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ANALYSIS OF THE COMMON POLYSACCHARIDE ANTIGENS FROM THE CELL ENVELOPE OF *Clostridium perfringens* TYPE A

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ABSTRACT

The major, common antigen of *Clostridium perfringens* type A, isolated and purified independently from three selected strains (Hobbs 5, Hobbs 9, and Hobbs 10), is composed of equimolar amounts of 2-acetamido-2-deoxy-D-mannose (ManNAc) and 2-acetamido-2-deoxy-D-glucose (GlcNAc). The purified antigen gave a strong immunoprecipitin line by double immunodiffusion in gel. Smith degradation of the major, common antigen caused decomposition of all of the GlcNAc, without concomitant loss in ManNAc, or a perceptible change in serological activity. Therefore, the serological activity of the major, common antigen depended solely on the presence of ManNAc. Data obtained by the ^{13}C-n.m.r.-spectral analysis of the Smith-degradation product revealed that it was a linear-backbone polysaccharide analogous to a *Rhodotorula glutinis* mannan, but composed of pairs of 2-acetamido-2-deoxymannopyranosyl residues alternately linked β-(1→3) and β-(1→4). The one-bond, carbon–hydrogen coupling-constant of 162 Hz for both anomeric centers was consistent with the proposed β-linkages. A similar, ^{13}C-n.m.r.-spectral analysis of the native, common antigen indicated that the GlcNAc residues were randomly connected to three of the four hydroxyl groups not already involved in linking the ManNAc backbone, the 4-hydroxyl group being the exception. A second, serologically inactive, polysaccharide composed of rhamnose, GalNAc, and galactose was identified, but not obtained in homogeneous state. The rhamnosyl residues were probably situated as nonreducing antennae, as they were quantitatively removed by Smith degradation without concomitant decomposition of the polymeric structure of the remaining residues.

INTRODUCTION

Clostridium perfringens type A is a known cause of several clinical infections,

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such as gas gangrene, septicemia, and uterine infections[1]. However, the major impetus for the extensive studies of *C. perfringens* conducted over the past 25 years has been its role as an important agent of human food-poisoning[2].

C. perfringens type A was subdivided by the serotyping system originally developed[3] by the Food Hygiene Laboratory (FHL), London, England. Initially, antisera were prepared against a small number of heat-resistant strains involved in food poisoning; these antisera led to the identification of distinct serotypes of *C. perfringens* type A. The number of specific antisera available has increased over the years through the cooperative efforts of several laboratories (e.g., Centers for Disease Control, Atlanta, Georgia[2] and FHL, London[4], 91 and 75 type-specific antisera, respectively), and yet many isolates remain untypable. The specificity of the antisera employed in the serotyping system was shown to reside in a cell-envelope component, a type-specific acidic heteropolysaccharide, particular to each strain[3,5–8].

Serological and chemical evidence has shown that, in addition to the type-specific heteropolysaccharides, common antigenic heteropolysaccharides occur as minor components of the *C. perfringens* type A cell-envelope[9,10]. The purification and chemical characterization of the major common antigen of three selected strains of *C. perfringens* serotype A have now been accomplished.

EXPERIMENTAL

Analytical methods. — Neutral carbohydrate was detected by the phenol–sulfuric acid method of Dubois *et al.*[10]. Hexosamine was quantitated after acid hydrolysis by the method of Elson and Morgan[11] as modified by Boas[12], and by the procedure of Smith and Gilkerson[13]. *N*-Acetylhexosamine was determined by the method of Reissig *et al.*[14]. Phosphate was estimated by the Ames and Dubin[15] modification of the procedure of Chen *et al.*[16]. Protein content was determined by a dye-binding assay reagent (Bio-Rad Laboratories 500-0006), and by amino acid analysis. The constituent monosaccharides were identified, and quantitated, by gas–liquid chromatography (g.l.c.) after acid hydrolysis by methods previously described[17].

Smith degradation[18] of polysaccharides is briefly described. Antigen (10–100 mg) was dissolved in freshly prepared 0.04M sodium periodate (5 mg per mL of reagent), and reaction allowed to proceed for 3 days at 4° in the dark. Ethylene glycol (15–150 mg) was added, and after 1 h at room temperature, sodium borohydride (20–200 mg) was added. The solution was refrigerated overnight, and then the pH was adjusted to 4.5 with glacial acetic acid. The sample was dialyzed *versus* de-ionized water, and then evaporated to dryness *in vacuo* below 40°, or lyophilized. A portion of the oxidized and reduced antigen was treated with 0.1M HCl for 16 h at 37°, dialyzed *versus* de-ionized water, and lyophilized. Identification of the hexosamines was accomplished as follows. A mixture of hexosamines (17 mg) was obtained by hydrolyzing the antigen with 2M HCl for 16 h. The sample was evapo-

rated *in vacuo* below 40°, and residual HCl was removed by repeated evaporation with absolute ethanol. The hexosamines were dissolved in 0.3M HCl (4 mL), and the mixture was resolved by chromatography on Dowex-50 X8-400 (H^+) ion-exchange resin according to Gardell[19], except that the column used was 97 × 2.3 cm. The identities of the individual hexosamine fractions were confirmed by the method of Stoffyn and Jeanloz[20] and by g.l.c.[17].

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra were recorded with a JNM/FX60Q n.m.r. spectrometer operated at 15.04 MHz and equipped with a 10-mm, ^1H–^{13}C dual probe, an NM-3975 foreground–background unit, and an NM-5471 temperature controller. The spectra were recorded at 70° by using the pulsed, fast Fourier-transform method, and by employing the deuterium resonance of the solvent, a buffer (pH 7.2) of sodium phosphate in deuterium oxide, as an internal lock. The spectra were 4-kHz wide, and were collected with 8-k data points in the f.i.d. and by use of a 45° pulse repeated at intervals of 1.1 s. Fourier-transformations were executed with application of an exponential, window function after zerofilling the f.i.d. by the addition of 8-k zero-intensity data-points; this resulted in 1 Hz broadening of the spectral lines. Chemical shifts were determined from spectra which were proton-decoupled by a 1-kHz noise-band centered at 48.00-kHz offset-frequency, and were measured relative to an external capillary containing tetramethylsilane.

Carbon–hydrogen coupling constants were obtained from spectra determined with gated, ^1H irradiation that provided no decoupling but gave nuclear Overhauser enhancement of signal intensities.

Serological procedures. — Serological relationships among the various polysaccharides and their derivatives were determined with specific rabbit antisera[9] by double immunodiffusion (i.d.) in gel[21]. Antigens were tested for their stability to oxidation by $NaIO_4$, as previously reported[6].

Polysaccharide antigens. — Unfractionated, cell-envelope antigens of *C. perfringens* type A, Hobbs 5, Hobbs 9, and Hobbs 10 were obtained as described previously[9]. Two extraction procedures, conducted consecutively, resulted in the isolation of the following three unfractionated, antigen preparations: (*1*) water-extractable, ethanol-precipitable, EP; (*2*) water-extractable, ethanol-nonprecitable, ES; and (*3*) 1% acetic acid-extractable, HA[9].

