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EFFECT OF SULFATED DERIVATIVES OF CHITOSAN ON SOME BLOOD COAGULANT FACTORS*

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ABSTRACT

The effect of some sulfated derivatives of chitosan on several blood-coagulant factors was examined in comparison with those of heparin (174 units/mg). The anticoagulant activity (units/mg) with respect to activated partial thromboplastin time was 331–379 for *O*-sulfated *N*-acetylchitosan (**2**), 190–287 for *N*,*O*-sulfated chitosan (**1**), and 21–31 for sulfated *O*-carboxymethylchitosan (**3**). The activity (units/mg) with respect to thrombin time was 70–87 for **2**, 44–70 for **1**, and 14–22 for **3**. The activity (units/mg) with respect to antithrombin activity was 4.9–9.0 for **2**, 4.7–8.7 for **1**, and 3.3–6.0 for **3**. No anti-(factor Xa) activity was observed with **1**–**3**. A 6-sulfate group in the hexosaminyl moiety was a main active site; although a 3-sulfate group was not essential, it promoted the activity of the 6-sulfate group. *N*-Sulfate was not a prerequisite for activity. The biological activities were also related to molecular weight in the sequence **2** (26,000) > heparin (21,000) > **1** (12,000) > **3** (540,000). For the Methylene Blue complexes, **1**, but neither **2** nor **3**, exhibited a negative, induced Cotton effect similar to that of heparin.

INTRODUCTION

Chitosan, an unbranched (1→4)-linked 2-amino-2-deoxy-β-D-glucan, is prepared by chemical *N*-deacetylation of chitin, which is the main structural element of the cuticles of crab, shrimp, and insects, and in the cell walls of bacteria. Sulfated derivatives of chitosan possess anticoagulant activity[1,2]. Whereas *O*-sulfated chitin has ~25% of the activity of heparin[3], *N*-sulfated chitosan has no activity[4]. Conversion of position 6 into a carboxyl group in *N*-sulfated chitosan gives a product with ~23% of the activity of heparin[5], and its *O*-sulfated derivative exhibits ~45% activity[6]. *O*-Sulfated *N*-carboxymethyl(CM)chitosan exhibits ~45% activity[7]. *N*,*O*-Sulfated chitosan has a low toxicity in mice[1] (LD_{50} 1.25–3.25 g/kg, *cf.* LD_{50} 1.50–2.00

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g/kg for heparin[8]). When position 6 of *N*-sulfated chitosan is converted into carboxyl, the product is much more toxic (LD_{50} 0.237 g/kg)[5].

Little is known about structure–activity relationships for sulfated derivatives of chitosan in relation to the coagulant factors. We now report on the effects of *N,O*-sulfated chitosan (**1**), *O*-sulfated *N*-acetylchitosan (**2**), and sulfated *O*-carboxymethyl(*O*-CM)chitosan (**3**) on four blood-coagulant factors.

EXPERIMENTAL

Chitosan. — Flonac-N (commercial chitosan of crab shell, Kyōwa Yushi Co., Chiba) was treated with aqueous 40% NaOH containing $NaBH_4$ (0.1 g/500 mL) at 110° for 5 h. The product had $[\alpha]_D^{20}$ −7° (*c* 1, aqueous 2% acetic acid), and a negligible signal for NAc at ~2 p.p.m. in the ^{1}H-n.m.r. spectrum (D_2O–DCO_2D, 9:1); the elemental analysis data agreed within 0.3% error with the theoretical value of the completely *N*-deacetylated product [C/N ratio 6.07 (calc. 6.00)].

N,O-*Sulfated chitosan* (**1**). — Chitosan, previously swollen with *N,N*-dimethylformamide, was treated with *N,N*-dimethylformamide–sulfur trioxide[9], and the resulting product was isolated (79%) as the sodium salt, $[\alpha]_D^{14}$ −11° (*c* 0.5, water); ν_{max}^{KBr} 3600–3400 (OH, NH), 1250 (S=O), 1080–1000 (C–O–C), and 810 cm^{-1} (eq. C–O–S).

