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ANALYTICAL CHARACTERIZATION OF LIPOPOLYSACCHARIDE ANTIGENS FROM SEVEN STRAINS OF *Pseudomonas aeruginosa**†‡

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ABSTRACT

Lipopolysaccharide antigens from seven different serotype strains (antigen immunotypes Nos. 1–7 in the classification of Fisher *et al.*[3]) of *Pseudomonas aeruginosa* have been analyzed for neutral carbohydrate, amino sugars, lipid, protein, 3-deoxy-*manno*-octulosonic acid, and phosphorus. The individual amino sugars were determined for each antigen type; all contained 2-amino-2-deoxy-D-glucose and -D-galactose, together with 2-amino-2,6-dideoxygalactose; the latter as isolated from the type 2 antigen was identified as the DL form. In addition, 2-amino-2,6-dideoxy-D-glucose was present in the types 3, 4, and 5 antigens. Mild, acid hydrolysis of the antigens gave the lipid A component containing all of the lipid and 2-amino-2-deoxy-D-glucose, together with lipid A-free polysaccharides that contained principally carbohydrate. The lipid A-free polysaccharides all contained L-rhamnose and D-glucose, together with 2-amino-2,6-dideoxygalactose in all except those from types 1, 5, and 7; that from type 6 also contained D-xylose.

INTRODUCTION

Pseudomonas aeruginosa is a potentially dangerous, opportunistic bacterial pathogen. Although the human immunological system is normally effective in protecting the healthy host against this organism, severe and frequently lethal infections are common in debilitated patients whose immunological defense-system has been weakened by illness or by trauma arising from severe burns or from surgery. Infections resulting from virulent strains of *P. aeruginosa* are especially troublesome if they become established in hospitals. Patients having extensive burns are parti-

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cularly prone to *Pseudomonas* sepsis and, as the common antimicrobial agents are largely ineffective in combating this Gram-negative organism, the vast majority of such patients fail to survive[4].

Reinforcement of the immunological defense of the susceptible host provides an alternative and adjunctive route for treatment of *Pseudomonas* infections. In principle, this reinforcement may be achieved prior to the onset of infection by active immunization with the bacterial antigen in order to stimulate the autoimmune response of the host. For treatment of established infections, the use of hyperimmune globulin raised in donors by administration of antigen preparations offers a second line for therapeutic control of *Pseudomonas* sepsis.

A major complication in the immunological approach arises from the fact that *Pseudomonas aeruginosa* is, serologically, exceedingly complex, and it is evident that a multiplicity of different antigens (capable of stimulating production of their specific, complementary antibodies) is to be expected among the many hundreds of strains of the organism that have been examined. Several schemes for bacteriological classification have been described[5–7] based on serological reactions *in vitro*, usually by whole-cell agglutination responses. However, these bacteriological serotypes bear no direct relation to the immunological behavior of each strain, and consequently, Fisher *et al.*[3] undertook development of a new classification scheme, based upon challenge protection in mice to whole-cell, killed vaccine from individual *P. aeruginosa* strains. This work led to the definition of a new immunotype classification that recognized seven distinct groups (immunotypes) of cross-protective homogeneity, into which almost all of the bacteriological serotypes could be accommodated. The utility of this immunotype schema for monitoring *P. aeruginosa* in high-risk patients and in the hospital environment has been demonstrated[8]. In the latter study, all but 4 of 755 strains examined were successfully classified[8] into the seven immunotypes (Types 1–7) of Fisher *et al.*[3], and comparisons of these type designations with some of the earlier bacteriological serotype classifications[5–7] were also presented.

The individual antigens isolated[1] from serotype strains representative of the seven immunoprotective types[3] were subsequently utilized, in combination, as a heptavalent vaccine[9] (Pseudogen®) for prophylactic use against invasive infection; administration of the vaccine stimulates a dose-related antibody response[10] that has been exploited to afford a dramatic decrease in the mortality rate from *Pseudomonas* sepsis in severely burned patients[4,10]. Similar immunization of patients suffering from neoplastic diseases has also demonstrated a significant decrease of *Pseudomonas*-associated deaths[11].

