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RATE, MECHANISM, AND IMMUNOCHEMICAL STUDIES OF LACTONISATION IN SEROGROUP B AND C POLYSACCHARIDES OF *Neisseria meningitidis**

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ABSTRACT

Meningococcal Serogroup B polysaccharide and colominic acid, which are (2→8)-α-linked homopolymers of sialic acid, undergo lactonisation at low pH at a rate which is dependent upon the molecular size and upon the salt form (Na^+ or Ca^{2+}). Meningococcal Serogroup C polysaccharide, a (2→9)-α-linked homopolymer of sialic acid with acetyl groups present at O-7 and/or O-8, reacts with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to give an *O*-acylisourea. The rate of formation of *O*-acylisourea does not differ substantially between *O*-acetylated (*O*-Ac^+) C, non-*O*-acetylated (*O*-Ac^-) C, and B polysaccharide††. ^{13}C-N.m.r. spectroscopy shows that, in the absence of *O*-acetyl groups, the majority of the activated carboxyl groups of C polysaccharide condense with an adjacent HO-8 to form a δ-lactone. Immunochemical studies show that the antigenicity of B polysaccharide is markedly reduced on lactonisation of <20%, as measured by a radioimmunoassay using an anti-B monoclonal antibody, and that low-molecular-weight colominic acid is poorly antigenic both before and after lactonisation, suggesting the presence of conformational determinants on B polysaccharide. In contrast, lactonisation and/or formation of *O*-acylisourea groups in the (*O*-Ac^+)-C polysaccharide does not cause a significant decrease in the antigenicity, which is consistent with a sequential (structural) determinant on the molecule.

INTRODUCTION

Neisseria meningitidis is a major cause of meningitis in humans, with >90% of the disease attributable to Serogroups A–C. The capsular polysaccharides from Serogroups B and C are homopolymers of sialic acid linked (2→8)-α and (2→9)-α, respectively[1], and which have different capacities for lactonisation[2]. At low pH or with a water-soluble carbodiimide, B polysaccharide forms a fully lactonised

*Capsular Polysaccharides of *Neisseria meningitidis*, Part II. For Part I, see ref. 3.

††*O*-Ac^+ and *O*-Ac^- connote *O*-acetylated and non-*O*-acetylated, respectively.

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polymer by condensation of the CO_2H group of one residue with HO-9 of an adjacent residue to give a six-membered ring[2]. Conversely, C polysaccharide does not undergo significant lactonisation unless treated with a carbodiimide. Moreover, the *O*-acetyl groups at C-7 and/or C-8 of most strains of C polysaccharide prevent lactonisation, thus implicating HO-7 and/or HO-8 in this process. We now report on the structure, rate, and mechanism of lactone formation in B and C polysaccharides.

The potential importance of lactonisation in B and C polysaccharides can be demonstrated in terms of the relative immunogenicity of the two polymers. Thus, modification of <10% of sialic acid residues in B polysaccharide is sufficient to reduce considerably its antigenicity[2], and *in vivo* lactonisation may help to explain its poor immunogenicity in humans. In contrast, C polysaccharide, which is less able to undergo lactonisation, is a relatively good immunogen. These facts are compatible with the hypothesis[3] that antibodies against B polysaccharide are directed against determinants present in the secondary or tertiary structure of the polymer (*i.e.*, conformational antibodies), whereas antibodies against C polysaccharide recognise a sequential (*i.e.*, structural) determinant. Further evidence in support of this hypothesis is now presented.

EXPERIMENTAL

Materials. — *N. meningitidis* serogroup B, (*O*-Ac$^-$)-C, and (*O*-Ac$^+$)-C polysaccharides (*O*-acetyl content, 1.7 μmol/mg) were prepared from strains CN 7630, CN 7869, and CN 7038, respectively, and purified as previously described[4]. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC), poly-L-lysine, chondroitin sulphate, bovine haemoglobin, methanesulphonic acid, and colominic acid were commercial products.

General methods. — Sialic acid was determined by the Svennerholm method[5], and counter-current immunoelectrophoresis (c.i.e.) was performed as previously described[2].

Spectroscopic methods. — The ^{13}C-n.m.r. spectrum of carbodiimide-treated (*O*-Ac$^-$)-C polysaccharide was recorded for a solution in $(CD_3)_2SO$ at 50 mg/mL, with a Bruker WM-250 spectrometer operating at 62.9 MHz and 80°.

I.r. spectra (KBr discs) were recorded with a Perkin–Elmer 580 spectrophotometer interfaced to a PDP11/34 mini-computer running time-sharing software. The extent of formation of lactone and *O*-acylisourea was estimated by comparing the intensities of the C=O and C–O stretching bands, in the 1750–1740, 1695, and 1190 cm^{-1} regions, respectively, with the Amide I band near 1650 cm^{-1} after computer subtraction of carboxylate absorbtion. Where necessary, Fourier domain resolution-enhancement was utilised to isolate overlapped bands followed by peak stripping and separate de-enhancement to restore the original band shape.

Lactonisation and/or formation of O*-acylisourea.* — (*a*) *With dilute acid.* The Na^+ salt of B polysaccharide was prepared by passage of B polysaccharide (Ca^{2+}

salt; 50 mg) through a column (0.9 × 14 cm) of Dowex 50 (Na^+) resin. Solid NaCl was added to the eluate to give 0.1M NaCl, and ethanol was added to a final concentration of 75%. The precipitate was collected by centrifugation at 10,000*g* for 10 min at 4°, washed once with dry ethanol, and redissolved in distilled water, and the solution was freeze-dried.

