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CHEMICAL MODIFICATION AND SEROLOGICAL PROPERTIES OF THE 3-DEOXY-α-D-*manno*-2-OCTULOSONIC ACID-CONTAINING POLYSACCHARIDE FROM *Escherichia coli* LP1092*

HAROLD J. JENNINGS, RENÉ ROY, AND ROSS E. WILLIAMS

Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A OR6 (Canada)

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ABSTRACT

The α-D configuration of the 3-deoxy-D-*manno*-2-octulosylonic acid residues in the *E. coli* LP1092 polysaccharide was definitively assigned by a comparison of its c.d. spectrum with those of methyl 3-deoxy-α- and -β-D-*manno*-2-octulopyranosidonic acid. The c.d. spectrum of the polysaccharide showed a negative n→π* transition at 211 nm, associated with carboxyl groups, which correlated well with the negative band at 218 nm exhibited in the c.d. spectrum of the methyl α-D-glycoside. In contrast, the methyl β-D-glycoside gave a positive band in the same region of its c.d. spectrum, at 221 nm. Treatment of the *E. coli* LP1092 polysaccharide with 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride yielded a modified polysaccharide containing *O*-acylisourea groups and intramolecular lactones, in addition to some unesterified carboxyl groups. Both forms of ester could be reduced to hydroxymethyl groups by sodium borohydride. Although immuno-precipitation of an antiserum specific for the *E. coli* LP1092 polysaccharide is not sensitive to the introduction of *O*-acylisourea groups into the polysaccharide, precipitation was completely eliminated when approximately half of the carboxyl groups of the polysaccharide were converted either into lactone or hydroxymethyl groups. Failure to precipitate the antiserum can be attributed to significant conformational changes in the polysaccharide brought about by the introduction of the latter two groups.

INTRODUCTION

The occurrence of 3-deoxy-D-*manno*-2-octulosonic acid (KDO) in bacterial capsular polysaccharides was first reported for that of *E. coli*[1] LP1092 and of *N. meningitidis* group[2] 29e, the KDO residue of the latter antigen being in the β-D configuration[3]. Further identification of KDO in polysaccharides of bacterial origin has also been reported in the *E. coli* K12, K13, K20, and K23 polysaccharides[4,5].

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In all of these polysaccharides, the KDO residues also adopt the β-D configuration[5–7]. The structure[8] of the KDO-containing *E. coli* LP1092 polysaccharide is composed of the linear-trisaccharide repeating-unit →2)-β-D-Rib*f*-(1→2)-β-D-Rib*f*-(1→7)-α-D-KDO*p*-(2→, and it was previously designated as the K6 antigen[4].

However, Messner and Unger[9] proposed a different structure for the K6 antigen, also reported to be obtained from *E. coli* LP1092, in which a branched structure is formed from the same trisaccharide repeating-unit, but the anomeric configuration of its KDO residues was not assigned[7,10]. The reason for this structural diversity has not yet been fully established; of importance, however, is the preliminary finding that a serological re-evaluation of the polysaccharide obtained from the *E. coli* LP1092 organisms used in our structural studies indicates that it is not, in fact, the K6 antigen, and that it will need to be redesignated[11].

The structure of our preparation of the *E. coli* LP1092 polysaccharide is of interest, because it contains the first reported case of KDO residues in the α-D configuration[8], thus implying that, unlike related sialic acid glycosides, those of KDO exist in Nature in both configurations. However, the accumulated evidence used to make this assignment, although highly supportive, was not definitive. Herein is described the application of circular dichroism spectroscopy to the LP1092 polysaccharide to assign the α-D configuration definitively to its KDO residues. Also described are the isolation, analysis, and serological properties of ester intermediates formed in the treatment of the LP1092 polysaccharide with 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (DEC), and their analogs.

RESULTS

Ozonolysis of the LP1092 polysaccharide. — In order to carry out circular dichroism studies on the LP1092 polysaccharide in the range of the carboxylate chromophore (200–270 nm), it was necessary to eliminate an interfering and very strong absorption band, at E_{max} 264 nm, given by the polysaccharide. This band is typical of the presence of a nucleic acid contaminant, although the failure to remove it enzymically, and by ion-exchange chromatography, indicated that it is tightly bound to the polysaccharide. The problem of removing it was finally solved by ozonolysis of the polysaccharide. Following treatment with ozone, a base, and sodium borohydride, the polysaccharide exhibited no absorption at 264 nm. However, although successful, this treatment caused substantial depolymerization of the polysaccharide, and it was recovered in a yield of only 15%. This could, perhaps, have been anticipated, as it is well known that degradation of polysaccharides does occur as a result of their oxidative cleavage[12], and ozone[13] is a potent oxidant.