As the polyvalent vaccine consists of endotoxins that have certain undersirable side-reactions when administered to human subjects[11], the use of globulin extracted from hyperimmune plasma raised in healthy human donors in response to the vaccine has also been evaluated[10,12] as a passive, immunochemical alternative or adjunct[10] for treatment of established, life-threatening *Pseudomonas* infections; the preparation employed[10,12] was obtained by fractionation of the plasma to provide a hyperimmune gamma-globulin containing IgG antibodies against the seven *Pseudo-*

monas immunotypes. This globulin proved strikingly effective in combating established *Pseudomonas* sepsis, especially in burn patients[10,12,13].

The antigens from *Pseudomonas aeruginosa*, immunotypes[3] 1–7, are high-molecular-weight lipopolysaccharides whose isolation from phenol-killed cell-pastes, their purification, and analytical composition have been described in preliminary reports[1,2]. This article now records details of the analytical characterization of the seven lipopolysaccharides, and further studies[14,15] (to be detailed later) are concerned with compositions of the individual antigens, and structural elucidation of component parts of each. These detailed chemical studies on the seven immunotype antigens were prompted by several considerations. First of all, knowledge of their chemical architecture might permit their modification to provide immunogenic materials exhibiting decreased toxic side-effects. In addition, chemical data on the antigens should provide a valuable tool for the rapid diagnostic identification and classification of *Pseudomonas* strains isolated from hospital subjects, as well as a basis for correlation of the immunotype classification[3] with classifications[5–7] based on bacteriological serotypes through comparison of data on these seven antigenic preparations with chemical data already recorded[16–20] for lipopolysaccharides isolated from a number of *Pseudomonas* serotypes. Furthermore, detailed structural characterization of these endotoxins would be useful in efforts to elucidate the molecular basis of the antigen–antibody interaction, and could also throw light on the interferon-stimulating activity of these antigens; the latter aspect could be very rewarding, especially in the areas of cancer and viral diseases.

RESULTS AND DISCUSSION

Each of the seven immunotype antigens[3], as purified and isolated for use in vaccine formulation[1], was obtained as a fluffy, freeze-dried solid that dissolved in water to give an opalescent solution. The Folin phenol assay[21] showed that all of them contained a small proportion (3–8%) of apparent protein (see Table I) whose presence may contribute to the pyrogenic reaction observed[8] on injection of the vaccine into human subjects. All of the antigens contained 2–4% of phosphorus (see Table I) associated with the lipid A component of the antigens. Analyses for 3-deoxy-D-*manno*-2-octulosonic acid[22] showed 4–5% of this component in each antigen (see Table I). Carbohydrate analyses on the seven lipopolysaccharides (see Table I) showed neutral carbohydrate ranging from a low of 12% (type 3) to a high of 18% (types 2 and 5), as determined by the phenol–sulfuric acid method[23]. Analyses for 2-amino-2-deoxy sugars in acid hydrolyzates of the antigens, by use of the Elson–Morgan reaction[24–26], gave values ranging from a low of 11% (type 6) to a high of 27% (type 4).

None of the foregoing analyses provide a clear-cut differentiation that would be useful as the basis of a rapid, chemical method for assigning the antigens into their respective immunotype classes. However, detailed examination of the distribution of amino sugars in the seven antigen types revealed distinctive, individual differences

TABLE I

ANALYTICAL COMPOSITIONS OF ANTIGENS OF *Pseudomonas aeruginosa* IMMUNOTYPES 1–7

Antigen immunotype[3]	*Percent composition*					
	Neutral carbohydrate[a]	*Amino sugars*[b]	*Lipid A*[c]	*KDO*[d]	*Protein*[21]	*P*
1	15	17	13	5	4	3
2	18	22	16	4	5	2
3	12	18	6	4	8	3
4	14	27	19	4	3	2
5	18	14	18	5	7	2
6	17	11	15	4	3	3
7	14	14	8	5	3	4

[a]Phenol–sulfuric acid method[23]. [b]Elson–Morgan reaction[24–26]. [c]After hydrolysis with acetic acid and centrifugation, or extraction with chloroform; see Experimental section for details. [d]3-Deoxy-D-*manno*-2-octulosonic acid[22].