Separate solutions of B polysaccharide (Ca^{2+} salt), B polysaccharide (Na^+ salt), and colominic acid (Na^+ salt) (125 μmol) in water (15.5 mL) were prepared (pH 6.9), and HCl (0.5 mL; 50 μmol) was added to give a molar ratio of CO_2^-: H^+ of 1:0.4. The pH dropped to 3.4, 4.0, and 3.7, respectively. Aliquots (2 × 0.3 mL) were removed at intervals (15, 30, 45, 60 min, 2, 4, 8, 24, and 48 h) and freeze-dried. Aliquots (1 mL) were also removed, incubated with KBH_4 (5 mg) at pH 9 for 2 h at room temperature, dialysed for 48 h at 4° against 0.01M ammonium carbonate (4 × 2 L), and freeze-dried.

(*b*) *With carbodiimide* (EDC). — To separate solutions of the Ca^{2+} salts of B, (*O*-Ac^+)-C, and (*O*-Ac^-)-C polysaccharides (110 μmol) in water (14 mL) was added EDC · HCl (44 μmol) to give a molar ratio of CO_2^-:EDC of 1:0.4. The pH was adjusted to, and maintained at, 4.75. At intervals (10, 20, 30, 60 min, 2, 4, and 24 h), aliquots (0.8 mL) were added to dry ethanol (3.5 mL) in 0.5M acetate buffer (pH 4.7, 0.2 mL). The precipitate was collected by centrifugation at 10,000*g* for 10 min at 4°, washed once with dry ethanol, and freeze-dried. Aliquots (1.2 mL) were also removed from the reaction mixture, incubated with KBH_4 (15 mg) at pH 9 for 2 h at room temperature, dialysed for 48 h at 4° against 0.01M ammonium carbonate (4 × 2 L), and freeze-dried.

Large-scale formation of lactone and/or *O*-acylisourea groups in (*O*-Ac^-)-C polysaccharide and chondroitin sulphate with excess of EDC was done essentially as described previously[2].

Methanolysis of the polysaccharides. — Partially carboxyl-reduced polysaccharides (0.5 mg) were treated with 2M methanesulphonic acid in dry methanol (1 mL). The samples were flushed with dry N_2, sealed in tubes, and heated at 65° for 15 h. Each solution was neutralised by vigorous mixing with Dowex 2 (HCO_3^-) resin, decanted, and concentrated to dryness. Each residue was *N*-acetylated and trimethylsilylated, and the products were subjected[6] to g.l.c. on 3% of OV-225 at 220°.

Gel filtration. — Molecular weight distributions were determined by gel filtration on a column (1.5 × 90 cm) of Sepharose CL-4B at 4°. The conditions of elution were as described previously[2].

Solid-phase radioimmunoassay. — Antigenic activity was measured in round bottom, soft plastic, microtitration plates (Dinatech) which were treated with poly-L-lysine [50 μg/mL in 0.01M sodium phosphate-buffered saline (0.85%), pH 7.4 (PBS)], rinsed, and then sensitised with meningococcal polysaccharides (1 μg/mL in PBS). Both incubations were for 1 h at 18° using 100 μL/well, and, after incubation, each plate was rinsed five times with PBS. Non-specific binding was blocked by incubation for 1 h with 5% bovine haemoglobin in PBS (150 μL/well) and rinsing

twice with PBS. Murine monoclonal antibodies MB34 (IgM) and MC45 (IgG2a) were used for specific binding to meningococcal B and C polysaccharides, respectively. The acquisition and properties of MB34 have been published elsewhere[7]. The same procedure, omitting the stimulation of mice with *C. parvum* vaccine, was used to obtain MC45. Binding of the antibodies was performed at 12 μg/mL for MB34 and 1.2 μg/mL for MC45 in 5% haemoglobin in PBS (50 μL/well, 5 min, 4°), followed by rinsing five times with PBS. When the inhibition studies were performed, various concentrations of the antigens were added to the fixed amount of antibody and preincubated for 20 min at 4°. Aliquots (50 μL) were then transferred to the antigen-coated plates and incubated for 5 min at 4°. The amount of mouse immunoglobulin specifically bound to the plate was estimated by further incubation (overnight at 4°) with immunopurified, ^{125}I-labelled, rabbit anti-mouse Ig[8] (60 μL/well, ~10^5 c.p.m./mL, 7–8 c.p.m./μg of Ig diluted in 5% haemoglobin in PBS). After incubation, each plate was washed five times with PBS and each well was counted. Inhibitory activity for each antigen was estimated by plotting the percent of radioactivity bound *vs.* concentration of antigen. A standard preparation of the native antigen was included in each experiment.