Anal. Calc. for $[C_6H_8NO_4(SO_3Na)_{2.63}(H)_{0.37} \cdot 1.60\ H_2O]_n$: C, 15.72; H, 2.54; N, 3.06; S, 18.4. Found: C, 15.44; H, 2.69; N, 3.10; S, 18.7.

The derivative (d.s. for sulfate, 1.32–1.80) has also been prepared by treatment of chitosan with chlorosulfonic acid[1,2].

O-*Sulfated* N-*acetylchitosan* (**2**). — *N*-Acetylchitosan (d.s. for *N*-acetyl, 1.0)[10] was sulfated as for **1**, and the resulting sodium salt of **2** (86%) had $[\alpha]_D^{18}$ −31° (*c* 1.2, water); ν_{max}^{KBr} 3600–3400 (OH, NH), 1650, 1560 (C=O and NH of NAc), 1240 (S=O), 1080–1000 (C–O–C), and 800 cm^{-1} (eq. C–O–S).

Anal. Calc. for $[C_8H_{11}NO_{11}S_2Na(H)_{0.45}(Na)_{0.54} \cdot 2.20\ H_2O]_n$: C, 22.00; H, 3.66; N, 3.21; S, 14.7. Found: C, 22.02; H, 3.98; N, 3.18; S, 14.7.

O-Sulfated chitin (d.s. for sulfate, 2.0) has been reported[3,11].

Sulfated O-*CM-chitosan* (**3**). — *O*-CM-chitosan (d.s. for CM, 0.9), $[\alpha]_D^{16}$ −7° (*c* 0.45, aqueous 5% NaOH) was prepared from *N*-acetylchitosan[12] and sulfated as for **1**. The resulting sodium salt of **3** (75%) had $[\alpha]_D^{14}$ −13° (*c* 0.5, aqueous 5% NaOH); ν_{max}^{KBr} 3600–3200 (OH, NH), 1590, 1410 (CO_2^-), 1250 (S=O), 1160–1000 (C–O–C), and 800 cm^{-1} (eq. C–O–S).

Anal. Calc. for $[C_6H_8NO_4(C_2H_2O_2Na)_{0.88}(SO_3Na)_{0.58}(H)_{1.54} \cdot 1.49\ H_2O]_n$: C, 30.71; H, 4.74; N, 4.61; S, 6.1. Found: C, 30.90; H, 5.04; N, 4.65; S, 6.4.

O-*Sulfated chitosan* (**4**). — A solution of *O*-sulfated chitosan (1.0 g) in 0.04M HCl (60 mL) was kept[13] at 100° for 2.5 h, and the product (0.5 g) was isolated as the sodium salt, $[\alpha]_D^{21}$ −6° (*c* 0.5, water); ν_{max}^{KBr} 3450 (OH, NH), 1630 (NH_2), 1250 (S=O), 1080–1000 (C–O–C), and 810 cm^{-1} (eq. C–O–S).

Anal. Calc. for $[C_6H_9NO_4(C_2H_3O)_{0.21}(SO_3Na)_{0.80}(SO_3H)_{0.32} \cdot 2.29\ H_2O]_n$: C,

24.16; H, 4.81; N, 4.88; S, 11.3. Found: C, 24.16; H, 4.81; N, 4.47; S, 11.3.

Sulfated, partially N-*acetylated chitosan* (**5**). — Partially *N*-acetylated chitosan (d.s. for NAc, 0.2)[14] was sulfated as for **1**, and the product (81%; d.s. for sulfate, 1.8) was isolated as the sodium salt, $[\alpha]_D^{21}$ −8° (*c* 1, water); ν_{max}^{KBr} 3500 (OH, NH), 1650, 1450 (C=O and NH of NAc), 1250 (S=O), 1700–1000 (C–O–C), and 800 cm^{-1} (eq. C–O–S).

General methods. — I.r. spectra were recorded with a Hitachi 215 grating spectrometer, ^{1}H-n.m.r. spectra with a Hitachi R-24 spectrometer, and optical absorption with a Hitachi 100-50 spectrometer. C.d. spectra were recorded with a Jasco ORD/UV-5 recorder, equipped with a CD apparatus. The absorption and induced Cotton effect were analysed (500–700 nm) after adding each sulfated derivative to dye solutions at various molar ratios of polyanionic site (P) and cationic dye (D)[15]. The molar absorptivity ε and molar ellipticity $[\theta]$ are expressed on the basis of dye concentration. Specific rotations were measured with a Jasco Dip-180 polarimeter. Sulfur content was calculated from the sulfate analysis by the barium chloroanilate method[16].