Circular dichroism of the LP1092 polysaccharide. — The c.d. spectrum of the ozone-treated LP1092 polysaccharide is shown in Fig. 1. The polysaccharide has a negative Cotton effect, with a band, at 212 nm, of $\Delta\varepsilon$ −0.25. Bands at similar wavelengths have been attributed to the carboxylate chromophore, the sign of those associated with 3-deoxy-D-*manno*-2-octulopyranosonic acid derivatives being

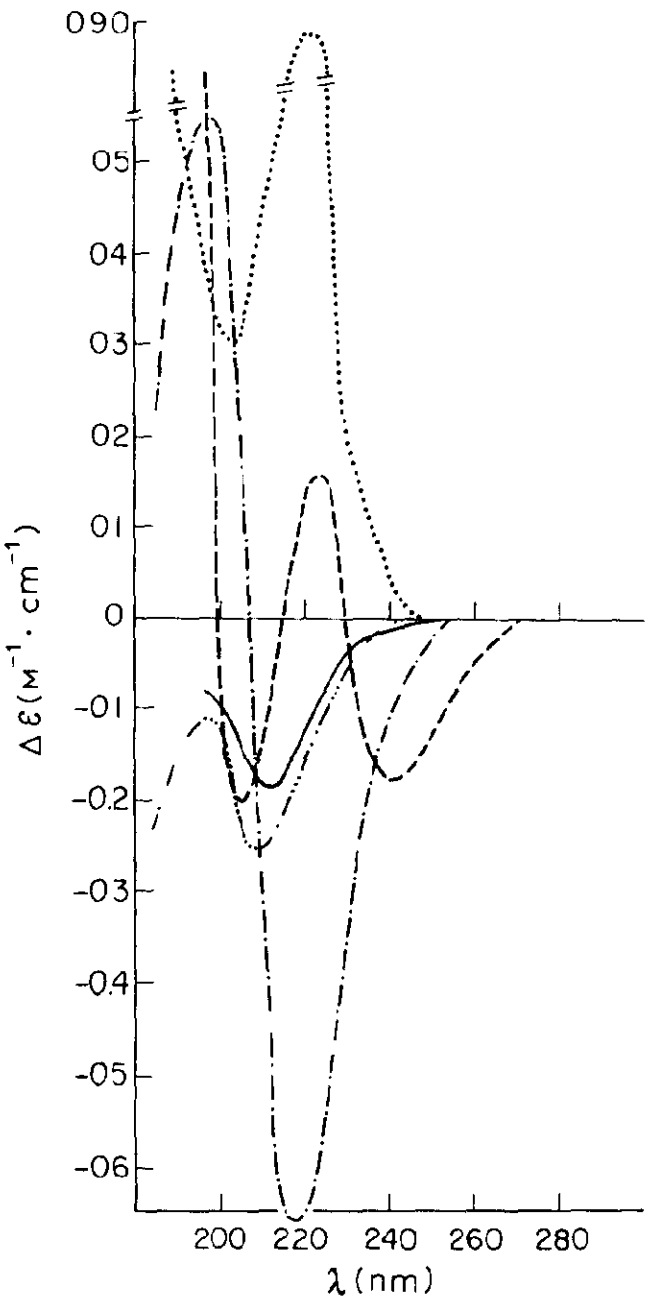


Fig. 1. The c.d. spectra of the LP1092 polysaccharide (— · · · —), the methyl ester of the LP1092 polysaccharide (———), the *E. coli* K23 polysaccharide (. . . . . .), methyl 3-deoxy-α-D-*manno*-2-octulopyranosidic acid (—·—), and methyl 3-deoxy-β-D-*manno*-2-octulopyranosidic acid (-------).

dependent on the anomeric configuration[14]. In comparison with the c.d. spectra of the model compounds methyl 3-deoxy-α- and -β-D-*manno*-2-octulopyranosidonic acids (see Fig. 1), the c.d. spectrum of the LP1092 polysaccharide is more similar to that of the methyl α-D-glycoside, which exhibits a negative band at 218 nm of $\Delta\varepsilon$ −0.66. The methyl β-D-glycoside gave a positive band in the same region of the spectrum, at 223 nm, of $\Delta\varepsilon$ +0.13, and an additional, negative band, at 240 nm, of $\Delta\varepsilon$ −0.18. On this evidence, the KDO residues of the LP1092 polysaccharide must be assigned the α-D configuration. Confirmatory evidence for this assignment is that the capsular polysaccharide of *E. coli* K23, which contains KDO residues in the β-D configuration[4], exhibits an absorption curve similar to that of the methyl β-D-glycoside. The K23 polysaccharide gave a positive band, at 218 nm, of $\Delta\varepsilon$ +0.87, and no negative band at 240 nm.