TABLE II

RELATIVE PROPORTIONS OF AMINO SUGARS IN THE ANTIGENS OF *Pseudomonas aeruginosa* IMMUNOTYPES 1–7

Antigen immunotype[3]	*Relative proportions of amino sugars*[a]			
	2-Amino-2,6-dideoxyglucose (1.51)[b]	*2-Amino-2,6-dideoxygalactose* (1.59)[b]	*2-Amino-2-deoxyglucose* (4.63)[b]	*2-Amino-2-deoxygalactose* (5.41)[b]
1	—	26	49	25
2	—	81	11	8
3	4	39	28	29
4	10	55	12	23
5	60	—	25	15
6	—	60	23	17
7	—	38	38	24

[a]Determined after vigorous hydrolysis with hydrochloric acid, borohydride reduction, acetylation, and determination by g.l.c.; see Experimental section for details. [b]G.l.c. retention-times of alditol acetates relative to that (1.00) of glucitol hexaacetate.

between the seven lipopolysaccharides. The antigens were hydrolyzed with 6M hydrochloric acid, and the resulting sugars (containing a high proportion of amino sugars), after conversion into their alditol acetates, were analyzed by quantitative g.l.c.[27]. Identities of amino sugars among the products were confirmed by their giving retention times and mass spectra identical to those given by authentic reference samples of the amino sugars that had been similarly treated. The relative, amino sugar compositions found are recorded in Table II. Neutral sugars present gave alditol acetates migrating more rapidly than the amino sugar derivatives. Some faster-

migrating products, probably arising from neutral dideoxy sugars, were also observed.

All seven immunotype antigens contained both 2-amino-2-deoxyglucose and 2-amino-2-deoxygalactose, and all except type 5 contained 2-amino-2,6-dideoxygalactose. Type 5 contained another amino sugar, one that was resolved only with difficulty from 2-amino-2,6-dideoxygalactose in the g.l.c. system employed; it was identified[28] as 2-amino-2,6-dideoxyglucose, and this amino sugar was also present in the types 3 and 4 antigens.

The amino sugar analyses recorded in Table II display a recognizably distinct and characteristic pattern for each of the seven antigens, a feature that might be utilized as the basis for a chemical method for assigning the individual lipopolysaccharides to the appropriate immunotype categories. The strains of *P. aeruginosa* used for producing the seven immunotype antigens were, in each instance, single bacteriological serotypes[29]. Chemical differences that may exist between the antigens from other serotypes belonging to the same immunotype remain to be established.

The fatty acid compositions of the seven antigens were examined by saponification, followed by g.l.c. analysis of the methyl esters of the resultant fatty acids. Five different acids were observed as major constituents of the mixture in each instance, and their g.l.c. behavior and relative proportions are recorded in Table III. Several trace-components were observed in most instances. Although these fatty acids showed some quantitative differences in their distribution according to antigen immunotype, they did not display the marked qualitative and quantitative differences that characterize the amino sugar distributions.

TABLE III

FATTY ACID COMPOSITIONS[a] OF *Pseudomonas aeruginosa* IMMUNOTYPE ANTIGENS, TYPES 1–7

Antigen immunotype[3]	*Relative amount of fatty acid (as percent of total fatty acid)*					*Number of trace peaks*
	A (13.45)[b]	*B* (15.35)[b]	*C* (15.6)[b]	*D* (18.2)[b]	*E* (20.1)[b]	
1	13	18	51	9	9	3
2	8	18	47	13	14	4
3	14	16	40	19	11	5
4	8	15	43	17	17	0
5	4	18	51	13	14	3
6	5	14	43	18	19	8
7	3	13	44	23	17	1

[a]See Experimental section for details. [b]Retention time (min) of methyl ester on a 1.3-m, glass column packed with 3% of OV-17, programmed from 60–300° at a rate of 10° per min; the major fatty acids (A–E) were not characterized by any direct method, but the retention times of their methyl esters were similar to those of the reference esters listed in the Experimental section.

Treatment of the antigens with 1% acetic acid for 1–2 h at 100° splits each lipopolysaccharide into the water-insoluble, lipid A component (compare ref. 30) and a water-soluble fraction containing lipid-free, polysaccharide material together with

components of low molecular weight. As shown in Table I, the content of lipid A ranged from a low of 6% (type 3) to a high of 18–19% (types 4 and 5). Most of the 2-amino-2-deoxyglucose present in the original lipopolysaccharides was removed in the lipid A fraction. The lipid A component presumably has a gross structure similar to that of that obtained from lipopolysaccharides of other Gram-negative bacteria[30–32], and the i.r. spectra of the lipid A from each of the seven antigens were essentially identical. Detailed structural analyses of these components have not yet been performed.