Molecular weight. — The molecular weights of **1–3** and heparin were estimated by gel chromatography on two columns of Bio-Gel P-300 (fine; 1.6 × 46.0 cm) and Toyopearl HW-65 (fine; 1.6 × 74.0 cm). Each column was equilibrated and eluted with 0.3M NaCl at room temperature. A calibration curve was obtained by analysis of relationship between Ve/Vo and the log of the molecular weights of standard pullulans (mol. wts. 853,000, 186,000, 100,000, and 12,200, Shodex) and dextrans (mol. wts. 40,100, 25,000, and 17,100; gifts from Professor K. Kawahara, Nagasaki University). The eluates were monitored by the phenol–sulfuric acid method[17] for pullulans and dextrans, the orcinol method[18] for heparin, and u.v. absorption at 194 nm for **1–3**.

Biological activities. — The anticoagulant activity was determined for human plasma with respect to activated partial thromboplastin time (APTT)[19], thrombin time (TT) using an aggrego-meter (Rika Denki Co., Tokyo)[20], antithrombin activity (AA) with a Testzyme®-AT-III kit using Phe–Pip–Arg–*p*NA (S-2238) as substrate (Dai-ichi Kagaku Yakuhin Co., Tokyo)[21], and anti(factor Xa) activity with a Testzyme®-Heparin kit using Bz–Ileu–Gly–Arg–*p*NA (S-2222) as substrate (Dai-ichi Kagaku Yakuhin Co., Tokyo)[22]. These activities were expressed as units/mg in relation to that of porcine intestinal heparin (174 units/mg, Lot No. CH-108M, Mitsui Seiyaku Kōgyō Co., Tokyo) as a standard.

RESULTS

Sulfated derivatives of chitosan. — The sulfation of *N*-acetylchitosan (1.0 g) was examined by three conventional methods involving conc. sulfuric acid[23] {0.72 g of product, d.s. for sulfate 1.1, $[\alpha]_D^{13}$ −20° (*c* 0.8, water)}, chlorosulfonic acid[24] {1.39 g of product, d.s. 1.9, $[\alpha]_D^{13}$ −21° (*c* 1, water)}, and *N*,*N*-dimethylformamide–sulfur trioxide[9] {1.8 g of product, d.s. 2.0, $[\alpha]_D^{18}$ −31° (*c* 1.2, water)}. The

TABLE I

METACHROMATIC BANDS AND INDUCED COTTON EFFECTS OF THE METHYLENE BLUE COMPLEXES WITH SULFATED DERIVATIVES OF CHITOSAN

Compound	*D.s.*		*P/D*[a]	*Metachromatic band* ($\lambda_{max}^{H_2O}$, *nm*)	*Induced Cotton effects*[b]	
	Sulfate	N-*Acetyl*			*λ (nm)*	$[\theta] \times 10^4$
1	2.6	0.0	1.5	561	567	−14
2	2.0	1.0	1.5	563		none
3	1.5[c]	0.0	1.2	562		none
4	1.1	0.2	1.5	561		none
5	1.8	0.2	1.2	557	567	−14
Heparin			1.5	566	569	−7

[a]Molar ratio of polymer anionic site (P) to Methylene Blue (D), [b]Molar ellipticity based on Methylene Blue. [c]Involving sulfate (0.6) and CM (0.9).