The methyl ester derivative of the LP1092 polysaccharide gave in its c.d.

spectrum a curve similar to that of the native polysaccharide, with a band, at 213 nm, of $\Delta\varepsilon$ −0.19. This, again, is consistent with its KDO residues's being in the α-D configuration because Charon and Szabó[14] showed, and we have confirmed, that the equivalent methyl (methyl 3-deoxy-β-D-*manno*-2-octulopyranosid)onate also gives a negative band in this region of the spectrum (224 nm), and the methyl ester of the methyl β-D anomer exhibits a positive band at 225 nm.

Reduction of the LP1092 polysaccharide. — Attempts were made to reduce the carboxyl groups of the KDO residues of the LP1092 polysaccharide by using the method of Taylor and Conrad[15,16]. Colorimetric analysis of the reduction product revealed that only half of the KDO residues had been reduced, and this proved to be a consistent result in subsequent, initial reductions of the LP1092 polysaccharide. The same degree of reduction (50%) was also obtained when the addition of sodium borohydride to the DEC derivative of the polysaccharide was made at pH 7.0, a modification suggested by Taylor and Conrad[15,16] to improve the reduction of acidic polysaccharides by their method.

That only half of the KDO residues were reduced was also consistent with the ^{13}C-n.m.r. spectrum of the reduced LP1092 polysaccharide. Some of the chemical shifts of signals pertinent to these studies, and their assignments, are shown in Table I. The ^{13}C-n.m.r. spectrum of the reduced polysaccharide still exhibited a signal, albeit of lessened intensity, at 100.9 p.p.m. previously assigned to the anomeric carbon atom (C-2) of the KDO residues of the LP1092 repeating-unit (**1**). The ^{13}C-n.m.r. spectrum of the reduced LP1092 polysaccharide exhibited an additional signal, of approximately equal intensity, at 102.6 p.p.m.; this could be assigned to C-2 of the reduced KDO residues. Thus, reduction of the carboxylate groups of the KDO residues caused a downfield displacement of +1.7 p.p.m. in their (anomeric) C-2 signals. Two other signals, 1A and 2A, of the LP1092 polysac-

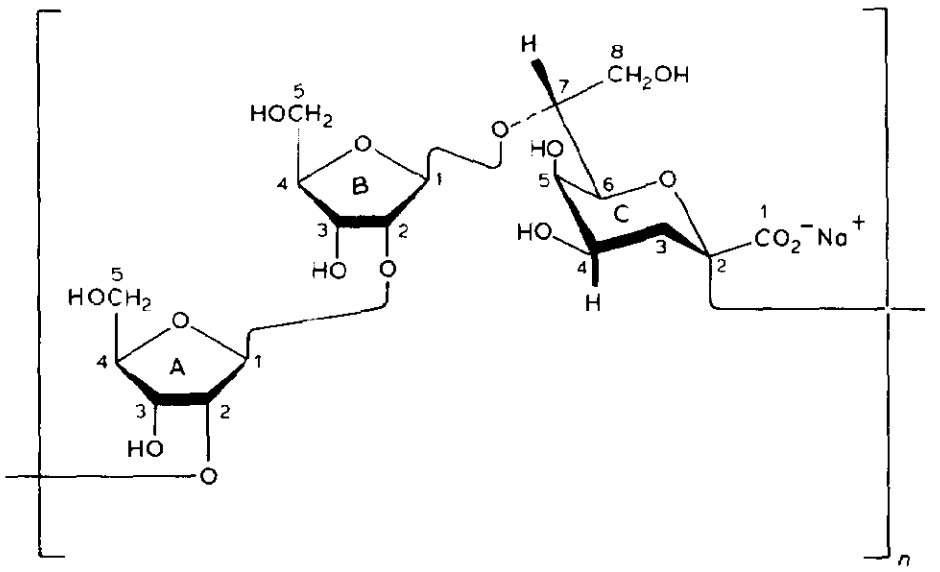
TABLE I

CHEMICAL SHIFTS OF SOME RELEVANT CARBON ATOMS IN THE ^{13}C-N.M R. SPECTRUM OF VARIOUS MODIFIED, *E. coli* LP1092 POLYSACCHARIDES

Modified polysaccharide	*^{13}C-Chemical shift (p.p.m.)[a] of*					
	1A	*1B*	*2C*	*2A*	*7C*	*1C*
O-Deacetylated[b]	108.0	104.7	101.0	77.2	75.6	176.1
Reduced (50%)	107.8	104.6	100.8	77.0	75.6	176.1
	108.5		102.5	74.8		
Reduced (100%)[c]	108.5	104.9	102.6	75.0	75.6	—
First DEC-treated	107.8	104.6	100.8	77.2	75.6	175.9
			102.1			171.4
Second DEC-treated	107.8	104.5	102.2	77.2	75.6	—[d]

[a]In parts per million from external Me_4Si. [b]Values taken from ref. 8. [c]Obtained by repetitive reductions. [d]Signal obscure.

charide were also similarly displaced by this reduction, half of each signal being displaced (by +0.6 and −2.1 p.p.m., respectively).