Dialysis of the lipid A-free, aqueous phase with a hollow-fiber apparatus having a nominal, molecular-weight cutoff of 5000 daltons gave a lipid A-free polysaccharide fraction in yields that ranged from 35% (type 1) to 49% (type 7) of the original lipopolysaccharide. The mild, acid hydrolysis by 1% acetic acid presumably[31,32] splits the lipopolysaccharides at the acid-labile, 3-deoxy-D-*manno*-2-octulosonic acid link. That fraction of each antigen not accounted for as non-dialyzable polysaccharide plus lipid A is evidently removed by dialysis as low-

TABLE IV

RELATIVE PROPORTIONS[a] OF NEUTRAL CARBOHYDRATES AND 2-AMINO-2,6-DIDEOXYHEXOSES IN LIPID A-FREE POLYSACCHARIDES FROM ANTIGENS OF *Pseudomonas aeruginosa* IMMUNOTYPES 1–7

Antigen immuno-type[3]	*Yield of lipid A-free polysac-charide* (%)	*Relative proportions in the lipid A-free polysaccharide*[b]				
		Rhamnose (0.23)[c]	*Xylose* (0.45)[c]	*Glucose* (1.00)[c]	*2-Amino-2,6-dideoxyglucose* (1.51)[c]	*2-Amino-2,6-dideoxygalactose* (1.59)[c]
1	35	56	—	44	—	—
2	45	6	—	38	—	56
3	43	14	—	70	2	14
4	41	17	—	25	8	43
5	45	51	—	37	12	—
6	31	24	18	31	—	26
7	49	24	—	76	—	—

[a]Determined by g.l.c. of the derived alditol acetates, after hydrolysis of the lipid A-free, polysaccharide fraction; see Experimental section for details. [b]Not including 2-amino-2-deoxygalactose, which is also present in this fraction. [c]G.l.c. retention-times of alditol acetates relative to that (1.00) of glucitol hexaacetate.

molecular-weight carbohydrates, degradation products, peptides, and inorganic constituents.

The lipid A-free polysaccharide components were inhomogeneous by gel-permeation chromatography on Sephadex G-75 and G-50; presumably, fractions corresponding[31,32] to "core polysaccharide" and "O-specific side-chains" are present, and detailed studies on the separation and structural characterization of these components[14,15] will be recorded in forthcoming reports[28]. Analysis of the sugar compositions of the entire, lipid A-free, polysaccharide fractions was made by acid hydrolysis and quantitative g.l.c. of the alditol acetates of the resultant mono-

saccharides. The results of these analyses, expressed as relative proportions of neutral sugars and 2-amino-2,6-dideoxyhexoses, are given in Table IV. In addition to 2-amino-2,6-dideoxygalactose (found in the polysaccharides from types 2, 3, 4, and 6) and 2-amino-2,6-dideoxyglucose (present in the polysaccharides from types 3, 4, and 5), all of the polysaccharides contained glucose (in proportions ranging from 25 to 76%) and rhamnose (in proportions ranging from 6 to 56%). That from type 6 was unique, in that it also contained xylose (18%). The identities of the sugar components were determined on the basis of their retention times and mass spectra; these techniques do not differentiate D and L enantiomorphs of the component sugars.

The immunotype 2 antigen contains the highest relative proportion of 2-amino-2,6-dideoxyhexose (see Tables I and II), and this sugar is the principal sugar constituent of the lipid A-free, polysaccharide fraction (see Table IV). This amino sugar was isolated from the lipid A-free polysaccharide by hydrolysis followed by separation from neutral sugars by use of ion-exchange chromatography. It was obtained analytically pure as its crystalline *N*-acetyl derivative, m.p. 189–191°, which showed negligible optical activity in water or pyridine, and whose diethyl dithioacetal gave a mass spectrum consistent[33] with formulation as a 2-acetamido-2,6-dideoxyhexose; the mass spectrum of its alditol peracetate also supported this structure. It was chromatographically indistinguishable from 2-acetamido-2,6-dideoxy-D-galactose (papergram and g.l.c. of the derived alditol peracetate), but the lack of significant optical activity contrasted sharply with that of the D enantiomer synthesized as a reference standard ($[\alpha]_D$ $+129 \rightarrow +92°$ in water).