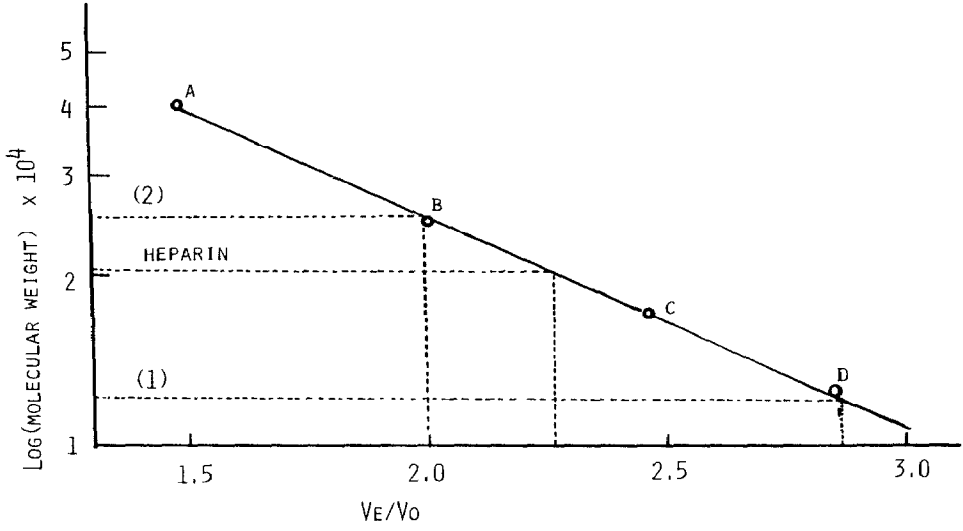


Fig. 1. Analysis of average molecular weight of **1, 2,** and heparin by gel filtration on a column (1.6 × 46.0 cm) of Bio-Gel P-300 (fine) by elution with 0.3M NaCl. The calibration curve was obtained by plotting Ve/Vo against the log of the molecular weight of standard saccharides (A, 40,100; B, 25,000; C, 17,000; and D, 12,000). The estimated molecular weights were 12,000 for **1**, 26,000 for **2**, and 21,000 for heparin

last method was superior in relation to d.s. for sulfate and yield of product, and was used to prepare **1–3** (Table I) and also **4** and **5**. The products were isolated as the sodium or sodium acid salts in yields of up to 86%. Each sulfated derivative had i.r. absorptions at 1240–1250 (S=O) and 800–810 cm^{-1} (eq. C–O–S). The d.s. value for *N*-acetyl was 1.0 in **2** and 0.2 in **5**, but the content of *N*-acetyl groups was negligible in **1, 3,** and **4**. The d.s. values for sulfate group were 2.6 in **1**, 2.0 in **2**, 1.8 in **5**, 1.1 in **4**, and 0.6 in **3** (*O*-CM, d.s. 0.9).

Distribution of sulfate groups in the hexosaminyl moiety was determined on

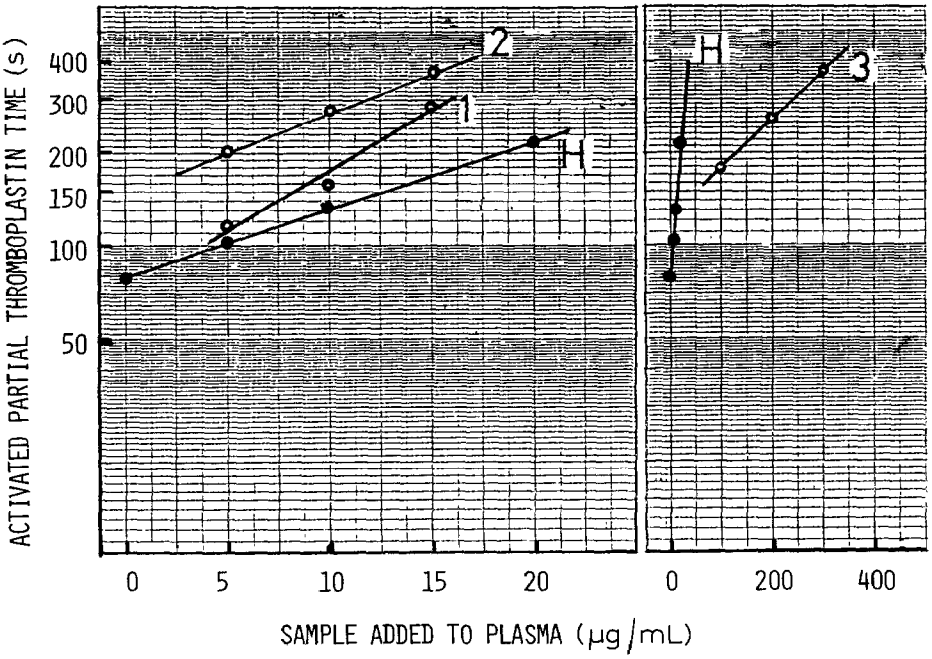


Fig. 2. Anticoagulant activities of **1–3** with reference to heparin (H) and APTT.