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A further reduction of the aforementioned, partially reduced LP1092 polysaccharide, using the same conditions, resulted in the reduction of more KDO residues. The ratio of the intensity of the anomeric signals at 102.6 and 100.9 p.p.m. was >9:1, indicating that >90% of the KDO residues of the LP1092 polysaccharide had been reduced. A similar, intensity ratio was also observed in the dual signals of 1A and 2A in the product of the second reduction; the chemical shifts and assignments of other signals of this product are given in Table I.

Reaction of the LP1092 polysaccharide with DEC. — The LP1092 polysaccharide was modified by treatment with DEC, and the amount of *O*-acylisourea formed was monitored by ^{1}H-n.m.r. spectroscopy. The ^{1}H-n.m.r. spectrum of the de-*O*-acetylated, native LP1092 polysaccharideis shown in Fig. 2. Inoue and Nagasawa[17], in studies on the treatment of glucosaminoglycuronans with DEC demonstrated that the molar quantity of 3-(3-dimethylaminopropyl)-1-ethylureido residue could be determined by integrating some of its characteristic methyl signals, at δ 1.19 ($NHCH_2CH_3$) and 2.80 [$N^+(CH_3)_2$], using assigned signals from the polysaccharide as reference signals.

The spectrum of the *O*-deacetylated LP1092 polysaccharide exhibits two anomeric protons, at δ 5.30 and 5.40, assigned to the two D-ribofuranosyl residues of its repeating unit (**1**), which could be used as reference signals. Integration of all of these signals in the spectrum of the product of the first DEC treatment of the LP1092 polysaccharide indicated that only 30% of the KDO residues were present as *O*-acylisourea derivatives. However, because half of the KDO residues of this modified LP1092 polysaccharide could be reduced with sodium borohydride, the presence in it of another reducible form of KDO must be assumed. This evidence is compatible with the ^{13}C-n.m.r. spectrum of the first DEC-treated polysaccharide, which exhibited a signal, at 100.8 p.p.m., assigned to the anomeric carbon atom of unesterified KDO residues, and another (new) anomeric signal, at 102.1