TABLE V

PROTON N.M.R.-SPECTRAL DATA AT 220 MHZ OF 2-ACETAMIDO-1,3,4-TRI-*O*-ACETYL-2,6-DIDEOXY-α,β-DL-GALACTOPYRANOSE

Proton	*α Anomer*[a]		*β Anomer*[a]	
	Chemical shift[b]	*Coupling (Hz)*	*Chemical shift*[b]	*Coupling (Hz)*
H-1	6.20 d	$J_{1,2}$ 3.8	5.70 d	$J_{1,2}$ 9.0
H-2[c]	4.69 m	$J_{2,3}$ 10.8	4.43 m	$J_{2,3}$ 11.5
H-3	~5.23 d	$J_{3,4}$ 3.4	5.10 d	$J_{3,4}$ 3.4
H-4	~5.27 d	$J_{4,5}$ <1	~5.27 d	$J_{4,5}$ <1
H-5	4.18 dd	$J_{5,6}$ 6.5	3.95 dd	$J_{5,6}$ 6.5
H-6	1.16 d		1.24 d	
Acetyl	1.95, 2.04, 2.28, 2.20		1.95, 2.02, 2.13, 20.2	

[a]Present as a 4:1, α,β mixture, as estimated from signal intensities. [b]In chloroform-*d*, p.p.m. relative to tetramethylsilane (δ scale); first-order values; d = doublet, m = multiplet. [c]Signal collapses to doublet of doublets on addition of deuterium oxide, with concomitant disappearance of low-field NH broadened doublet ($J_{2,NH}$ 9.5 Hz).

The 220-MHz, proton n.m.r. spectrum (see Table V) of the peracetylated amino sugar indicated an α,β-anomeric mixture of 2-acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxyaldohexopyranoses in the $^4C_1$(D) [$^1C_4$(L)] conformation, in about 4:1 ratio.

The terminal *C*-methyl group gave high-field (δ 1.16 and 1.24) doublets for the α and β anomers, respectively, and observation of the H-2 signals (α, δ 4.69; β, δ 4.43) at relatively high field indicated[34] that the acetamido group was at C-2; confirmation was provided by deuterium exchange, which effected simplification of the H-2 signals by removal of the NH, H-2 coupling. The spin-couplings for the anomers were, in principle, concordant with either *altro* or *galacto* stereochemistry. The non-identity of the observed specific rotation of the *N*-acetyl derivative with the literature data[35] for 2-acetamido-2,6-dideoxy-D-galactose prompted the synthesis[36] of the D-*altro* derivative as a reference compound, but the peracetate of the latter gave n.m.r. chemical-shift data that differed from those recorded here (see Table V) for the peracetylated amino sugar from the type 2 *Pseudomonas* antigen. Subsequently, the demonstrated identity of the latter sugar derivative with that of a synthetic, reference sample of the D enantiomorph in all respects, except for lack of optical activity in the former, led to formulation of the amino sugar in the type 2 antigen as 2-amino-2,6-dideoxy-DL-galactose.

Although 2-amino-2,6-dideoxy-D-galactose has frequently been encountered as a constituent of bacterial lipopolysaccharides, and the L form has also been reported[35], the occurrence of the DL form is unusual, although its presence has been reported[19] in hydrolyzates of cells of *Pseudomonas aeruginosa* serotype N 10. A detailed comparison of the analytical data for these seven immunotype antigens with literature data[16–20] for preparations obtained from various *P. aeruginosa* serotypes will be reported separately.

EXPERIMENTAL

General methods. — Evaporations were performed under diminished pressure at temperatures below 50°. Melting points were determined with a Thomas–Hoover apparatus and are uncorrected. A Perkin–Elmer Model 141 polarimeter and 1-dm tubes were used for measuring optical rotations. N.m.r. spectra were recorded at 220 MHz with a Varian HR-220 spectrometer, and the chemical shifts refer to an internal standard of tetramethylsilane ($\delta = 0.00$). Mass spectra were recorded with an AEI MS-9 double-focusing, high-resolution spectrometer operating at an ionizing potential of 70 eV and an accelerating potential of 8 kV, and the direct-inlet source was maintained at 150°. G.l.c. was performed on Beckman GC-5 and Hewlett–Packard 5720-A gas chromatographs, both equipped with flame-ionization detectors, and 3% of ECNSS-M on Gaschrom Q (Applied Science Laboratories, State College, Pa.) was used as the liquid phase. Column temperatures were maintained isothermally at 190° for analysis of neutral sugars, and programmed from 190 to 225° for amino sugars. Photometric analyses were performed with a Beckmann model DU spectrophotometer. Paper chromatography was performed on Whatman No. 1 paper with 10:4:3 ethyl acetate–pyridine–water as the developer, and indication with ammoniacal silver nitrate. Elemental analyses were performed by W. N. Rond.