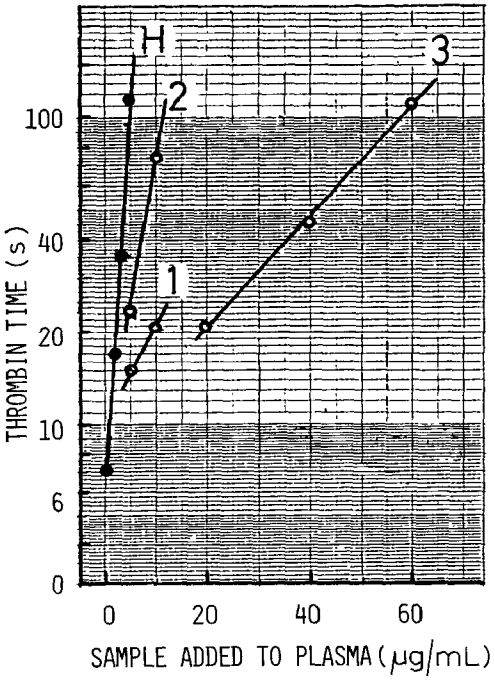


Fig. 3. Anticoagulant activities of **1–3** with reference to heparin (H) and TT

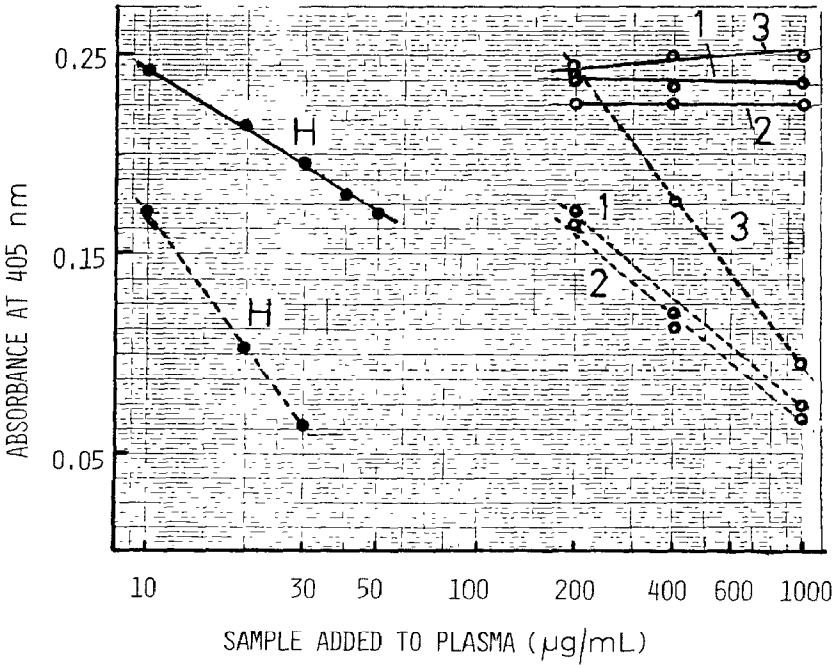


Fig. 4 Activities of **1–3** with reference to heparin (H) and AA(------) and anti(factor Xa) activity(——).

the basis of the reactivity sequence 6-sulfate > 3-sulfate, elemental and sulfate analyses, and i.r. spectra. Thus, **1** contained *N*- (d.s. 1.0), 6- (d.s. 1.0), and 3-sulfate (d.s. 0.6), **2** contained 6- (d.s. 1.0) and 3-sulfate (d.s. 1.0), **3** contained *N*-sulfate (d.s. 0.6), **4** contained 6- (d.s. 1.0) and 3-sulfate (d.s. 0.1), and **5** contained *N*- (d.s. 0.8) and 6-sulfate (d.s. 1.0).