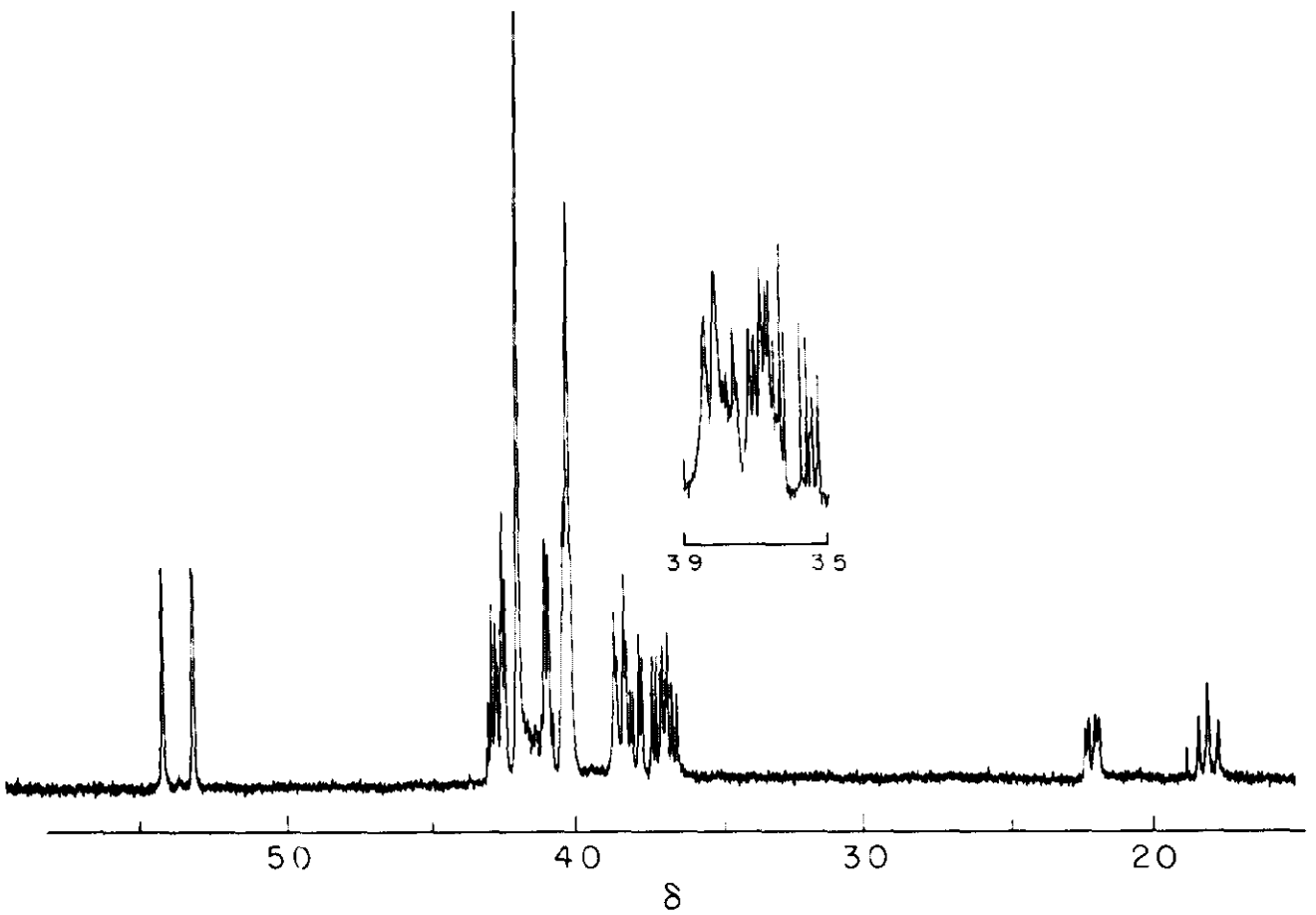


Fig. 2. High-resolution, 400-MHz, ^{1}H-n.m.r. spectrum of the LP1092 polysaccharide. [The inset is the region of the high-resolution, ^{1}H-n.m.r. spectrum, from δ 3.5 to 3.9, of the product of the second treatment of the LP1092 polysaccharide with DEC.]

p.p.m., not identical with that of reduced KDO residues (102.6 p.p.m.). These signals were in the ratio of ~1:1, and the signal at 102.1 p.p.m. could be the superimposed, anomeric signals of two differently esterified KDO residues.

The DEC-treated LP1092 polysaccharide was subjected to a further reaction with DEC, using conditions identical to those in the first reaction, and this gave a product that had undergone more-extensive esterification. From an analysis of signals, previously referred to, in its ^{1}H-n.m.r. spectrum, it was deduced that ~50% of its KDO residues were present as *O*-acylisourea derivatives. The fact that >90% of the KDO residues of the product of the second DEC treatment could be reduced with sodium borohydride indicated the presence also of another reducible form of KDO (~40%). This is again consistent with the ^{13}C-n.m.r. spectrum of the product, which exhibited an intense signal at 102.1 p.p.m., previously assigned to the (anomeric) C-2 atoms of esterified KDO residues, this signal and that of the anomeric carbon atoms of the unesterified KDO residues being in the ratio of 9:1.

Serological properties of the native and modified LP1092 polysaccharide. — Immunodiffusion experiments were conducted by using the native and modified LP1092 polysaccharides and an antiserum made to the *E. coli* LP1092 organisms; the results are shown in Fig. 3. The native and *O*-deacetylated LP1092 polysaccharides gave a precipitin line of identity, thus indicating that a small proportion of *O*-acetyl (0.2 mol per repeating unit[8]) has little effect on the serological properties of the polysaccharide. The product of the second reduction of the polysaccharide,

