jozefonvicz, J. (1997) Kinetic features of the carboxymethylation of dextran and the structure of the reaction product. *Polymer Science, Ser. A.* **39**(1), 74–80.

Jozefonvicz, J. and Jozefowicz, M. (1990) Interaction of biospecific functional polymers with blood proteins and cells. *J. Biomater. Sci. Polymer Edn.* **1**, 147–165.

Letourneur, D., Logeart, D., Avramoglou, T. and Jozefonvicz, J. (1993) Antiproliferative capacity of synthetic dextrans on smooth muscle cell growth: the model of derivatized dextrans as heparin-like polymers. *J. Biomater. Sci. Polymer Edn.* **4**, 31–434.

Letourneur, D., Champion, J., Slaoui, F. and Jozefonvicz, J. (1993) *In vitro* stimulation of human endothelial cells by derivatized dextrans. *In Vitro Cell. Dev. Biol.* **29A**, 67–72.

Lindhal, U., Bäckström, G., Thunberg, L., Leder, I. (1980) Evidence for a 3-O-sulfated-D-glucosamine residue in the antithrombin-binding sequence of heparin. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6551–6555.

Logeart, D., Avramoglou, T. and Jozefonvicz, J. (1994) Inhibitory effect of functional dextrans on rat arterial smooth muscle cell growth. *Colloids and Surfaces B: Biointerfaces* **2**(1994), 315–320.

Mauzac, M., Maillet, F., Jozefonvicz, J. and Kazatchkine, M.D. (1985) Anticomplcmentary activity of dextran derivatives. *Biomaterials* **6**, 61–63.

Mauzac, M. and Jozefonvicz, J. (1984) Anticoagulant activity of dextran derivatives. Part I: Synthesis and characterization. *Biomaterials* **5**, 301–304.

Meyer, S., Lamberts, B.L. and Egan, R.S. (1979) Anticoagulant activity of dextran derivatives. Part I: Synthesis and characterization. *Carbohydr. Res.* **66**, 33–42.

Ramirez, J.C., Sanchez-Chaves, M. and Arranz, F. (1994) Functionalization of dextran with chloroacetate groups: immobilization of bioactive carboxylic acids. *Polymer* **35**, 2651–2655.

Ramirez, J.C., Sanchez-Chaves, M. and Arranz, F. (1993) Dextran functionalized by cyclic carbonate groups as support of bioactive compounds. *Angew. Makromol. Chem.* **206**, 77–85.

Seymour, F.R., Knapp, R.D. and Bishop, S.H. (1976) Determination of the structure of dextran by 13C-nuclear magnetic resonance spectroscopy. *Carbohydr. Res.* **51**, 179–194.

Seymour, F.R., Knapp, R.D., Bishop, S.H. and Jeanes, A. (1979) High-temperature enhancement of 13C-N.M.R. chemical-shifts of unusual dextrans, and correlation with methylation structural analysis. *Carbohydr. Res.* **68**, 123–140.

Seymour, F.R., Knapp, R.D. and Bishop, S.H. (1979) Correlation of the structure of dextrans to their 1H-N.M.R. spectra. *Carbohydr. Res.* **74**, 77–92.

Seymour, F.R., Knapp, R.D. (1980) Structural analysis of dextrans, from strains of leuconostoc and related genera, that contain 3-O-α-D-glucosylated α-D-glucopyranosyl residues at the branch points, or in consecutive, linear positions. *Carbohydr. Res.* **81**, 105–129