As shown in Fig. 1, the average molecular weights of **1**, **2**, and heparin were estimated to be 12,000, 26,000, and 21,000, respectively, by gel filtration on Bio-Gel P-300, and that of **3** to be 540,000 by gel chromatography on Toyopearl HW-65.

Biological activities of the sulfated derivatives. — *(a) On APTT.* As shown in Fig. 2, a straight line was observed by plotting, semilogarithmically, APTT (log) *versus* each sulfated derivative (μg/mL). APTT was significantly prolonged with **2**, and its activity at 5–15 μg/mL was 1.9–2.2 times that of heparin. APTT was also prolonged with **1**, and its activity was 1.1 (5 μg/mL), 1.2 (10 μg/mL), and 1.6 (15 μg/mL) times that of heparin. APTT was slightly prolonged with **3**, and its activity at 100–300 μg/mL was 20–30% of that of heparin. The anticoagulant activity (units/mg) with respect to APTT was 331–379 at 5–15 μg/mL for **2**, 190–287 at 5–15 μg/mL for **1**, and 21–31 at 100–300 μg/mL for **3**. The plotted line of APTT for **2** was almost parallel to that of heparin, but those of **1** and **3** were not parallel.

(b) On TT. — A straight line was observed by plotting, semilogarithmically, TT (log) *versus* each sulfated derivative (μg/mL) as shown in Fig. 3. TT was prolonged with **2**, and its activity was 40% (5 μg/mL) and 50% (10 μg/mL) of that of

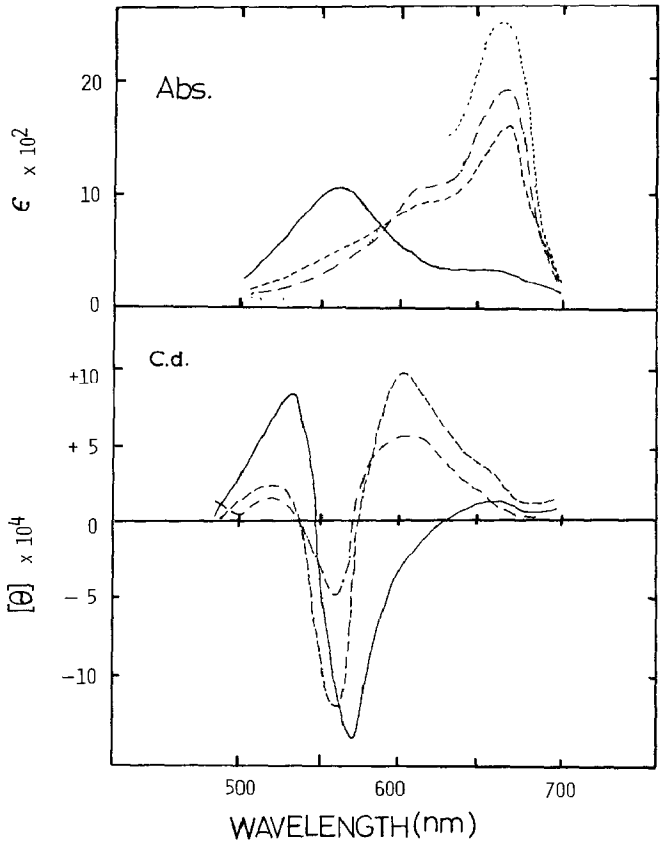


Fig. 5. Metachromatic bands and negative, induced Cotton effects of the Methylene Blue complexes with **1** at various P/D ratios obtained by adding **1** to a solution of Methylene Blue. The top panel shows the absorptions, and the bottom panel shows the c.d. spectra (500–700 nm). Molar absorptivity ε and molar ellipticity $[\theta]$ are based on Methylene Blue. The metachromatic band appeared at 557 nm. The negative, induced Cotton effect appeared at 557 nm with $[\theta]$ -6×10^4 at P/D 0.2 (— · —), at 560 nm with $[\theta]$ -12×10^4 at P/D 0.4 (------), at 567 nm with $[\theta]$ -14×10^4 at P/D 1.2 and 0.8 (——), and Methylene Blue (· · · · · ·).

heparin. TT was prolonged with **1**, and its activity was 30% (5 μg/mL) and 40% (1.0 μg/mL) of that of heparin. TT was also prolonged with **3**, and its activity was 10% (20 μg/mL), 20% (40 μg/mL), and 30% (60 μg/mL) of that of heparin. The anticoagulant activity (units/mg) with respect to TT was 70–87 at 5–10 μg/mL for **2**, 44–70 at 5–10 μg/mL for **1**, and 14–22 at 20–60 μg/mL for **3**. The plotted lines of TT were not parallel to that of heparin.