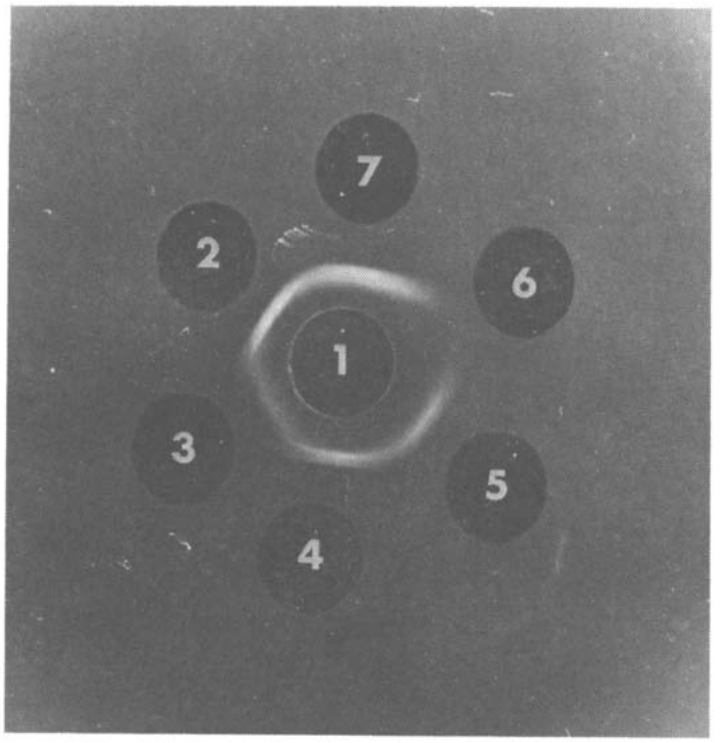


Fig. 3. Double diffusion, in agar, of the anti-LP1092 capsular polysaccharide rabbit sera (well 1), with the native LP1092 capsular polysaccharide (wells 2 and 4), product of the second DEC treatment of the LP1092 polysaccharide (well 3), the *O*-deacetylated LP1092 polysaccharide (well 7), the partially reduced LP1092 polysaccharide having ~50% of the KDO residues reduced (well 6), and the product of the first DEC treatment of the LP1092 polysaccharide (well 5).

having >90% of its KDO residues reduced, failed to react with the antiserum, and this was also true of the product of the first reduction, having only 50% of its KDO residues reduced. Thus, modification of half of the KDO residues was sufficient to eliminate all of the determinants from the LP1092 polysaccharide. This also proved to be the case when approximately half of the polysaccharide KDO residues were converted into intramolecular lactones.

The product of the second DEC treatment of the LP1092 polysaccharide, in which 40% of its KDO residues were present as intramolecular lactone and 50% as *O*-acylisourea derivatives, reacted only weakly with the antiserum, and, on storage or mild heating, it lost its ability to precipitate the antiserum completely. Presumably, during these procedures, a small increase in the proportion of lactone is brought about by conversion from the *O*-acylisourea derivative. Treatment of the aforementioned, DEC-treated polysaccharide with base then restored its ability to precipitate the antiserum. That the KDO residues present as *O*-acylisourea derivatives were not as important to the serological properties of the modified polysaccharides as the intramolecular lactone can be deduced from the precipitin reaction of the product of the first DEC treatment of the LP1092 polysaccharide. Despite half of its KDO residues's being esterified (20% of intramolecular lactone and 30% of *O*-acylisourea), it gave a precipitin line of identity with the native polysaccharide.

DISCUSSION

Before attempting studies on the chemical modification of the *E. coli* LP1092 polysaccharide, we needed to establish its structure without ambiguity. Although the structure of this polysaccharide had been reported[8], due to the lack of an anomeric proton, the anomeric configuration of its 3-deoxy-D-*manno*-2-octulopyranosidic residues (assigned as α-D) could be established only by using ^{13}C- (carboxylate carbon atom) and ^{1}H- (H-3 atoms) n.m.r., chemical-shift data. Although these data have so far proved to be a reliable indicator of anomeric configuration[3,8], they do not provide a definitive assignment. The recent assignment of the α-D configuration to KDO residues in the lipopolysaccharide of a heptose-less mutant of *E. coli,* based on the chemical shifts of their anomeric carbon atoms, must also be considered to be even more speculative[18]. The assignment[8] of the α-D configuration to the KDO residues of the LP1092 polysaccharides by the measurement of their heteronuclear, $^{3}J_{\text{H-3}a,\text{C-1}}$ value, which yielded unequivocal assignments in the case of the equivalent, monomeric methyl glycosides[19] and the *E. coli* K13 polysaccharide[7], was also not without ambiguity. The doublet was unresolved in the coupled spectrum of the polysaccharide, thus necessitating an approximate measurement of the $^{3}J_{\text{H-3}a,\text{C-1}}$ value at the half-height of the signal.

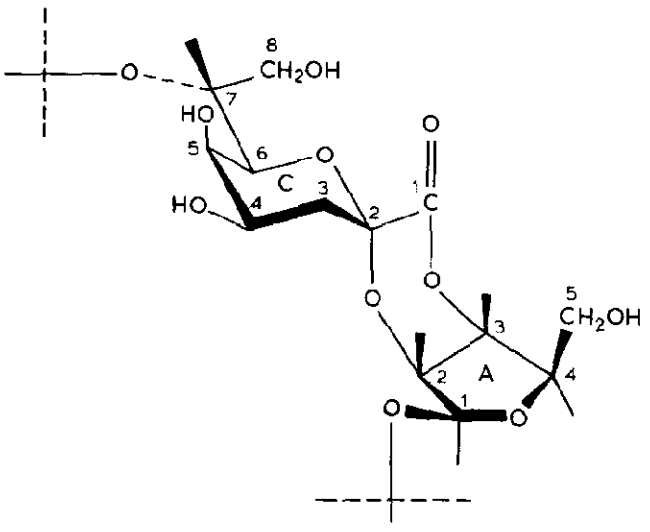
Further to substantiate our initial assignment of the α-D configuration of the KDO residues of the LP1092 polysaccharide, we compared its circular dichroism spectra with those of the methyl α- and β-D-glycoside of KDO. It has been demonstrated, both for sialic acid[20] and for KDO[14], that the $n\rightarrow\pi^*$ transition of the carboxyl chromophore is sensitive to differences in anomeric configuration, and that, despite these molecules's existing in different conformations, the sign of the Cotton effect at ~220 nm is negative for their α-D anomers and positive for their β-D anomers. Charon and Szabó[14] used the methyl ester derivatives to establish the anomeric configuration of a number of monomeric 3-deoxy-2-glyculosonic acids by using this technique. Because we were interested in applying this technique to polysaccharides, it was convenient, although not mandatory, to use the free carboxylate group as the chromophore.