Protease digestion. — A selected, unfractionated antigen was dissolved in 0.01M sodium (ethylenedinitrilo)tetraacetate (EDTA)–0.01M L-cysteine buffer, pH 6.5 (10 mg/mL), containing 1 mg of papain per 100 mg of antigen. The solution was incubated for 24 h at 50°, at which time a second portion of enzyme (0.5 mg/100 mg of antigen) was added, and the incubation continued for an additional 24 h. The digest was dialyzed *versus* de-ionized water, centrifuged to remove any insolule material, and lyophilized.

Column chromatography. — Antigen (200 mg in 20 mL of de-ionized water) was applied to a column (2.5 × 30 cm) of DEAE-Sephadex A 25 (Cl^-) (Pharmacia), and the column was washed with water at a flow rate of 25 mL/h. The ef-

fluent was analyzed (*1*) by continuous monitoring at 206 nm, (*2*) for hexosamine content, and (*3*) for serological activity by i.d. Column fractions possessing common antigen were combined, dialyzed *versus* de-ionized water (Spectrapore 3 dialysis tubing, molecular weight cutoff of 3500), and lyophilized.

The DEAE-derived antigen (50–100 mg) was dissolved in 0.05M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) buffer, pH 7.6 (5.0 mL), and fractionated by gel-permeation chromatography (g.p.c.) on a column (93 × 2.5 cm) of Sepharose CL 6B (Pharmacia) equilibrated with the same buffer. The column was eluted at a flow rate of 20 mL/h, and the effluent was analyzed as described for DEAE-Sephadex. Appropriate fractions obtained from several experiments were combined, dialyzed, and lyophilized.

Treatment with hydrofluoric acid. — Antigen (40 mg/mL) was dissolved in ice-cold, 48% hydrofluoric acid (2 mL), and the mixture was kept in a refrigerator for 16 h, after which time the acid was carefully neutralized with cold, M LiOH. After 30 min, the neutral suspension was centrifuged at 4° to remove the lithium salts that had precipitated. The supernatant liquor was decanted, and the precipitate was washed with cold water (3 mL). The supernatant liquor and wash were pooled, dialyzed *versus* de-ionized water, and the product (28 mg) isolated by lyophilization.

Succinylation of polysaccharide[22]. — A solution of polysaccharide (50 mg) in water (0.5 mL) was chilled in an ice bath. Succinic anhydride (50 mg) was dissolved in anhydrous acetone (0.7 mL), and the volume was adjusted to 3.0 mL with cold water. The solution of succinic anhydride was immediately transferred to the vessel containing the polysaccharide, and the pH of the reaction was maintained at 6.0 by careful addition of 20% NaOH. After 10–15 min, the pH became stable, and the mixture was then kept for 5 h at 4°, dialyzed *versus* de-ionized water, and the lyophilized retentate applied to a column (1 × 15 cm) of DEAE-Sephadex A-25 (Cl^-) ion-exchange resin. The column was washed with water, and then eluted with M NaCl, the effluent being monitored continuously at 206 nm.

Hydrolysis with alkaline borohydride. — The antigen fraction (5 mg) derived by gel-permeation chromatography (g.p.c.) was dissolved in M NaOH (0.5 mL) which contained $NaBH_4$ (5 mg). The mixture was kept at 100° and, after 3.5 h, it was cooled and M HCl (0.5 mL) was added. The sample was diluted with water to 2 mL, the pH was adjusted to 7.0, and then it was dialyzed (Spectrapore 6, 1000 MWCO) against distilled water. A portion of the sample was reserved, and the rest was acetylated with acetic anhydride in aqueous methanol[16] and dialyzed. The products were analyzed by i.d.

Partial hydrolysis with acid. — G.p.c. antigen fractions (3–5 mg) were dissolved in 0.1M HCl (1 mL), heated for 15 min at 100°, cooled, evaporated under diminished pressure, and freed of residual HCl by repeated evaporation with absolute ethanol. The residues were dissolved in 0.1M glycine buffer, pH 10 (1 mL), and a portion of each was reserved for *N*-acetylhexosamine and inorganic phosphate analyses. The remainders of the samples were digested with *E. coli* alkaline phos-

phatase (10 μL, Sigma P4252) for 3 days at 37°, and the two analyses were repeated. The treated, g.p.c. antigens were dialyzed, and analyzed by g.l.c. after hydrolysis with trifluoroacetic acid.

RESULTS

Column chromatography. — Unfractionated ES antigens (5, 9, and 10) and papain-digested HA antigens (5, 9, and 10) were individually processed by passage through columns of DEAE-Sephadex A-25. The common antigens were not retained by the DEAE-Sephadex, and were therefore recovered from the effluents. Prior equilibration of the column with various buffers did not alter the binding

TABLE I

SUMMARY COMPOSITION OF THE COMMON ANTIGEN FRACTIONS

Strain	*Antigen fraction*	*Hexosamine*[a]	*% (by weight)*		
			Neutral sugar[b]	*Phosphate*	*Protein*
Hobbs 5	ES[c]	22	12	4.8	2
	DEAE effluent	48	6	1.4	nil
	GPC II	12	2	1 9	nil
	GPC III	49	3	0.3	nil
	HA[d]	24	12	1.3	8
	DEAE effluent	36	11	2.7	nil
	GPC II	24	17	8.5	nil
	GPC III	38	12	4.3	nil
Hobbs 9	ES	24	6	1.2	14
	DEAE effluent	37	5	1.6	nil
	GPC II	40	15	1.0	nil
	GPC III	64	2	4.4	nil
	HA[d]	18	18	5.0	10
	DEAE effluent	39	13	5.4	nil
	GPC II	17	28	7.7	nil
	GPC III	46	11	2.2	nil
Hobbs 10	ES	6	11	3.4	7
	DEAE effluent	21	7	0.9	nil
	GPC II	16	4	1.3	nil
	GPC III	37	4	0.2	nil
	HA[d]	9	15	4.1	14
	DEAE effluent	29	14	4.7	nil
	GPC II	18	27	8.3	nil
	GPC III	35	7	2.8	nil
GPC II Refractionated		23	27	7.5	nil

[a]Determined colorimetrically after hydrolysis with acid. [b]Determined with the phenol–sulfuric acid reagent. [c]Key to abbreviations: ES, water-extractable, ethanol-nonprecipitable antigen; HA, 1% acetic acid-extractable antigen; GPC II and GPC III, combined column peaks obtained by gel-permeation chromatography; arabic numerals 5, 9, and 10 pertain to the *C perfringens* Hobbs type used for the production of antigen. [d]HA antigens were predigested with papain before chromatography on DEAE-Sephadex A-25 (Cl^-) resin. Analysis of the digests was conducted, but the results are not shown.