(c) On AA and anti(factor Xa) activity. — As shown in Fig. 4, a straight line was observed by plotting the absorption of released *p*-nitroaniline (*p*NA) at 404 nm *versus* each sulfated derivative [log(μg/mL)]. AA was 3–5% of that of heparin. The biological activity (units/mg) at 200–1,000 μg/mL was 4.9–9.0 for **2**, 4.7–8.7 for **1**, and 3.3–6.0 for **3**. However, no anti(factor Xa) activity was observed even at 200–1,000 μg/mL.

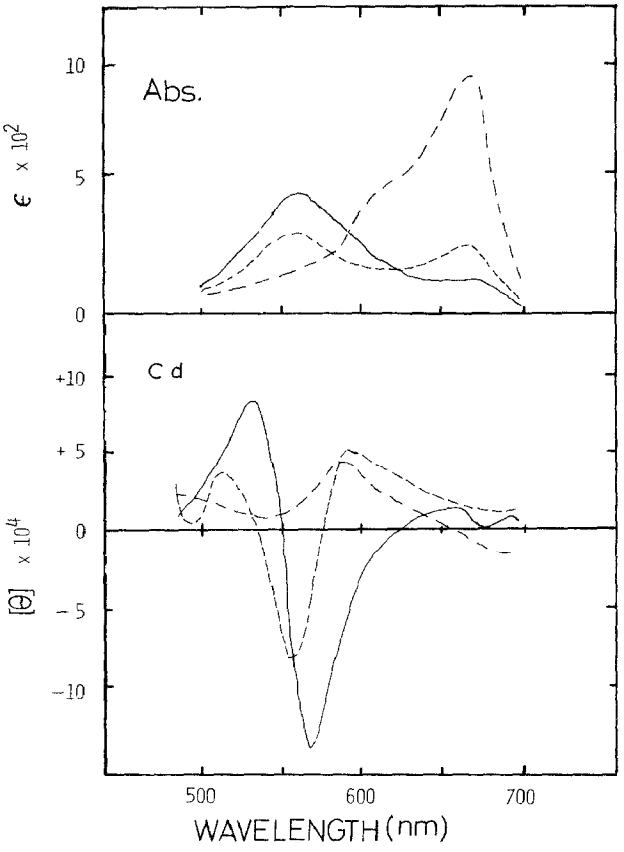


Fig. 6. Effects of urea on the metachromatic bands and the induced Cotton effects of the Methylene Blue complexes with **1**. The top panel shows the absorptions and the bottom panel shows the c.d. spectra of the Methylene Blue complexes with **1** (P/D 1.5) in water (——) with $[\theta]$ -14×10^4 at 565 nm, in 2M urea (------) with $[\theta]$ -8×10^4 at 552 nm, in 7M urea (— · —).

Metachromatic absorption of the dye complexes of the sulfated derivatives. — Each sulfated derivative exhibited unique metachromasy with cationic dyes, as indicated by the shift of λ_{max} from 665 to 557 nm with Methylene Blue, from 492 to 477 nm with Acridine Orange, and from 635 to 534 nm with Toluidine Blue. The top panel in Fig. 5 shows the molar absorption of Methylene Blue and its complex with **1** at P/D 0.2, 0.4, and 1.2. The maximum absorbance appeared at P/D 1.2–1.5 with Methylene Blue and at P/D 0.7–1.0 with Acridine Orange and Toluidine Blue.