RESULTS AND DISCUSSION

Rate of lactonisation of B polysaccharide at low pH. — The Na^+ salts of B polysaccharide and colominic acid readily form[2] lactones at low pH. The rate of lactonisation has now been determined in these polysaccharides, and also in the Ca^{2+} salt of B polysaccharide, by incubating solutions in dilute HCl and subjecting the products to i.r. spectroscopy. The degree of lactonisation was estimated from the intensities of the C=O stretching band near 1750 cm^{-1} and the C–O vibration near 1190 cm^{-1}, both of which are characteristic of lactonisation in these materials[2]. The degree of lactonisation was also determined by borohydride reduction of the lactones followed by methanolysis of the partially carboxyl-reduced polysaccharides and analysis[6] of the trimethylsilylated products by g.l.c.; the results are given in Table I. Acceptable agreement between the three methods for the determination of lactone formation was found for the Na^+ salts of B polysaccharide and colominic acid, the latter having a higher rate of lactonisation which probably reflected the lower molecular weight and greater chain flexibility. However, during incubation of the Ca^{2+} salt of B polysaccharide at low pH, the i.r. spectra revealed a broad band between 1760 and 1720 cm^{-1}, consistent with the presence of both carboxylic acid (C=O band near 1725 cm^{-1}) and lactone (C=O band near 1750 cm^{-1}) residues, thus rendering this method unsuitable for determining the extent of lactone formation. Furthermore, estimation of the degree of lactonisation was consistently greater by the g.l.c. than by the i.r. method for measuring the intensity of the C–O band. Although it is not known why the latter method gave low results, it seems to be a phenomenon associated with the presence of Ca^{2+} ions in the polysaccharide (see below).

TABLE I

DEGREE OF LACTONISATION OF B(Ca^{2+} SALT) AND B(Na^+ SALT) POLYSACCHARIDES AND COLOMINIC ACID (Na^+ SALT) FOLLOWING INCUBATION AT LOW pH, AS DETERMINED BY THE G L.C AND I R METHODS (SEE EXPERIMENTAL)

Time (h)	*Degree of lactonisation (%)*								
	B(Ca^{2+} salt)			*B(Na^+ salt)*			*Colominic acid (Na^+ salt)*		
	G.l.c.	*I.r.*		*G.l.c.*	*I.r.*		*G.l.c.*	*I.r.*	
		C–O	*C=O*		*C–O*	*C=O*		*C–O*	*C=O*
0.25	2.4	1.9	16.1[a]	1.7	3.7	3.4	2.0	4.7	7.5
0.5	4.1	3.3	18.1[a]	3.9	5.2	6.7	5.6	6.2	8.9
0.75	4.8	4.0	18.3[b]	5.0	6.5	9.4	7.8	7.1	10.6
1.0	5.9	4.0	19.0[b]	7.0	7.3	10.2	9.1	9.2	12.8
2.0	9.0	6.4	20.8[b]	10.8	11.9	14.0	12.3	11.7	14.2
4.0	13.1	8.0	23.6[c]	17.5	16.2	17.1	18.3	17.8	20.4
8.0	15.9	10.0	26.2[c]	18.9	19.9	18.0	19.3	22.0	23.7
24.0	17.6	13.8	28.5	21.7	22.3	21.2	24.6	25.3	21.6
48.0	19.6	15.7	29.4	23.1	24.1	24.9	26.7	27.6	23.4

[a]C=O band centred near 1735 cm^{-1}. [b]C=O band centred near 1740 cm^{-1}. [c]C=O band centered near 1745 cm^{-1}.

G.l.c. of the products of methanolysis of the Na^+ and Ca^{2+} salts of B polysaccharide shows that they react at different rates (Table I), emphasising the importance of the salt form in determining the rate of lactone formation. Following incubation of B polysaccharide in the free-acid form at room temperature for 1 h, extensive lactonisation occurred (~75%), implying that the rate-limiting step involved the formation of free acid with subsequent fast lactonisation. The i.r. spectra of the Na^+ salts of B polysaccharide or colominic acid after incubation at low pH did not show a carboxylic acid band at 1725 cm^{-1}, in accord with this mechanism[2]. However, after the Ca^{2+} salts of B polysaccharide had been incubated at low pH, the i.r. spectra contained, within 10 min, a significant carboxylic acid band, indicating that, following fast formation of acid, the rate-limiting step was lactonisation. This may be because Ca^{2+} ions[9], as well as other divalent and trivalent cations[10], coordinate directly to hydroxyl groups in sialic acids, implying that HO-9 may be involved in a complex with Ca^{2+} ions, thereby preventing rapid lactonisation.

Lactonisation and/or formation of O-acylisourea derivatives of C polysaccharide with carbodiimide. — Treatment of (*O*-Ac^+)-C polysaccharide with carbodiimide (EDC), followed by i.r. spectroscopy of the products, has shown[2] the absence of a C=O band near 1750 cm^{-1} and of a C–O vibration near 1190 cm^{-1}, both of which are characteristic of lactonisation in B and *O*-deacetylated C polysaccharide; this was interpreted as indicating the absence of lactone formation. However, after borohydride reduction and methanolysis, g.l.c. showed that 75% of the carboxyl groups had been reduced. Similarly, treatment of (*O*-Ac^-)-C poly-

saccharide afforded a product in which 83% of the carboxyl groups had been reduced. The i.r. spectra of native (*O*-Ac$^+$)-C and (*O*-Ac$^-$)-C polysaccharides (Figs. 1A and 2A, respectively) were similar, apart from the C–O and C=O stretching bands near 1250 and 1725 cm^{-1}, respectively, which are characteristic of *O*-acetyl groups present in C polysaccharide (Fig. 1A). However, following treatment of (*O*-Ac$^+$)-C polysaccharide with carbodiimide, the i.r. spectrum (Fig. 1B) showed a strong absorption in the carbonyl region near 1695 cm^{-1}, whereas that of (*O*-Ac$^-$)-C polysaccharide (Fig. 2B) showed a strong C=O band near 1740 cm^{-1} characteristic of lactonisation, and only a weak band near 1695 cm^{-1}; a diminished carboxylate peak near 1610 cm^{-1} was observed in each spectrum. Alkaline hydrolysis of both EDC-treated polysaccharides (0.1M NaOH, 4 h, 37°) re-established the carboxylate peak and removed the carbonyl-region bands near 1695 and 1740 cm^{-1}.