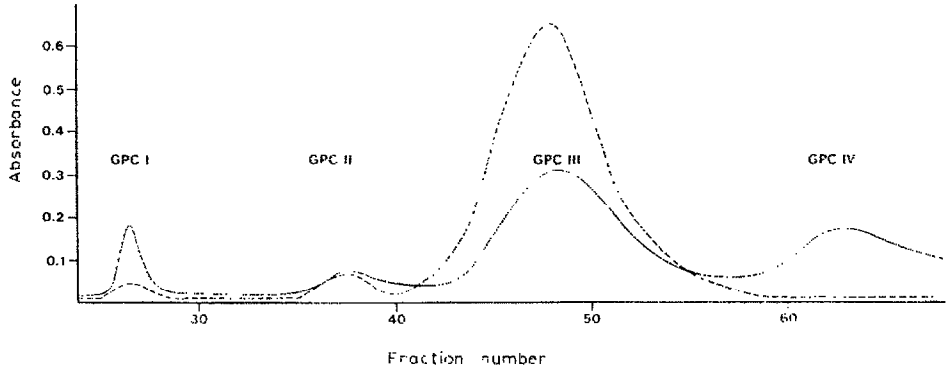


Fig. 1. Chromatography of ES 9 on Sephacryl S-200. (Each fraction contained 6.5 mL of eluate, collected at 18-min intervals.) (Key: ——, at 206 nm; and ----- hexosamine, at 540 nm.)

properties of the common antigens. Comparative analysis, by i.d., of the DEAE-Sephadex effluents with the unfractionated antigens showed serological identity between the two, except for the disappearance of the immunoprecipitin lines representing the type-specific acidic heteropolysaccharides[9]. The ultraviolet absorption spectra of the DEAE-Sephadex effluents showed no characteristic features between 240 and 300 nm, whereas the unfractionated antigens showed absorption spectra typical of nucleic acid. The general, comparative, chemical composition between common antigen fractions is shown in Table I. The unfractionated EP antigens were comprised mainly of type-specific polysaccharides; they were reserved for future analysis.

Gel-permeation chromatography of the DEAE-Sephadex effluents from each common antigen fraction gave similar elution patterns (see Fig. 1). Typically, four peaks absorbing at 206 nm were detected. GPC I, not always observable, contained the smallest proportion of recoverable antigen (0–3%). It was weakly active in i.d., showing an immunoprecipitin line of identity with the weakest of three immunoprecipitin lines observed in the unfractionated antigens, ES 5 and ES 10 (see Fig. 2). The scarcity of GPC I prevented additional study thereof at present.

GPC IV was serologically inactive and was devoid of carbohydrate (see Fig. 1). This peak usually comprised 5–10% of the sample applied to the column. The material in Peak IV was reserved, and not studied further.

GPC III (see Fig. 1), the largest of the four fractions isolated, typically consisted of 45 to 60% of the applied sample. It was rich in hexosamine (see Table I, and Fig. 1 at 540 nm), and by i.d. produced a single immunoprecipitin line which corresponded to the major line observed with the unfractionated antigen (see Fig. 2).

GPC II (see Fig. 1), derived from the ES and HA samples, respectively contained an average of 8 and 20% of the sample applied. This peak contained considerably less hexosamine than GPC III, but contained significantly larger proportions of neutral sugar (see Table I). GPC II was serologically active by i.d., and showed

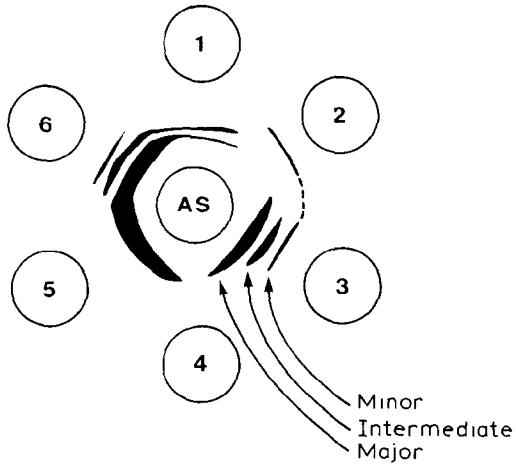


Fig. 2. A composite diagram of i.d. of selected antigens in gel. (Well 1 contained ES 9 GPC II, well 2, ES 9 GPC I; well 3, ES 10; well 4, ES GPC IV; well 5, ES 9 GPC III; and well 6, ES 5. The central well contained specific antisera against *C. perfringens* Hobbs 5.)

an immunoprecipitin line of identity with the intermediate precipitin line observed with the unfractionated antigen (see Fig. 2). A second immunoprecipitin line, corresponding to the major antigen (GPC III), was also observed; this was caused by incomplete separation of GPC II and GPC III. When individual fractions comprising GPC II were analyzed by i.d., only the intermediate immunoprecipitin line was evident in the ascending limb. The GPC II fractions could not be studied independently, as the quantity of material available was limited; therefore, various samples showing obvious serological and chemical identity were combined, and refractionated by g.p.c. The appropriate fractions were combined and analyzed (see Table I, GPC II Refractionated); GPC II Refractionated gave a single immunoprecipitin line of identity with GPC III by i.d. An antigen component corresponding to the intermediate immunoprecipitin line (see Fig. 2) was not recovered.

Monosaccharide analysis. — The chemical composition of the GPC III fractions, derived from the six cell-envelope extracts, prompted their division into two discrete groups that were directly related to the original isolation procedures employed. ES 5, ES 9, and ES 10 comprised the first group, and each contained 2-amino-2-deoxy-D-glucose (GlcN) and 2-amino-2-deoxy-D-mannose (ManN) in almost equimolar ratio as determined by g.l.c. (see Table II). The identities of the major hexosamines were determined by isoating GlcN and ManN by ion-exchange chromatography (see Fig. 3), followed by g.l.c. analysis of the resolved peaks. Treatment of the resolved hexosamines with ninhydrin produced only arabinose[20], thereby confirming their identities. A third component was isolated in low yield and was identified as 2-amino-2-deoxy-D-galactose (GalN; see Fig. 3).

The second group of GPC III fractions consisted of HA 5, HA 9, and HA 10, but, in this case, the carbohydrate composition was more complicated. In addition

TABLE II

MOLAR RATIOS OF THE CARBOHYDRATE CONSTITUENTS OF THE MAJOR COMMON ANTIGENS[a]

Fraction analyzed	*Molar ratios*					
	Rha	*GlcN*	*GalN*	*ManN*	*Gal*	*Phosphate*
ES 5 GPC III[b]	nil	0.92	nil	1	nil	0.02
ES 9 GPC III	nil	0.79	nil	1	nil	0.17
ES 10 GPC III	0.04(1.22)[c]	0.92	0.03(**1**)	1	0.04(1.39)	0.01
HA 5 GPC III	0.28(1.71)	1.02	0.16(**1**)	1	0.27(1.79)	0.30(3.9)
HA 9 GPC III	0.18(1.09)	0.89	0.16(**1**)	1	0.20(1.29)	0.10(1.6)
HA 10 GPC III	0.51(1.19)	0.85	0.43(**1**)	1	0.59(1.37)	0.20(1.1)
GPC II Refrac.	1.73(1.10)	0.88	1.57(**1**)	1	1.89(1.20)	(1.78)

[a]Except for phosphate, all analytical data were obtained by g.l.c. [b]See Table I for key to abbreviations. [c]The numbers in parentheses are compared to GalN taken as unity.

to GlcN and ManN, substantial quantities of rhamnose (Rha), GalN, and galactose (Gal) were present (see Table II). This was reflected in the higher neutral-sugar content observed for this group of antigens (see Table I).