Analyses on the intact antigens. — Samples of each of the lipopolysaccharide

antigens of immunotypes[3] 1–7 were analyzed for percent of phosphorus, and the protein content of each antigen was determined by the method of Lowry *et al.*[21]. The content of 3-deoxy-D-*manno*-2-octulosonic acid in each was determined by the method of Weissbach and Hurwitz[22]. The results are shown in Table I.

Neutral carbohydrates were determined, on accurately weighed samples (~1 mg) of the antigens dissolved in water (10 ml), by the phenol–sulfuric acid colorimetric method, with measurement of color production at 490 nm, according to the procedure of F. Smith *et al.*[23]. At least three independent determinations were made for each sample, and the values obtained (see Table I) were found reproducible to within ±1.5%. The percentages recorded in Table I relate to the values (taken as 100%) given by standard samples of D-glucose, cellobiose, and cellulose, all of which gave essentially identical values (after correction for net sugar content).

For determination of the total content of 2-amino-2-deoxy sugars, accurately weighed samples of the antigens (~1 mg) in 3M hydrochloric acid (3 ml) were heated in sealed vials for 16 h at 100°, and additional analyses were performed with 6M hydrochloric acid for 3 h at 100°. The solutions were then evaporated directly, and 1-propanol was several times added to, and evaporated from, the residues to remove all of the acid. The residues were then dissolved in water (10 ml), and 4-ml aliquots of this solution were analyzed for amino sugars by the Blix[24] modification[25] of the Elson–Morgan[26] procedure. A standard curve was prepared by using 2-amino-2-deoxy-D-glucose hydrochloride, and the optical absorbances of the final solutions from the antigen analyses fell within the linear range of the standard curve. Three independent determinations were made for each sample, and these proved reproducible to within ±1.5%; no significant differences were observed in the results from the two hydrolytic procedures employed. The values thus determined are given in Table I.

Analyses of the amino sugar compositions of the antigens. — Samples of the antigens[3] (~10 mg) were hydrolyzed with 3M and 6M hydrochloric acid as just described. After evaporation of the acid, the residues were dissolved in water (10 ml), and sodium borohydride (30 mg) was added to each. After 2 h at ~25°, the excess of borohydride was decomposed by adding a few drops of acetic acid. The solutions were evaporated to dryness, and methanol and acetic acid were several times added to, and evaporated from, the residue to remove boric acid. Pyridine (1.5 ml) and acetic anhydride (1.5 ml) were then added, and the mixtures were heated for 45 min at 110°, and poured into ice–water, and the aqueous solutions were extracted with chloroform. The extracts were dried (sodium sulfate) and evaporated, and the residues dissolved in ethyl acetate (0.5 ml). These solutions were used as stock solutions for the g.l.c. investigations. The aminodeoxyalditol acetates displayed retention times (see Table II) much longer than those of the alditol acetates; all seven antigens gave peaks corresponding in retention time to the alditol acetates of 2-amino-2,6-dideoxygalactose, 2-amino-2-deoxygalactose, and 2-amino-2-deoxyglucose; immunotypes 3, 4, and 5 also contained a product corresponding to 2-amino-2,6-dideoxyglucose, whose retention time was very similar to that of the galactose analogue. Each product

gave a retention time and a mass spectrum identical to that of an authentic, reference standard that had been processed similarly and injected into a coupled g.l.c.–mass spectrometer (DuPont C.E.C.). The relative percentages of the amino sugars, as determined by peak-area integration, and assuming equal response-factors, are given in Table II.