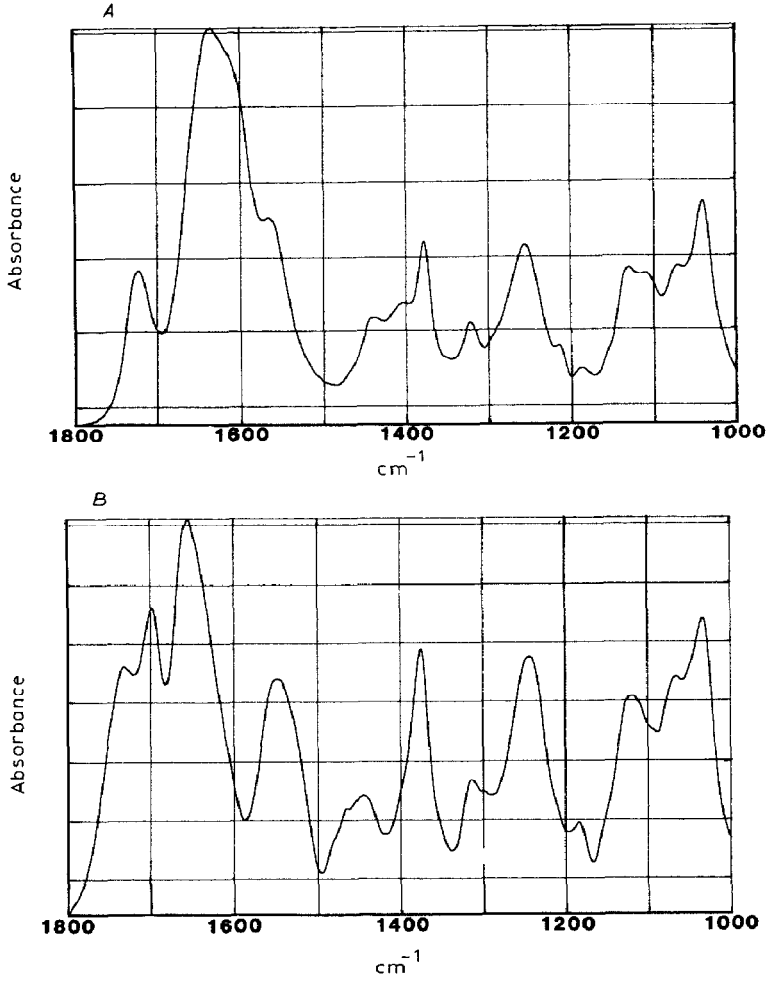


Fig. 1. I.r. spectrum of (*O*-Ac$^+$)-C polysaccharide (Ca^{2+} salt) before (*A*) and after (*B*) treatment with carbodiimide.

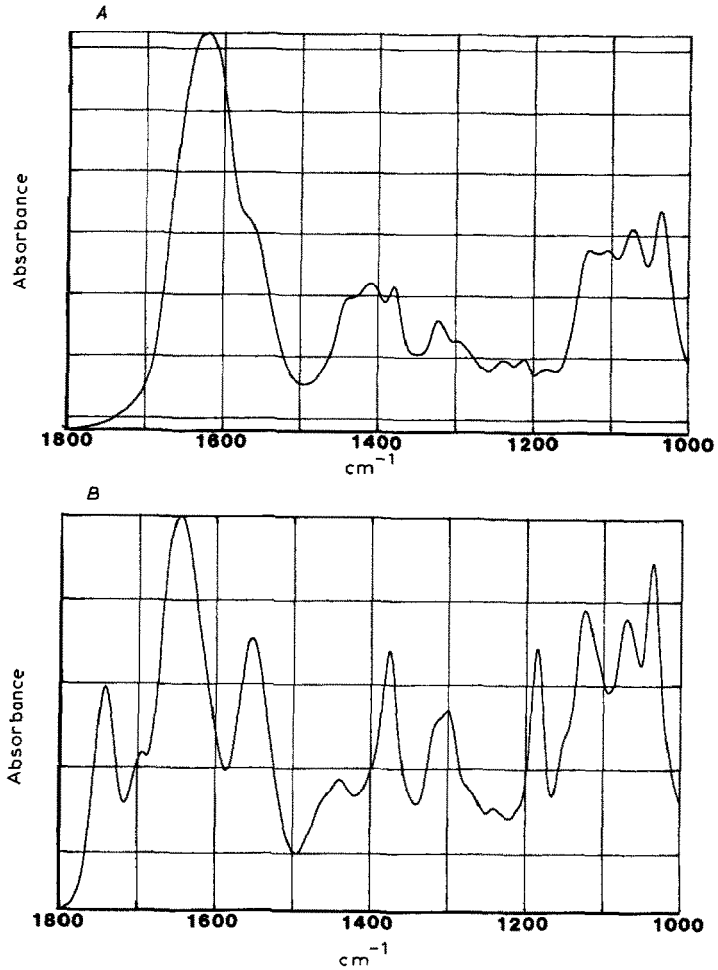
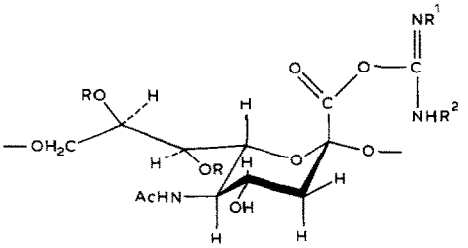


Fig. 2. I.r. spectrum of (*O*-Ac⁻)-C polysaccharide (Ca^{2+} salt) before (*A*) and after (*B*) treatment with carbodiimide.