Induced Cotton effects of the Methylene Blue complexes. — The bottom panel in Fig. 5 shows the induced Cotton effects of Methylene Blue complexes with **1** at P/D 0.2, 0.4, and 1.2 in the 500–700 nm range. The induced Cotton effects shifted slightly to shorter wavelength with increasing P/D up to 1.5, giving the maximum molar ellipticity ($[\theta]$ -14×10^4) at 557 nm. As shown in Table I, an analogous, negative, induced Cotton effect was observed in the 557–567 nm range with **1, 5,**

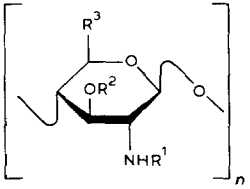
and heparin. A noticeable induced Cotton effect was not observed with **2** and **3** in the 500–700 nm range.

As shown in Fig. 6, addition of urea to the Methylene Blue complex of **1** resulted in lowering of both the molar absorptivity and ellipticity. The induced Cotton effect weakened in 2M urea and disappeared in 7M urea, indicating dissociation of the bound dye or changes in the conformation.

DISCUSSION

The biological activities were in the sequence **2** > **1** > **3** with respect to APTT, TT, and AA. These sulfated derivatives of chitosan did not inhibit factor-Xa activity. The pattern of these biological activities is similar to that of dermatan sulfate[25–28], but differs from that of heparin[28–31]. Table II summarises the structure–activity relationships for the sulfated derivatives of chitosan. It is considered

TABLE II
STRUCTURE–ACTIVITY RELATIONSHIPS



Derivative (Mol. wt.)	*R¹*	*R²*	*R³*	*Activity*[a]	*Ref.*
1 (12,000)	SO_3Na	H, SO_3Na	CH_2OSO_3Na	110–160[b]	
				30–40[c]	
				3–5[d]	
				0[e]	
				14–52[f]	1
				40[f]	2
2 (26,000)	Ac	SO_3Na	CH_2OSO_3Na	190–220[b]	
				40–50[c]	
				3–5[d]	
				0[e]	
				25[f]	3, 11
3 (540,000)	SO_3Na	H	$CH_2OCH_2CO_2Na$	20–30[b]	
				10–30[c]	
				3–5[d]	
				0[e]	
	SO_3Na	H	CH_2OH	0[f]	4
	SO_3Na	H	CO_2Na	23[f]	5
	SO_3Na	SO_3Na	CO_2Na	45[f]	6
	CH_2CO_2Na	H	CH_2OSO_3Na	45[f]	7

[a]Based on the anticoagulant activity of heparin (174 units/mg = 100). [b]Activated partial thromboplastin time. [c]Thrombin time. [d]Antithrombin activity. [e]Anti(factor Xa) activity. [f]USP assay.

that a 6-sulfate group in the hexosaminyl moiety is a main active site, and there is no activity when it is absent[4]; the activity is maintained more or less when position 6 is CO_2H or $CH_2OCH_2CO_2H$. A 3-sulfate group in the hexosaminyl moiety promotes the activity when position 6 is an acidic group, but it is not essential. The important contribution of the *N*-sulfate group to the activity has been pointed out[1,2], but its presence is not a prerequisite for the activity, as demonstrated in the present study with respect to APTT. In fact, dextran sulfate, which has no hexosaminyl moiety, has anticoagulant activity[32], and the *N*-acetyl group is a minor component of heparin[33]. It is also clear that molecular weight is an important factor for the anticoagulant activity, as reflected by the sequence of activities **2** (26,000) > heparin > **1** (12,000) > **3** (540,000) with respect to APTT, TT, and AA.

The induced Cotton effect in the metachromatic band of Methylene Blue complexes depends on the stereospecific helical array of acidic groups present in polymers and has been demonstrated with right- and left-handed polypeptides[34]. The negative, induced Cotton effect of the Methylene Blue complexes with **1** and **5** is analogous to those of heparin[35] and poly(D-glutamic acid)[34]. Derivatives **2–4** exhibited no induced Cotton effect in the c.d. spectra, indicating the absence of the stereospecific array of acidic groups. No apparent correlation between the induced Cotton effect and the biological activity was found. This finding indicates that the biological activity is not due to the stereospecific array of sulfate groups in the whole polymer, but is due to specific sulfate groups in a small region of the polymer, as observed with heparin[36–39].

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