Analyses of the fatty-acid compositions of the antigens. — The individual antigens (~10 mg) were saponified by heating in 3M aqueous sodium hydroxide for 5 h at 100°. The cooled solutions were acidified, and the fatty acids extracted with chloroform. The dried (magnesium sulfate) extracts were evaporated, and the residues were converted into their methyl esters by treatment with methanol containing a little boron trifluoride etherate. The products were analyzed by g.l.c. on an F and M gas chromatograph equipped with a 1.3-m, glass column packed with 3% of OV-17, and the column was programmed from 60–300° with a 10° per min rise of temperature. Sharp peaks (A–E, see Table III) were observed having retention times closely similar to those of reference samples of the methyl esters of dodecanoic, 12-methyltridecanoic, tetradecanoic, hexadecanoic, and octadecanoic acids, respectively; additional, minor peaks were observed for most of the antigens. Table III records the relative percentages of the principal fatty acids, as determined by peak-area integration and also lists relative retention-times of the principal components, and the incidence of trace peaks.

Cleavage of the antigens into lipid A and lipid A-free polysaccharide. — The lipopolysaccharide antigen samples of each of the seven immunotypes[3] (100 mg) in 1% aqueous acetic acid (50 ml) were heated for 45–120 min at 100°. The exact time used was adjusted to the minimum necessary for complete cleavage of the lipid A. The reaction was judged to be complete when no additional precipitation of lipid A could be observed. The mixtures were then cooled, and centrifuged at a relative centrifugal force of 6500 *g* for 1 h at ~25°. This procedure permitted recovery of almost all of the lipid A from immunotypes 2, 5, 6, and 7; for immunotypes 1, 3, and 4, it was necessary to extract the supernatant, aqueous solutions with three 100-ml portions of chloroform to recover all of the lipid A.

The precipitated lipid A was in each instance dissolved in chloroform, and the chloroform extracts from the aqueous phase were added to these solutions in the case of immunotypes 1, 3, and 4. The solutions were then washed with water (100 ml), dried (sodium sulfate), and evaporated to give the lipid A in the form of yellow to brown, amorphous solids. Material having better handling characteristics was obtained by dissolving the products in benzene and freeze-drying the solutions; the products were obtained as fluffy solids very similar in appearance to the samples of the original lipopolysaccharides that had been freeze-dried from water. The products all had i.r. spectra that were essentially identical and that corresponded to that reported for lipid A by Burton and Carter[30]. Table I gives the percent content of lipid A (±1.5%, mean of several determinations) for each of the seven antigens.

The supernatant aqueous solutions, the aqueous phases from the extractions, and the aqueous washings were pooled in each instance, dialyzed with a Bio Fiber 50

membrane (Bio-Rad Laboratories, New York) against running, distilled water for 2 h, and then freeze-dried to give the lipid A-free, polysaccharide components. The net yields of these polysaccharide components from the seven antigens are recorded in Table IV.

Analysis of the sugar compositions of the lipid A-free polysaccharides. — Samples of each lipid A-free, dialyzed, polysaccharide component from the preceding experiment (~10 mg), together with an accurately weighed amount (~5 mg) of D-mannose as an internal standard, in 0.25 or 0.5M sulfuric acid (3 ml) were heated in sealed vials for 16 h at 100°. The solutions were then made neutral with barium carbonate, and evaporated. These products were reduced to their alditols, which were acetylated, and the acetates examined by g.l.c. by exactly the same procedure as already described for analysis of the individual amino sugars. All determinations were repeated at least three times, and no significant differences were observed in the results from the two different concentrations of acid used. The products all contained glucose and rhamnose; 2-amino-2,6-dideoxygalactose and 2-amino-2,6-dideoxyglucose occurred frequently, and that from immunotype 6 also contained a product corresponding to xylose. The relative proportions of these components, as determined from peak areas on the chromatograms and corrected by the response factor (1.2) for the 2-amino-2,6-dideoxyhexoses, were found reproducible to within ±3%, and are recorded in Table IV.