1 R = H, Ac ; R^1, R^2 = Et , $(CH_2)_3\overset{+}{N}HMe_2$

The evidence is consistent with activation of the carboxyl group by the formation of *O*-acylisourea (**1**) during the treatment with carbodiimide, with significant lactonisation occurring subsequently only in (*O*-Ac$^-$)-C polysaccharide. The formation of *O*-acylisourea was further suggested by a 2.0- and 1.4-fold rise, respectively, in the nitrogen content of (*O*-Ac$^+$)-C and (*O*-Ac$^-$)-C polysaccharide, following treatment with a 10-mol excess of EDC. Since treatment with borohydride is known to give ~80% of carboxyl-reduced polysaccharide, and alkaline hydrolysis generates the free carboxylate, the possibility of any significant formation[11] of *N*-acylurea is precluded and, thus, the i.r. peak at 1695 cm^{-1} (Figs. 1B and 2B) can be assigned to the C=O stretching vibration of an activated carboxylic ester group (**1**). *O*-Acylisourea groups are formed on treatment[12] of chondroitin 4-sulphate with EDC, although no i.r. spectroscopic evidence was presented. We have found that treatment of chondroitin sulphate with EDC gave a product having a strong i.r. carbonyl absorption near 1704 cm^{-1}, not given by the native polysaccharide, thus confirming our assignment of the band at 1695 cm^{-1} in (*O*-Ac$^+$)-C polysaccharide. No additional prominent bands in the C–O region were observed, either with (*O*-Ac$^+$)-C polysaccharide or with chondroitin sulphate, which suggested this to be characteristic of *O*-acylisoureas.

Acetyl groups at O-7 and/or O-8 of C polysaccharide presumably prevent nucleophilic attack of a hydroxyl group at the activated carboxyl group, a reaction that occurs in (*O*-Ac$^-$)-C polysaccharide. However, a weak band near 1695 cm^{-1} in the i.r. spectrum of EDC-treated (*O*-Ac$^-$)-C polysaccharide (Fig. 2B) suggested some of the secondary hydroxyl groups available for lactonisation to be only weakly nucleophilic and/or not favourably located with respect to the activated carboxyl groups. This latter possibility supports our conclusions based on molecular mechanics calculations[3], which showed that the carboxyl and hydroxyl groups are in closer proximity and, hence, more favourably disposed for lactonisation in B than in C polysaccharide.

Rate of lactonisation and/or formation of O-acylisoureas of B and C polysaccharides with carbodiimide. — The rate of formation of lactone or *O*-acylisourea derivatives of B, (*O*-Ac$^-$)-C, and (*O*-Ac$^+$)-C polysaccharides on incubation with a limited amount of EDC (0.4 mol/mol of sialic acid) was monitored. G.l.c. of the products obtained after carboxyl-reduction and methanolysis revealed rapid initial formation of lactone or *O*-acylisourea (<1 h) for each polysaccharide, followed by slow hydrolysis (after 4 h), presumably when the carbodiimide was exhausted (Fig. 3). This hydrolysis was more pronounced in EDC-treated B and (*O*-Ac$^-$)-C polysaccharides than in EDC-treated (*O*-Ac$^+$)-C polysaccharide, implying that the lactones were hydrolysed faster than the *O*-acylisourea linkages. This situation was indicated by the fact (see Table II) that there was no i.r. band near 1695 cm^{-1} in EDC-treated B and (*O*-Ac$^-$)-C polysaccharide (*i.e.*, only lactone formation occurred on reaction with a limited amount of EDC), but that a strong band near 1695 cm^{-1} was present in (*O*-Ac$^+$)-C polysaccharide (*O*-acylisourea), with only a weak band near 1190 cm^{-1} (lactone).

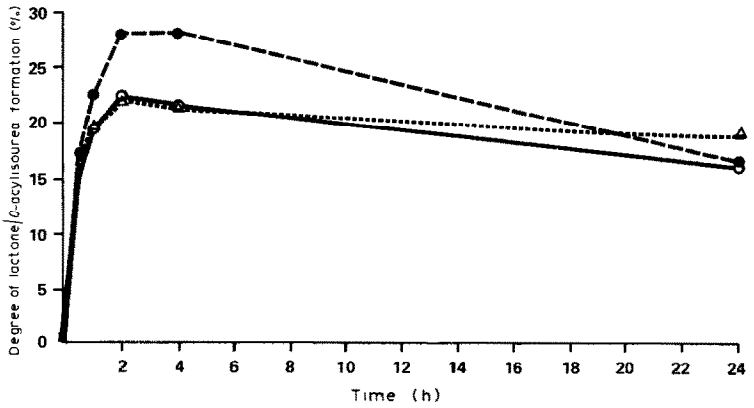


Fig. 3. Rate of lactonisation and/or formation of *O*-acylisoureas of B polysaccharide (—○—), (*O*-Ac^+)-C polysaccharide (---△---), and (*O*-Ac^-)-C polysaccharide (– – –●– – –) following treatment with a limited amount of carbodiimide (molar ratio CO_2^-: EDC of 1:0.4).