Analysis by g.l.c. of the GPC II Refractionated showed that a similar, qualitative relationship existed between it and the HA group of Peak III antigens; but, quantitatively, Rha, GalN, and Gal were most abundant, with GlcN and ManN assuming a secondary, quantitative position (see Table II). Comparison of the five carbohydrate constituents of the HA GPC III and the GPC II Refractionated anti-

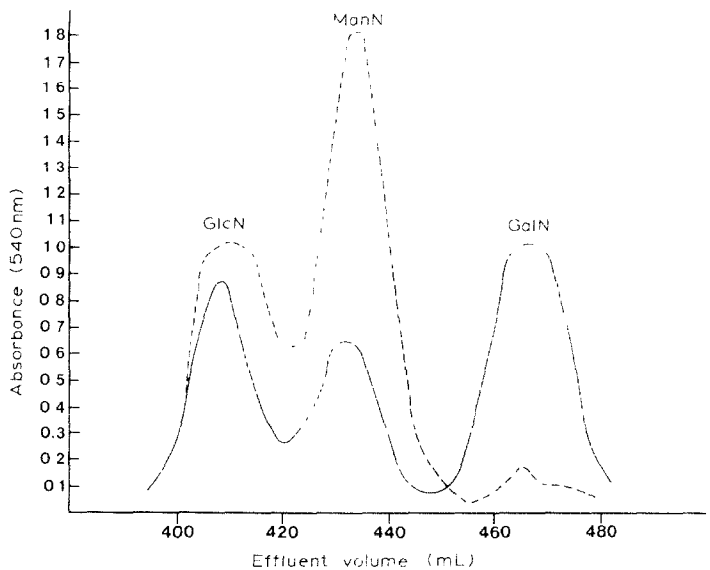


Fig. 3. Separation of GlcNAc, ManN, and GalN by ion-exchange chromatography on Dowex-50 X8-400 (H^+) ion-exchange resin. (Key: ——, standard mixture, and -----, antigen hydrolyzate.)

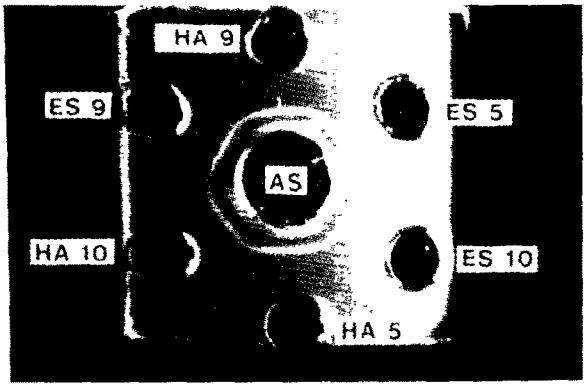


Fig. 4. Analysis, by i.d. in gel, of sodium periodate-treated GPC III antigens.

gens, with ManN arbitrarily assigned a value of unity, showed that a relatively constant, molar ratio existed only with GlcN (see Table II). However, when the data were compared on the premise that two distinct polysaccharides were present, the molar ratios of Rha, GalN, and Gal, with GalN arbitrarily assigned a value of unity, proved to be in fair agreement as well (see Table II, data in parentheses).

G.p.c. peaks compared by i.d. — The six GPC III fractions (ES 5, ES 9, ES 10, HA 5, HA 9, and HA 10) were serologically indistinguishable by i.d., although they were chemically divisible into 2 discrete groups. The GPC II Refractionated was not serologically distinct, and was equivalent to the major, immunoprecipitin line (GPC III).

Smith degradation. — Oxidation of any ES GPC III fraction resulted in the disappearance of GlcN and almost total recovery of ManN. The same experiment conducted with any HA GPC III fraction resulted in the complete oxidation of Rha, and major decomposition of GlcN (90%), with a lesser effect on GalN (30%), and Gal (50%). ManN was essentially unaffected.

Analysis by i.d. of the sodium periodate-treated antigens showed serological identity for all six fractions (see Fig. 4). However, the presence of an immunoprecipitin line close to the antiserum well for the HA fractions was rarely observed (see Fig. 4). Although the GPC antigens were subjected to drastic, chemical alteration, no apparent change in their serological activity was observed when they were compared, by i.d., with the corresponding, untreated antigens.

Treatment with hydrofluoric acid. — The DEAE-Sephadex antigens of ES 10 and HA 10 were treated with 48% hydrofluoric acid. The immunoprecipitin line corresponding to the major antigen was still observable by i.d. for both of the HF-treated samples. However, the intermediate immunoprecipitin line was not detected for either antigen. Analysis of the hydrofluoric acid-treated antigens showed that the percentage of hexosamine was essentially unchanged, whereas there was a significant decrease in phosphate content (see Table III). Analysis by g.l.c. of the HF-treated HA 10 antigen (DEAE-Sephadex-treated only) indicated no qualita-

TABLE III

ANALYSES OF ANTIGENS TREATED WITH HYDROFLUORIC ACID

	% (by weight)			
	ES 10[a]		*HA 10*	
	Control	*HF*	*Control*	*HF*
Hexosamine	34	33	30	30
Phosphate	1.1	0.3	4.8	1.7
Neutral sugar	6.8	5.3	14.1	11.5

[a]DEAE-Sephadex-derived, common-antigen fractions. See Table I for key to abbreviations.

TABLE IV

GENERAL COMPOSITION OF THE POLYSACCHARIDE DERIVED BY PARTIAL HYDROLYSIS WITH ACID

Fraction analyzed	*% (by weight)*			
	Acid hydrolyzate		*Alkaline phosphatase*	
	Phosphate	*Hexosamine*[a]	*Phosphate*	*Hexosamine*
ES 5 GPC III	—	2.7	0.2(67)[b]	2.9(5.9)
ES 9 GPC III	0.2	2.3	0.4(40)	2.4(3.8)
ES 10 GPC III	0.1	0.9	— —	1.2(3.2)
HA 5 GPC III	0.2	2.5	2.5(58)	2.4(6.3)
HA 9 GPC III	0.2	2.3	1.1(50)	2.4(5.2)
HA 10 GPC III	0.1	2.7	1.5(54)	2.6(7.4)
GPC II Refrac.	0.1	1.4	5.6(75)	1.2(5.2)

[a]Determined spectrophotometrically with GlcNAc as the standard. [b]The numbers in parentheses represent the percentage released, compared to the original concentration of the constituent in the untreated sample. See Table I for key to abbreviations.

tive difference in composition as compared to a control sample. However, an equimolar decrease in Rha and Gal was observed, but the decrease was not quantitated.