Initially, the LP1092 polysaccharide preparations could not be used for these studies, because they had a strong u.v. absorption at 264 nm, presumably due to the presence of contaminating, or possibly covalently attached, nucleic acid. This material was not removed by physical methods, and could only be eliminated by using a more-drastic, chemical procedure involving the ozonolysis of the polysaccharide. Although this procedure also caused extensive depolymerization of the polysaccharide, enough survived dialysis to permit satisfactory circular dichroism studies to be performed.

On the basis of these studies, the α-D configuration was assigned to the KDO residues of the LP1092 polysaccharide, consistent with previous determinations[8]. The LP1092 polysaccharide gave a negative band at 212 nm, similar to that of the methyl α-D-glycoside of KDO (218 nm), and the methyl β-D-glycoside of KDO

gave a positive band at approximately the same wavelength (223 nm), although it did exhibit an additional negative band at 240 nm (see Fig. 1). For further confirmation of this assignment, the *E. coli* K23 polysaccharide, which contains KDO residues in the β-D configuration (as deduced from n.m.r. studies[4]), gave a positive band at 218 nm like that exhibited by the equivalent methyl β-D-glycoside of KDO. Interestingly, unlike the equivalent monomer, the *E. coli* K23 polysaccharide did not exhibit a negative band at 240 nm. In the circular dichroism spectrum of the methyl ester of the LP1092 polysaccharide, the absorption curve was only slightly displaced from that of its carboxylate form (see Fig. 1); this is consistent with the conclusion of Melton *et al.*[20] that esterification also has no major influence on the circular dichroism curves of sialic acid.

Our interest in studying the intermediate, DEC-treated *E. coli* LP1092 polysaccharide stemmed from the observation that the initial reduction of the polysaccharide, using the method of Taylor and Conrad[5,6] resulted in the reduction of only half of its KDO residues. Consistent with this observation, n.m.r. studies on the intermediate indicated that half of its KDO residues were, in fact, unesterified, and, of the reducible KDO residues (50%), only 30% could be detected that were present as *O*-acylisourea derivatives. Therefore, it is necessary to invoke the presence of another type of esterified KDO residue, present to the extent of 20% in the intermediate, of which an intramolecular ester is a likely candidate. Inspection of CPK models indicated that the most probable site of lactonization in the repeating unit of polysaccharide **1** is between the carboxyl group of a KDO residue and OH-3 of a neighboring, D-ribofuranosyl residue, forming a six-membered lactone-ring. The lactone (**2**) is depicted in one of its possible conformations.



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Similar intramolecular lactones involving sialic acid residues have been identified in the DEC-treated, groups B and C meningococcal polysaccharides[21] and sialyl gangliosides[22]. Unfortunately, the formation of this six-membered lactone in the LP1092 polysaccharide does not provide unambiguous evidence for the α-D configuration of its KDO residues, because models demonstrated that a similar lactone could also be formed from KDO residues in the β-D configuration. In their ini-

tial studies, Taylor and Conrad[15] proposed that their reduction proceeded through lactones, and did not involve intermediate esters. However, Inoue and Nagasawa[17] have recently shown that DEC treatment of heparin and chondroitin 6-sulfate results in the exclusive formation of *O*-acylisourea derivatives that could be quantitatively reduced with sodium borohydride. Our evidence demonstrates that both forms of ester can be involved in the reduction, the formation of lactones obviously being dependent on favorable stereochemistry.

Further treatment of the first intermediate, DEC product with DEC yielded another intermediate, in which >90% of the KDO residues were reducible. This product contained 10% of unesterified KDO residues and 50% of KDO *O*-acylisourea derivatives, the remaining 40% of reducible KDO residues presumably being in the form of intramolecular lactones. Although no direct evidence of lactone formation could be obtained, that previously assembled by inference and the following additional observations are highly supportive of the presence of intramolecular lactones. Both the first and second DEC intermediates were converted into the original polysaccharide by treatment with a base.