The data in Table II show there is a good correlation between g.l.c. and i.r. methods for determining the degree of lactonisation of EDC-treated B and (*O*-Ac^-)-C polysaccharides. However, estimation of the intensity of the C–O band near 1190 cm^{-1} showed that, although satisfactory results were obtained with the Ca^{2+} salt of (*O*-Ac^-)-C polysaccharide, anomalously low values were obtained with the Ca^{2+} salt of B polysaccharide. This appeared to be due directly to the presence of Ca^{2+}, since treatment of the Na^+ salt of B polysaccharide with carbodiimide resulted in close agreement between the methods based on the C=O and C–O bands.

TABLE II

DEGREE OF FORMATION OF LACTONE AND *O*-ACYLISOUREA OF THE Ca^{2+} SALTS OF B, (*O*-Ac^-)-C, AND (*O*-Ac^+)-C POLYSACCHARIDES FOLLOWING INCUBATION WITH EDC, AS DETERMINED BY THE G.L.C. AND I.R METHODS (SEE EXPERIMENTAL)

Time (min)	*Degree of lactone/O-acylisourea formation (%)*								
	B			*(O-Ac⁻)-C*			*(O-Ac⁺)-C*		
	G.l.c.	*I.r.*		*G.l.c.*	*I.r.*		*G.l.c.*	*I.r.*	
		C–O[a]	*C=O*[b]		*C–O*[a]	*C=O*[b]		*C–O*[a]	*C=O*[c]
10	4.5	0.6	3.2	6.7	8.7	9.7	6.8	1.9	2.5
20	7.7	3.4	5.7	11.3	11.8	15.7	9.2	3.3	6.7
30	13.7	6.1	10.8	17.2	18.1	16.7	14.8	4.6	8
60	19.4	9.6	18.3	22.7	20.7	23.3	19.7	4.8	17.4
2 h	22.7	13.9	24.1	28.0	24.3	27.0	22.1	5.6	17.5
4 h	21.7	13.2	22.7	28.3	22.9	26.2	21.6	6.5	17.5
24 h	16.5	10.2	16.4	17.3	11.5	14.9	19.3	5.6	18.1

[a]C–O band centred near 1190 cm^{-1} measures lactonisation. [b]C=O band centred between 1740–1750 cm^{-1} measures lactonisation. [c]C=O band centred near 1695 cm^{-1} measures *O*-acylisourea formation (**1**).

TABLE III

PROPERTIES OF B, (*O*-Ac$^+$)-C, AND (*O*-Ac$^-$)-C POLYSACCHARIDES BEFORE, AND AFTER, TREATMENT AT LOW pH AND WITH A CARBODIIMIDE (EDC)

		Degree of lactone/O-acylisourea formation (%)				*C.i.e.*		*Molecular size*[d]	
		G.l.c.	*I.r.*			*Before alkaline hydrolysis*	*After alkaline hydrolysis*	$\%K_D=O$	$\%K_D \leq 0.5$
			C=O (~1745 cm^{-1})	*C=O (~1695 cm^{-1})*	*C–O (~1190 cm^{-1})*				
(*O*-Ac$^+$)-C	Salt form	0	[a]	0	0	+	+	61	92
	Free-acid form	0	[a]	0	[b]	+	+	18	81
	EDC-treated	75	[a]	56	5	−	+	75	98
(*O*-Ac$^-$)-C	Salt form	0	0	0	0	+	+	18	80
	Free-acid form	0	[a]	0	[b]	+	+	2	58
	EDC-treated	83	50	22	56	−	+	72	95
B	Salt form	0	0	0	0	+	+	43	90
	Free-acid form	76	79	0	82.3	−	+	9	45
	EDC-treated	95	102	0	100	[c]	+	[c]	[c]

[a]Not determined due to the presence of *O*-acetyl and/or carboxylic acid carbonyl bands near 1725 cm^{-1}. [b]Not determined due to the presence of a carboxylic acid C–O band near 1180 cm^{-1}. [c]Not determined due to the water-insoluble nature of the polysaccharide. [d]$K_D = (V_e - V_o)/(V_t - V_o)$, where V_o = void volume, V_t = total volume, and V_e = elution volume.

It is evident from the data in Table II that lactonisation and the formation of *O*-acylisourea groups in EDC-treated (*O*-Ac$^+$)-C polysaccharide can be determined separately by analysis of their i.r. spectra, thus forming a method complementary to that based on g.l.c., which reflected total lactone and *O*-acylisourea formation.

The reaction of glycosaminoglycuronans with EDC involves the formation of a stable *O*-acylisourea[12], and not a lactone as suggested previously[13]. Our results suggest that, following the formation of an *O*-acylisourea (**1**), the polysaccharide only undergoes lactonisation when a nucleophilic hydroxyl group is in close proximity to the activated carboxyl group. Thus, with a limited amount of EDC, although the rate of formation of *O*-acylisourea for each polysaccharide was similar (Fig. 3), the i.r. spectra of B and (*O*-Ac$^-$)-C polysaccharides, only, showed a subsequent high rate of lactonisation, indicating the activated ester **1** to be highly reactive. However, following reaction with an excess of EDC, only B polysaccharide underwent full lactonisation, whereas the i.r. spectra indicated (*O*-Ac$^-$)-C polysaccharide to contain both lactone (C=O band near 1740 cm^{-1}) and *O*-acylisourea groups (C=O band near 1695 cm^{-1}).