*Isolation and characterization of 2-amino-2,6-dideoxy-*DL*-galactose from the lipid A-free polysaccharide of immunotype 2 antigen.* — The lipid A-free polysaccharide (50 mg) from immunotype 2 was heated in 0.25M sulfuric acid for 6 h at 100°. The solution was made neutral with barium carbonate, solids were removed by centrifugation, and the supernatant liquor was applied to a column (1 × 10 cm) of Dowex 50W-X8 (H^+) resin. Neutral sugars were eluted with water (75 ml), and the amino sugar component was eluted with M hydrochloric acid (100 ml). The second eluate was evaporated, and the resulting solid 2-amino-2-deoxy-DL-galactose hydrochloride (35 mg) was dissolved in water (2 ml). The solution was cooled to 5°, acetic anhydride (0.5 ml), methanol (0.5 ml), and Dowex 1-X8 (CO_3^{2}) resin (1 g) were added, and the mixture was stirred for 2 h at 5°. Additional acetic anhydride (0.5 ml) was then added, and stirring was continued for 2 h at 5° and for 2 h at ~25°. The mixture was filtered, the filtrate was concentrated, and the concentrate was applied to a column (1 × 5 cm) of Dowex 50W-X8 (H^+) resin. The column was eluted with water (50 ml), and the effluent was evaporated, to give crude product (26 mg) that, on crystallization from acetone, gave pure *2-acetamido-2,6-dideoxy-*DL*-galactose*, m.p. 189–191°, $[\alpha]_D^{20}$ −0.5° (*c* 1, pyridine); R_{GlcNAc} 1.27; *m/e* 206 (0.1, MH^+), 187 (0.5, $M-H_2O$), 170 (0.2), 160 (0.8), 143 (18, $M-H_2O-CH_3CHO$), 114 (28), 101 (12), 72 (50), 59 (44), and 43 (100).

Anal. Calc. for $C_8H_{15}NO_5$: C, 46.87; H, 7.32; N, 6.83. Found: C, 46.79; H, 7.12; N, 6.81.

The negligible $[\alpha]_D$ value observed was reproducibly demonstrated in 4 independent repetitions of the entire experiment. The product was indistinguishable by

paper chromatography and mass spectrum from a reference sample of 2-acetamido-2,6-dideoxy-D-galactose[35] (m.p. 196–197°, $[\alpha]_D^{20}$ +129 → +92° in water, $[\alpha]_D^{20}$ +91° in pyridine). The corresponding L enantiomer has been reported[35] to have m.p. 195–198°, $[\alpha]_D$ −116 → −83° (*c* 0.2, water).

Reduction of the product (3.5 mg) with sodium borohydride, and subsequent acetylation with acetic anhydride–pyridine by the general procedure described in the analytical experiment, gave *2-acetamido-1,3,5-tri-*O*-acetyl-2,6-dideoxy-*DL*-galactitol* (5.0 mg), which could be obtained crystalline from ether–petroleum ether (b.p. 30–60°), m.p. 142–143°, $[\alpha]_D^{20}$ 0° (*c* 0.5, chloroform); T_R by g.l.c. 1.60 (relative to D-glucitol hexaacetate); *m/e* 375 (0.04, M^+), 332 (0.05, M−Ac), 302 (12, $M-CH_2OAc$), 260 (3, $M-CH_2OAc-CH_2{=}C{=}O$), 242 (8, $M-CH_2OAc-HOAc$), 201 (9), 195 (11), 182 (8), 144 (29, $AcOCH_2\text{-}\overset{+}{C}H\text{-}NHAc$), 140 (22), 102 (30), 98 (23), 84 (40, $CH_2{=}\overset{+}{C}\text{-}NHAc$), and 43 (100).

Treatment of the acetamido sugar with ethanethiol–hydrochloric acid by the conventional procedure[33,37] gave *2-acetamido-2,6-dideoxy-*DL*-galactose diethyl dithioacetal*, m.p. 140–145°; *m/e* 311 (3, M^+), 282 (3, M−Et), 2.52 (3, $M-AcNH_2$), 188 (4), 177 [95, $(EtS)_2CCH{=}\overset{+}{C}HOH$], 146 (18, $EtS^+H\text{–}CH{=}CHNHAc$), 135 [100, $(EtS)_2\overset{+}{C}H$], 117 (84), 102 (70), 99 (65), 75 (52), 60 (100), 57 (85), and 43 (100).

*2-Acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxy-α,β-*DL*-galactopyranose.* — Conventional acetylation of 2-acetamido-2,6-dideoxy-DL-galactose with acetic anhydride–pyridine gave the peracetate, m.p. 242–243° (from chloroform–petroleum ether, b.p. 30–60°), $[\alpha]_D^{20}$ −1 ±1° (*c* 1, chloroform); for n.m.r.-spectral data, see Table V.

Anal. Calc. for $C_{14}H_{21}NO_8$ (331.32): C, 50.75; H, 6.39; N, 4.23. Found: C, 51.00; H, 6.26; N, 4.04.

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