Hydrolysis with alkaline borohydride. — Treatment with alkaline borohydride completely eliminated the immunoprecipitin line commonly observed by i.d. However, *N*-acetylation of the treated samples restored the original, serological activity; a precipitin line of identity was observed when the acetylated sample was compared directly to an untreated control.

Partial hydrolysis with acid. — The small percentages (3–7%) of the total *N*-acetylhexosamine content of the GPC antigens, released by partial acid-hydrolysis, were essentially unchanged by subsequent treatment with alkaline phosphatase (see Table IV). Of the phosphate present in the untreated GPC antigens, ~10% was immediately released by mild hydrolysis with acid, but 40–75% of the phosphate was released by treatment of the hydrolyzates with alkaline phosphatase.

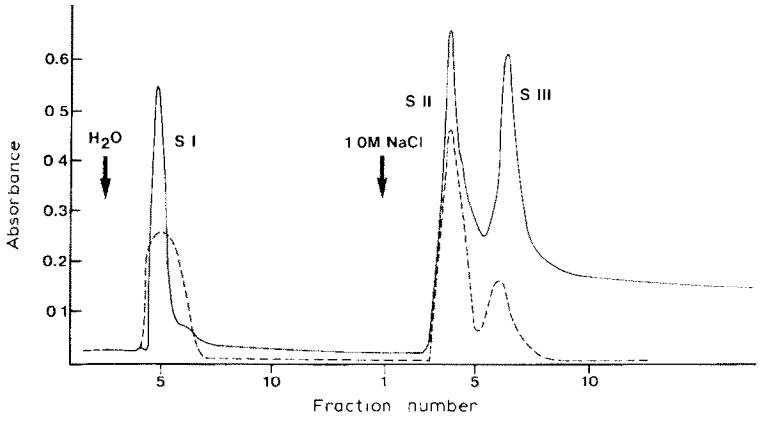


Fig. 5. Chromatography of succinylated, DEAE-Sephadex-derived, HA 10 antigen on DEAE-Sephadex A-25 (Cl^-) (Key: ——, at 206 nm; and -----, hexosamine, at 540 nm.)

After dialysis and total hydrolysis with acid, g.l.c. indicated that no significant change had occurred in the GlcN and ManN composition of all of the GPC III antigens. However, 40–60% of the Rha, GalN, and Gal was lost from the HA GPC III antigens. In addition, the precipitin line observed by i.d. was unchanged when all of the treated GPC antigens were compared to their corresponding, untreated controls.

Succinylated antigen. — The succinylated antigen (DEAE-Sephadex HA 10; 21 mg) gave three discrete peaks (SI, SII, and SIII) when applied to, and eluted from, DEAE-Sephadex (see Fig. 5). The first peak was not retained by the column,

TABLE V

COMPOSITION OF SUCCINYLATED ANTIGENS FRACTIONATED BY DEAE-SEPHADEX CHROMATOGRAPHY[a]

	Antigen HA 10[b] *Eluant*		*Antigen HA 9*[b] *Eluant*	
	Water (SI)	*M NaCl (SII)*	*Water (SI)*	*M NaCl (SII)*
	Percent by weight			
Hexosamine	29	23	39	27
Phosphate	3.6	0.6	3.5	1.3
	Molar ratios			
Rha	2.79(1.49)[c]	0.97(1.20)	n.d.[d]	0.26(1.51)
GlcN	1.42	1.00	n.d.	0.92
GalN	1.44(**1**)	0.81(**1**)	n.d.	0.21(**1**)
ManN	1.00	1.00	n.d.	1.00
Gal	2.16(1.51)	1.1(1.37)	n.d.	0.26(1.51)

[a]The molar ratios were determined by g.l.c. [b]DEAE-Sephadex-derived antigens. See Table I for key to abbreviations. [c]The numbers in parentheses are compared to GalN taken as unity. [d]N.d., not determined.

and was immediately eluted with water. The second and third peak were eluted with M NaCl (see Fig. 5). All three peaks contained hexosamine, and the cumulative, total recovery constituted 90% of the hexosamine applied to the column (see Table V). Peak SI and Peak SII respectively accounted for 28 and 53% of the experimental sample on a dry-weight basis. Peak SIII was not recovered in an amount sufficient for detailed chemical characterization; however, analysis indicated that Peak SIII contained 9% of the hexosamine originally applied to the DEAE-Sephadex column.

Peak SI formed a precipitin line of identity with the major, common-antigen fraction. Peak SII was serologically identical to the untreated antigens; two discrete immunoprecipitin lines of identity were observed that corresponded to the major and intermediate antigen fractions (see Fig. 2).

Analysis of the succinylated antigen showed that the peak eluted with water contained significantly higher concentrations of phosphate and hexosamine compared to the peak eluted with sodium chloride (see Table V). Comparison of the analytical results from peaks SI and SII (see Table V) showed constancy in chemical content, and only minor, quantitative differences.

Amino acid analysis. — Analysis of the DEAE-Sephadex-derived antigens revealed the presence of 3–5% of peptide. No constant pattern of amino acid composition was observed. Examples of analyses are given in Table VI.

^{13}*C-N.m.r. spectroscopy.* — The proton-decoupled spectrum of the major

TABLE VI

AMINO ACID ANALYSIS OF SELECTED FRACTIONS OF ANTIGEN

Amino acid	*HA 5* (*μmol/mg*)	*ES 9* (*μmol/mg*)
Alanine	0.0190	0.0647
Arginine	0.0126	—
Aspartic acid	0.0380	0.0128
Cystine/2	—	—
Glutamic acid	*0.0363*	*0.0371*
Glycine	0.0277	0.0340
Histidine	0.0052	0.0295
Isoleucine	0.0173	0.0037
Leucine	0.0157	0.0053
Lysine	0.0239	
Methionine		0.0326
Phenylalanine	—	—
Proline	0.0137	0.0029
Serine	*0.0176*	*0.0139*
Threonine	0.0130	0.0053
Tryptophan	—	—
Tyrosine	—	—
Valine	0.0256	0.0018
Peptide (%)	3.3 ±1	3.0 ±1