Properties of B and C polysaccharides following the formation of lactone and/or O-acylisourea groups. — Table III shows some of the properties of B, (*O*-Ac$^+$)-C, and (*O*-Ac$^-$)-C polysaccharides before, and after, treatment at low pH or with a carbodiimide. Both the i.r. and g.l.c. methods revealed that lactonisation did not occur in the salt form of the polysaccharides. It was deduced[2] from the i.r. spectra of the free-acid forms of (*O*-Ac$^+$)-C and (*O*-Ac$^-$)-C polysaccharides that <7% of lactonisation occurred, although the absence of a C=O band near 1740 cm^{-1} could not be established, due to the carboxylic acid band near 1725 cm^{-1}. G.l.c. of the products of methanolysis of the carboxyl-reduced polysaccharides confirmed the absence of lactonisation (Table III). In contrast, the free-acid form of B polysaccharide underwent rapid and extensive lactonisation.

Counter-current immunoelectrophoresis (c.i.e.) indicated a complete loss of immunoprecipitating capacity following the formation of lactone and/or *O*-acylisourea groups in B and C polysaccharides. After alkaline hydrolysis (0.1M NaOH, 4 h, 37°), however, antigenicity was fully restored (Table III).

The results of gel filtration, on Sepharose CL-4B, of the native, free-acid, and carbodiimide-treated forms of B and C polysaccharide are shown in Table III. The apparent molecular weight of each polymer was characterised by the material eluted in the void volume ($K_D = 0$) and by the material eluted before $K_D = 0.5$. For each polymer, the free-acid form had an apparent molecular weight lower than that of the native form of the polymer. It is highly unlikely that the decrease in molecular weight (real or apparent) caused the decrease in antigenicity, since alkaline treatment resulted in a complete recovery of the capacity to precipitate with antibody in the c.i.e. test. Although (*O*-Ac$^+$)-C and (*O*-Ac$^-$)-C polysaccharides showed an increase in apparent molecular weight following treatment with carbodiimide (which may suggest that some intermolecular cross-linking occurred),

TABLE IV

^{13}C-N.M R DATA FOR (*O*-Ac$^-$)-C POLYSACCHARIDE AND ITS CARBODIIMIDE (EDC)-TREATED ANALOGUE

Carbon atom	*(O-Ac$^-$)-C polysaccharide*	*EDC-treated (O-Ac$^-$)-C polysaccharide*	*Difference (p.p.m.)*
C-1	174.8	168.4 (174.1[b])	−6.4
C-2	101.3	98.1	−3.2
C-3	41.1	42.4	+1.3
C-4	69.4[a]	68.0	−1.4
C-5	53.2	54.2	+1.0
C-6	73.3	74.8	+1.5
C-7	69.5[a]	68.0	−1.5
C-8	71.3	77.9	+6.6
C-9	66.1	59.6	−6.5
N-Ac(CH_3)	23.3	23.3	0.0
N-Ac(C=O)	176.0	176.0	0.0

[a]These assignments may be reversed. [b]Minor peak.

much of the material (~75% and ~50%, respectively) was retained on the column. This may have been due to the formation of *O*-acylisourea groups in these polymers, the presence of which in *N*-acetylchondrosine retards[12] its elution from Sephadex G-15.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. data for solutions in $(CD_3)_2SO$ of EDC-treated (*O*-Ac$^-$)-C polysaccharide are given in Table IV, together with the assignments for the native and modified polysaccharide. The latter polymer showed a broad spectrum with 11 major resonances. Some minor peaks were present but were not assigned, and may have been due to unmodified residues (~20%) and to the formation of *O*-acylisourea groups (**1**). Of the major peaks, the upfield shift of the resonances at 174.8 to 168.4 p.p.m. is consistent[2] with lactonisation at C-1. A residual peak at 174.1 p.p.m. is evidence that a minority of carboxyl groups remained unmodified. The three possible sites for lactonisation (C-4,7,8) have such similar chemical shifts in the native polysaccharide that it is not possible to assign the signal at 77.9 p.p.m. in the lactonised analogue. However, the large upfield shift of the resonance for C-9 from 66.1 to 59.6 p.p.m. is indicative of lactonisation involving HO-8. This is analogous to the chemical shift differences between native and lactonised B polysaccharide[2], where lactonisation involving HO-9 produced an upfield shift (7.2 p.p.m.) of the signal for C-8, arising from a combination of shielding effects and ring strain. Furthermore, the upfield shift (4.3 p.p.m.) of the signal for C-2 due to ring formation in B polysaccharide was also observed in (*O*-Ac$^-$)-C polysaccharide (3.2 p.p.m.). Thus, lactonisation occurred in the latter polymer, predominantly through condensation of the carboxyl group of one residue with HO-8 of an adjacent residue, to form a six-membered ring, as previously postulated[2]. Acetylation at HO-8 of C polysaccharide, therefore, prevents lactonisation and accounts for the formation of *O*-acylisourea groups (**1**).

Inhibition of antigenicity. — Various partially acid- and EDC-treated B and

TABLE V

CORRELATION BETWEEN DEGREE OF LACTONE/*O*-ACYLISOUREA FORMATION AND ANTIGENICITY OF B AND (*O*-Ac$^+$)-C POLYSACCHARIDES

Polysaccharide	*Incubation conditions*[a]	*Lactone/O-acylisourea (%) by g.l.c.*	*Concentration giving 50% inhibition before borohydride reduction (μg/mL)*	*Concentration giving 50% inhibition after borohydride reduction (μg/mL)*
B(Na$^+$ form)	—	0	8.0 (1.0)[b]	8.0 (1.0)
	15 min	1.7	5.0 (0.6)	17 (2.1)
	pH 4.0 1 h	7.0	4.5 (0.6)	34 (4.2)
	4 h	17.5	51.0 (6.4)	160 (20.0)
B(Ca^{2+} form)	—	0	7.4 (1.0)	7.4 (1.0)
	15 min	2.4	30 (4.1)	70 (9.5)
	pH 3.4 1 h	5.9	27 (3.6)	~275 (~37)
	4 h	13.1	51 (6.9)	~1000 (~135)
Colominic acid	—	0	~130 (1.0)	[c]
(Na$^+$ form)	15 min	2.0	>500 (>3.8)	[c]
	pH 3.7 1 h	9.1	>500 (>3.8)	[c]
	4 h	18.3	>500 (>3.8)	[c]
B(Ca^{2+} form)	—	0	1.5 (1.0)	1.5 (1.0)
	20 min	4.5	1.8 (1.2)	26 (17.3)
	EDC 1 h	19.4	0.9 (0.6)	55 (36.7)
	4 h	21.7	0.9 (0.6)	58 (38.7)
(*O*-Ac$^+$)-C (Ca^{2+} form)	—	0	0.9 (1.0)	0.9 (1.0)
	20 min	9.2	1.0 (1.1)	0.05 (0.06)
	EDC 1 h	19.7	1.4 (1.6)	0.45 (0.5)
	4 h	21.6	3.0 (3.3)	0.9 (1.0)

[a]See Tables I and II and Experimental for conditions. [b]Figures in parentheses show the relative concentrations giving 50% inhibition. [c]Not determined.

(*O*-Ac$^+$)-C polysaccharides, both before and after carboxyl-reduction, were tested for inhibition of antigenicity in a radioimmunoassay. The results in Table V show that a modest degree of lactonisation (<20%) in the Na$^+$ and Ca^{2+} salts of B polysaccharide generally resulted in a substantial loss of antigenicity. This is particularly evident following borohydride reduction of the lactone residues in the polymer. Thus, lactonisation of B polysaccharide at low pH or in the presence of EDC, followed by carboxyl reduction, resulted in a 20–>100-fold reduction in antigenicity, whereas non-lactonised samples were unaffected by treatment with borohydride. This loss of antigenicity was far greater than would be expected for an antibody which recognised a linear (*i.e.*, structural) determinant, but was consistent with the presence of (a) conformational determinant(s) on B polysaccharide[3]. Thus, a low degree of lactonisation is likely to disrupt substantially the three-dimensional structure of the polysaccharide with a concomitant reduction in antigenicity. This hypothesis is supported by the low affinity of colominic acid ($\bar{M}_w$ ~10^4) for the anti-B antibody, and the fact that only 2% lactonisation caused a further loss of antigenicity.

In contrast, (*O*-Ac$^+$)-C polysaccharide showed only a modest decrease in an-

tigenicity following the formation of lactone and *O*-acylisourea groups and an insignificant change in antigenicity on borohydride reduction of these groups, thus suggesting the presence of a structural determinant on the molecule. (O-Ac$^-$)-C polysaccharide binds poorly to this particular anti-C monoclonal antibody, thus implicating the acetyl group at O-7 and/or O-8 of (O-Ac$^+$)-C polysaccharide in the binding site (*i.e.*, as the immunodominant moiety). It was not possible, therefore, to test the influence of lactonisation of (O-Ac$^-$)-C polysaccharide on its antigenicity with this monoclonal antibody.

The observation that potentially, highly reactive *O*-acylisourea groups were formed on treatment of C polysaccharide with carbodiimide (EDC) may be extrapolated to the studies of Beuvery *et al.*[14], in which they added tetanus toxoid to this reaction mixture to obtain C polysaccharide–tetanus toxoid conjugates as a potential human vaccine. However, substantial amounts of free polysaccharide were present in the conjugate they described, and the ability of the group-C polysaccharide component to react with specific antisera was reduced. It is highly probable, therefore, that the conjugate preparation they described contained a drastically modified C-polysaccharide component, consisting of *O*-acylisourea (major) and lactone (minor) forms. The presence of *O*-acylisourea groups in this conjugate may have adverse effects on its use as a vaccine because of indiscriminate binding to host tissue *in vivo* and the presence of the intrinsic irritant effect of EDC. The first of these possibilities has been demonstrated[15] with periodate-oxidised dextran, which induced a marked suppression of anti-dextran antibody (tolerance) when injected into mice, probably due to the reaction of the aldehyde groups on the oxidised dextran with the amino groups on the dextran-specific B lymphocyte precursors. Thus, vaccination, rather than conferring protection, may have the opposite effect by inducing tolerance in the host. Therefore, it seems essential that any conjugate resulting from reaction of an acidic polysaccharide with a carbodiimide be critically evaluated for the presence of *O*-acylisourea groups.

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