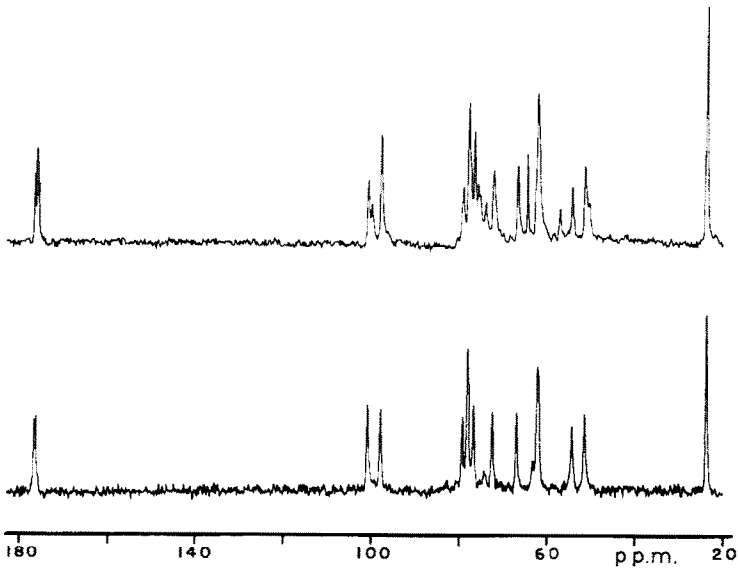


Fig. 6. The ^{13}C-n.m.r. spectra of major common antigen (MCA, top) and Smith-degraded, major common antigen (S-MCA, bottom) of *C. perfringens* type A.

common antigen (MCA) constituting the pooled fractions (ES 5 and 9, GPC III) is given in Fig. 6, together with the corresponding spectrum of the product obtained by Smith degradation of the major common antigen (S-MCA). Both of these spectra exhibited resonances characteristic of polysaccharides containing *N*-acetylhexosamine residues.

The most-upfield and -downfield signals in each spectrum occurred where resonances from the *N*-acetyl group would be expected to appear. The most-intense and most-upfield signal (~23.6 p.p.m.) in each spectrum was probably due to unresolved, overlapping resonances of almost identical methyl groups. In the spectrum of MCA, there was, at 23.9 p.p.m., an almost resolved signal having an intensity approximately one third of that of the resonance at 23.5 p.p.m. The most-downfield signal for the S-MCA consisted of two closely spaced resonances of equal intensity, at 176.5 and 176.2 p.p.m., and a low-intensity, poorly resolved shoulder at slightly higher field. These are characteristic of carbonyl absorptions, and the two signals corresponded almost exactly to signals for the native material. In the spectrum of MCA, there also appeared at slightly higher field at least three additional carbonyl resonances (175.9, 175.8, and 175.5 p.p.m.), not well resolved from the pair observed for the S-MCA.

In the spectral region where anomeric carbon atoms absorb (~100 p.p.m.), the spectrum of S-MCA exhibited two well resolved resonances of equal intensity, and that of MCA had additional resonances, with poorer resolution, and unequal intensities. Four definitive signals, apparently representing at least five resonances, were observed at 100.6, 99.9 (minor intensity), 99.5 (lowest intensity), and 97.7 p.p.m. (double intensity) for the MCA polysaccharide.

The degradation product, S-MCA, also exhibited a remarkably simple spectrum in the absorption region characteristic of resonances for the other carbon atoms of the pyranose rings. This region contained ten additional, major signals, including two overlapping sets (78.0 and 77.9 p.p.m.; 62.1 and 61.9 p.p.m.). These ten signals were of approximately equal intensities. There also occurred a poorly resolved, minor signal just downfield from the composite set at 62 p.p.m. In this spectral region, the major common antigen exhibited a greater number of signals, with varied intensities and poorer resolution. Five signals were observed for the MCA polysaccharide in the spectral region where C-2 atoms should absorb. These signals had the following chemical shifts: 56.9, 54.2, 51.3, 50.9 (shoulder), and 50.5

TABLE VII

CHEMICAL SHIFTS[a] AND COUPLING CONSTANTS[b] FOR THE SMITH-DEGRADATION PRODUCT OF THE NATIVE POLYSACCHARIDE, ES GPC III

Carbon atom	*Anomeric configuration*	D-*ManNAc*[c]	*ES GPC III*[d] *IO_4 oxidation*	*Chem. shift*[e] *difference*	*Mannan*[f] *chemical-shift difference*	*Difference*[g] *in chemical-shift difference*
1	α	94.7				
	β	94.7	97.7d (162)	3.0	3.5	−0.5
2	α	54.9				
	β	55.7	51.4d (140)	−4.3	−3.4	−0.9
3	α	70.5				
	β	73.7	79.1d (144)	5.4	6.4	−1.0
4	α	68.7				
	β	68.4	66.9d (146)	−1.5	−1.7	0.2
5	α	73.7				
	β	78.0	77.9d (145)	−0.1	0.1	−0.2
6	α	62.3				
	β	62.3	62.1t (146)	−0.2	0.0	−0.2
1′	β		100.7d (162)	6.0	6.4	−0.4
2′	β		54.2d (146)	−1.5	−0.8	−0.7
3′	β		72.3d (139)	−1.4	−1.5	0.1
4′	β		77.9d (145)	9.5	9.9	−0.4
5′	β		76.6d (146)	−1.4	−1.0	−0.4
6′	β		62.1t (146)	−0.2	−0.4	0.2
CH_3	α	23.6				
	β	23.7	23.6q (129)			
C=O	α	176.2				
	β	177.1	176.2s, 176.5s			

[a]Chemical shifts, measured at 70°, in p.p.m. downfield from an external capillary sample of tetramethylsilane. The values are believed to be accurate to within ±0.03 p.p.m. See the Results section of the text for analogous chemical-shifts of the native polysaccharide, ES GPC III. [b]Apparent coupling-constants, $^1J_{C\text{-}1,H\text{-}1}$, in Hz. Values are in parentheses. [c]Chemical-shift assignments for 2-acetamido-2-deoxy-D-mannopyranose are given by analogy to ref. 32, or are based on relative intensities. [d]The Smith-degradation product from ES GPC III. [e]The chemical-shift differences of the carbon atoms of the polysaccharide and the analogous atoms of 2-acetamido-2-deoxy-β-D-mannopyranose. [f]The chemical-shift differences of the carbon atoms of the D-mannan from *R. glutinis*, and the analogous atoms of β-D-mannopyranose, as given in ref. 31. [g]The differences between analogous pairs of values from footnotes *e* and *f*.

p.p.m. The eleven following additional signals were observed for the native polysaccharide: 79.0, 77.7, 77.3 (shoulder), 76.5, 75.6 (minor), 75.2 (minor), 72.0, 73.8 (minor), 66.7, 64.4, and 62.0 p.p.m. The signal at 64.4 p.p.m. was atypically narrow, and may have been due to a low-molecular-weight contaminant (probably ethylene glycol) which was incompletely removed by dialysis.

Table VII contains the chemical shifts for 2-acetamido-2-deoxy-β-D-mannopyranose, and of the S-MCA as obtained for the spectrum presented in Fig. 6, all measured under isometric conditions. Also given in parentheses, for the S-MCA, are the one-bond, carbon–hydrogen coupling-constants obtained from nuclear Overhauser-enhanced spectra.

DISCUSSION

In a previous study, Dayalu *et al.*[9] presented evidence for the presence of common antigens in the cell envelope of several serotypes of *C. perfringens* type A. The unfractionated antigens, obtained by the extraction of whole cells with water or acetic acid at 100°, were compared by i.d. Their results showed that a maximum of three precipitin lines were formed by any one antigen preparation, and that none of the precipitin lines was unique to any one antigen fraction[9]. Several of the antigen fraction possessed all three precipitin lines, whereas others formed only one or two. The major, serologically active, common antigen has now been resolved by gel-permeation chromatography. The order of elution from the g.p.c. column was correlated with the rate of diffusion of the various components present in each antigen fraction; *e.g.*, the material responsible for forming the precipitin lines having the slowest and fastest rates of diffusion were respectively eluted early (GPC I) and late (GPC III) from the g.p.c. column (see Figs. 1 and 2).

The major, common-antigen fraction (MCA), ES GPC III, obtained from *C. perfringens* type A, Hobbs 5, Hobbs 9 and Hobbs 10, was a neutral polysaccharide composed mainly of ManNAc and an almost equal proportion of GlcNAc. Smith degradation of this species resulted in destruction of its constituent GlcNAc, but not in the destruction of the ManNAc. The serological specificity observed was associated with ManNAc residues, as the S-MCA, which completely lacked intact GlcNAc, gave a precipitin line of identity with the untreated, ES GPC III antigen. The *N*-acetyl group appeared to be required for the formation of a precipitin line, as antigen treated with alkali–borohydride was not serologically active by i.d unless it was re-*N*-acetylated with aqueous acetic anhydride.

The second group of common antigens, HA GPC III, was purified by the same procedures used in the isolation of the ES GPC III fractions. The serological and physical properties of the two groups of antigens (ES GPC III and HA GPC III) were identical, and it was natural to assume that their carbohydrate composition would also match. However, this proved not to be the case, as all of the HA GPC III fractions contained Rha, GalN, and Gal, in addition to GlcN and ManN. The molar ratios of Rha, GalN, and Gal were not constant when compared to

ManN, but their molar ratios were consistent when compared to GalN (see Table II). Therefore, the HA GPC III antigens were most probably comprised of two separate polysaccharides: (*1*) ManNAc and GlcNAc were the components of the first polysaccharide (MCA), and it exhibited serological activity; (*2*) Rha, GalNAc, and Gal were the constituents of the second polysaccharide (RGG), and it exhibited no serological activity. The chemical composition and physical properties of the RGG polymer closely resembled those of a polysaccharide isolated from a strain of *C. perfringens* type A as described by Pickering[23].

The third common-antigen group consisted of one member, GPC II Refractionated. GPC II Refractionated was similar in composition to HA GPC III, except that it had a higher apparent average molecular weight and was composed mainly of the RGG polysaccharide. However, ManNAc and GlcNAc were also detected, and, presumably, the MCA polysaccharide was present. The occurrence of MCA was probably responsible for the precipitin line of identity (observed by i.d.) that formed with the ES GPC III and HA GPC III fractions; both contained the MCA polysaccharide. The carbohydrate molar ratios (see Table II) and serological properties of GPC II Refractionated supported the postulated existence of two separate polysaccharides, MCA and RGG.

The observation that treatment with HF did not alter the appearance of the major immunoprecipitin line formed by i.d., for ES 10 and HA 10, indicated that phosphate was not an essential participant in the covalent structure of the backbone of the major common antigen (MCA). Retention of the major immunoprecipitin line formed by i.d., even with the partial loss in Rha and Gal due to treatment of HA 10 with HF, was consistent with the postulated occurrence of the serologically active MCA and the serologically inactive RGG (see preceding).

The hexosamine composition and the i.d. patterns for ES GPC III and HA GPC III antigens were essentially unaffected by the combination of partial hydrolysis with acid and digestion with phosphatase. However, a large concomitant release of inorganic phosphate occurred. This behavior supported the previously stated conclusion regarding the nonessential nature of phosphate in the covalent structure of the MCA.

Carboxyl functional groups were successfully introduced into the covalent structure of the common antigen by treatment with succinic anhydride. The comparative carbohydrate composition of the DEAE-Sephadex-bound, S II, and the DEAE-Sephadex-unbound, SI, gave additional support for the presence of two separate polymers, MCA and RGG, as the molar ratios were consistent only if two separate polymers existed (see Table V). Unfortunately, succinylation did not aid in resolving the MCA and RGG mixture.

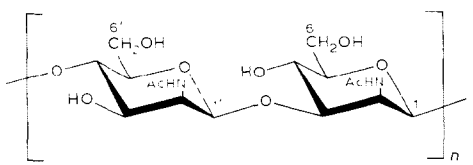
HA GPC III and ES GPC III contained between 3 and 4% of peptide. The amino acid composition of the analyzed fractions seemed to be randomly produced during their isolation and purification, as the molar ratios of the individual residues rarely agreed. However, serine and glutamic acid appeared to be present in constant proportions. The importance of this observation as it pertains to the identity

of a possible site of linkage, if one truly exists between polysaccharide and peptide, is not known.

The ^{13}C-n.m.r. spectrum of S-MCA exhibited sixteen major signals having similar intensities. Some of the signals occurred as closely spaced, or even as unresolved, pairs. There were two equal-intensity resonances due to two different, anomeric carbon atoms; the chemical shifts and carbon–hydrogen coupling-constants observed (162 Hz) for both of these signals were consistent with the presence of axial anomeric hydrogen atoms[24–26], as would be anticipated for 2-acetamido-2-deoxy-β-mannopyranosyl residues. Although the one-bond, carbon–hydrogen coupling-constants for the anomers of 2-acetamido-2-deoxymannopyranose itself have apparently not yet been reported, those for the α and β anomers of 2-amino-2-deoxy-D-mannopyranose have been given as 170 and 165 Hz, respectively[27]. The absence of signals near 73.7 p.p.m. also suggested that the ManN residues present were not in the α-pyranoid configuration. This resonance was prominent in the spectrum of 2-acetamido-2-deoxy-α-D-mannopyranose (see Table VII) and had been observed relatively unchanged in that of an *O*-deacetylated polysaccharide from *Neisseria meningitidis* containing this pyranose[28], but was absent from those of other polysaccharides containing the analogous β-pyranose[29,30].

These factors, along with the occurrence of eight pairs of carbon resonances and the absence of significant numbers of minor signals, suggested the presence of a linear polysaccharide composed of pairs of differently linked 2-acetamido-2-deoxy-β-mannopyranosyl residues. Because Smith degradation failed to decompose the ManNAc, these monosaccharide units must have had substituents at either O-3 or O-4. This fact, and the relative simplicity of the spectrum, led to the postulate that S-MCA must consist of a polysaccharide having alternating β-(1→4)- and β-(1→3)-linked 2-acetamido-2-deoxymannopyranosyl units, although random or block sequences, with a nonalternating order of attachment, could not be unequivocally precluded.

Fortuitously, the ^{13}C-n.m.r. spectrum of a structurally well characterized polysaccharide involving analogous D-mannopyranoside linkages had previously been assigned through selective, isotopic enhancement. Gorin[31] reported the detailed assignment of the twelve ^{13}C-n.m.r. signals in the spectrum of the D-mannan from *Rhodotorula glutinis*, as well as the chemical-shift differences between the signals of the D-mannan and β-D-mannopyranose, measured at 70°. Application of these chemical-shift differences to the corresponding chemical-shifts of 2-acetamido-2-deoxy-β-D-mannopyranose as measured in this investigation, under conditions identical to those used for the spectra in Fig. 6 (given in Table VII), permitted assignment of the signals from S-MCA to specific carbon atoms in **1** .



1

These assignments were based on the assumptions that the chemical shifts of 2-acetamido-2-deoxy-β-D-mannopyranose reported previously[32] were assigned correctly, and that the chemical-shift differences between 2-acetamido-2-deoxy-β-D-mannopyranose and the 2-acetamido-2-deoxy-β-mannopyranosyl residues in **1** are comparable to those between β-mannopyranose and the D-mannosyl residues in the D-mannan from *R. glutinis*. The assignments thus established are given in Table VII, as are the chemical-shift differences observed between the assigned resonances and those of 2-acetamido-2-deoxy-β-D-mannopyranose. The observed differences compare very favorably with those reported by Gorin[31] for the corresponding twelve signals in the D-mannan from *R. glutinis*. The average difference between the two sets of chemical-shift differences is 0.4 p.p.m. The average is exceeded substantially by only three of the twelve values, namely, those for the signals of C-2, C-2′, and C-3. The C-2 and C-2′ atoms contrast most significantly with the corresponding atoms of D-mannose, as the OH-2 group in D-mannose is replaced in the former by the 2-acetamido substituent, which is not only different in electrical and hydrogen-bonding characteristics, but is significantly larger.

It was, therefore, not surprising that the signals for these carbon atoms in the spectrum of this 2-acetamido-2-deoxymannose polysaccharide exhibit a larger upfield displacement from those of the corresponding monosaccharide (due, in part, to steric compression resulting from either adjacent O-1 or O-3 substitution) than was the case for the mannose analogs. Substitution at O-3 results in a downfield displacement of the C-3 resonance by 6.4 p.p.m. for the *R. glutinis* mannan, and in a similar, but slightly smaller, displacement of 5.4 p.p.m. in that of the polysaccharide described herein. This could result from a steric compression produced by the close proximity of the relevant carbon atom to the acetamido group on C-2. The assigned chemical shifts given in Table VII, although not identical to, seem to be in satisfactory agreement with, those for carbon atoms in somewhat similar 2-acetamido-2-deoxy-β-D-mannopyranosyl residues reported recently by other investigators[29–31]. When significant differences exist, they may be attributable to the various other residues to which the 2-acetamido-2-deoxy-D-mannopyranosyl units were attached, the various media in which the spectra were determined, and the various techniques employed in referencing the chemical shifts in the literature cited.

The several factors aforementioned constitute rather convincing evidence supporting **1** for the structure of the polysaccharide that remained after Smith degradation of MCA in the ES GPC III fractions. This S-MCA polysaccharide probably constitutes the backbone structure of the native material. It is not, however, possible at present to propose a structure for the native MCA as satisfactorily supported by ^{13}C-n.m.r. data. In the native MCA, there occurs almost as much GlcNAc as there is ManNAc. Because all of the GlcNAc residues were decomposed by the periodate oxidation, they did not have substituents attached to O-3 or O-4. They could have had substituents at O-6, have been part of a separate polysaccharide that was not separated by the various purification procedures, been

(1→6) self-linked, or been attached as self-linked chains or as individual units linked to various positions on the backbone polysaccharide.

From the two spectra in Fig. 6, it appeared that the backbone O-4 atom was not frequently substituted in the native MCA. The C-4 resonance, at 66.9 p.p.m., of the Smith-degradation product was strongly and sharply present in both spectra. If O-4 of S-MCA were substituted in the native material, the associated C-4 resonance should be displaced downfield by several p.p.m., and there appears to be no other resonance in the degradation product, or from GlcNAc, that could have replaced it. In the spectrum of the native material were found signals that could be rationalized in terms of partial substitution at all of the other backbone positions available (O-3′, O-6, and O-6′). Unfortunately, no exclusive substitution-pattern for the native polysaccharide emerged from consideration of the resonances of the 2-acetamido-2-deoxy-D-mannopyranosyl residues.

Consideration of the resonances expected for 2-acetamido-2-deoxy-D-glucopyranosyl residues also did not lead to a hypothesis regarding the structure of the native material. In the spectrum of the native material, there was one broadened resonance at 72.1 p.p.m. The absence of substantial intensity of resonances in this part of the spectrum, such as should be characteristic of the chemical shifts of C-3, C-4, and C-5 of 2-acetamido-2-deoxy-α-D-glucopyranosyl residues, strongly suggested that the GlcNAc residues had the β, not the α, configuration. Contrary to this concept, however, was the absence of a C-1 resonance below 100 p.p.m., such as we have observed in the spectrum of chitotriose (C-1 resonances: 103.0, 102.8, 96.5, and 92.1 p.p.m.), which has 2-acetamido-2-deoxy-β-D-glucopyranosyl linkages. In the native MCA, GlcNAc residues may often be attached to a ManNAc residue and, therefore, their comparison with chitotriose may not be valid.

In the spectral regions for resonances of carbonyl carbon, anomeric carbon, and C-2 atoms in the native material, there was chemical-shift or intensity evidence for the presence of at least five different, principal kinds of pyranosyl residues.

The conclusions that may be drawn from the foregoing considerations are as follows. (*1*) Assuming the structure assigned to the D-mannan from *R. glutinis*[31] was correct, S-MCA is structurally analogous, with alternating (1→4)- and (1→3)-linked 2-acetamido-2-deoxy-β-mannopyranosyl residues. (*2*) In the native MCA, O-4 (see **1**) of ManNAc remained unsubstituted. (*3*) Some, but not necessarily all, of the GlcNAc residues have the β configuration. Probably, most of them are β, but difficulties remain in reconciling the chemical shifts observed with those expected for their C-1 and C-2 atoms. (*4*) In the native MCA polysaccharide, there is probably no regular, highly repeating connection of the GlcNAc residues to the ManNAc backbone. The GlcNAc may be randomly connected, as there was a lack of significant intensity for any particularly identifiable resonances. (5) A second, serologically inactive polysaccharide (RGG), composed of Rha, GalNAc, and Gal was identified, but it was not obtained homogeneous. The Rha residues were probably situated as nonreducing antennae, as they were quantitatively removed by Smith degradation without concomitant decomposition of the polymeric structure

of the remaining residues. The possible participation of the 3% of peptide, or of the phosphate, in the covalent structure of the two polysaccharides was not determined.

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