On heating the second DEC intermediate for 3 h at 60°, 30% of the *O*-acylisourea disappeared (^{1}H-n.m.r. evidence); however, because 90% of the KDO residues of the heat-treated intermediate were still reducible, it is possible to deduce that these esters were converted into intramolecular lactones. In the ^{1}H-n.m.r. spectrum of the second DEC intermediate, a quartet at δ 3.52, not present in that of either the original polysaccharide (see Fig. 2) or the reagent (DEC), and which disappeared on base treatment of the intermediate, could be assigned to one of the protons of **2**. The most likely protons would be H-2 or H-3 of the D-ribofuranosyl residue; however, the former was eliminated when the quartet remained unaffected by irradiation of either of the D-ribofuranosyl anomeric protons, at δ 5.30 and 5.40. The intensity of the quartet, ~50% of that of either of the anomeric protons, and the $^3J_{H,H}$ values of 6.3 and 11.6 Hz are also consistent with the assignment of this quartet to H-3 of the D-ribofuranosyl residues involved in the lactone ring. Lactonization of the LP1092 polysaccharide caused an upfield displacement in the H-3 signal, presumably due to shielding of H-3 by the carbonyl group of the lactone.

The immunological properties of the modified LP1092 polysaccharide were dependent on both its degree of lactonization and reduction. Interestingly, in both of these modifications, the conversion of approximately half of the KDO residues prevented the modified polysaccharides from precipitating with an antiserum specific for the original polysaccharide (see Fig. 3). The presence of *O*-acylisourea to the extent of 30%, in addition to 20% of intramolecular lactone did not, however, prevent precipitation. This phenomenon can be explained by the possibility that glycosidic, bond-angle changes between half of the KDO residues and their D-ribofuranosyl "aglycons" induce sufficient conformational change in the polysaccharide to make it unrecognizable by the antibodies.

Although the formation of *O*-acylisourea derivatives of the KDO residues

does not appear to cause the bond-angle changes, they occur of necessity in the formation of **2**, and chemical-shift differences[23,24] between signals in the spectra of the *O*-deacetylated and reduced LP1092 polysaccharides, particularly those associated with 1A and 2A of the "aglyconic" D-ribofuranosyl residues of KDO (see Table I) would also suggest that they might occur in the reduced polysaccharide. Whether these conformational changes are restricted to local sites, or induce further, long-range, conformational disturbances, is not known. Certainly, the former case would probably demand at least a predominance of the conversion of alternate KDO residues into *O*-acylisourea derivatives in the original activation of the polysaccharide, in order that the formation of intramolecular lactones or hydroxymethyl groups could eliminate most of the determinants possible.

EXPERIMENTAL

Isolation of the LP1092 polysaccharide. — The *Escherichia coli* LP1092 organisms were kindly supplied by Dr. P. W. Taylor of the School of Medicine, University of Leeds, England, and were grown, as previously described, with control of the pH at 7.5, in a synthetic medium[8]. The polysaccharide was isolated, according to previously published methods, by using a modified, phenol-extraction procedure on the disrupted cells[8]. The polysaccharide was partially purified by precipitation from the aqueous phase of the phenol extraction with Cetavlon, and, finally, by ion-exchange chromatography. The purified LP1092 polysaccharide exhibited a u.v. absorption at 264 nm that could not be eradicated by using the methods just described.

Materials. — The *O*-deacetylated LP1092 polysaccharide was obtained as previously described[8], and the *E. coli* K23 polysaccharide was kindly provided by Dr. W. F. Vann of the Office of Biologics, Food and Drug Administration, Bethesda, MD 20205, U.S.A. The ammonium salt of KDO was prepared by a slight modification of a procedure described by Unger[10]. Methyl 3-deoxy-α- and β-D-*manno*-2-octulopyranosidic acid were synthesized by previously published procedures[3], and their methyl ester derivatives, plus that of the ozonolyzed LP1092 polysaccharide, were prepared by use of an Aldrich MNNG diazomethane kit for the generation of an ether solution of diazomethane. This solution was added to a methanol solution of the acids until the persistence of a yellow coloration denoted the presence of an excess of diazomethane.

Analytical methods. — Solutions were evaporated under diminished pressure <40°. Quantification of KDO was performed by the method of Weisbach and Hurwitz[25]. A Bausch and Lomb Spectronic u.v. scanning spectrophotometer was used for u.v. measurements made on the LP1092 polysaccharide (concentration 100 μg/mL).

Circular dichroism spectra were recorded with a calibrated Cary–Varian spectropolarimeter model 61. The quartz cells (0.05-cm path) were thermostated at 27 ±0.5°. Solutions (~1 mg/mL) were made up in de-ionized, glass-distilled water.

In the case of the polymers, molar absorption coefficients ($\Delta\varepsilon$) were determined by using the mean residue molecular weight, which was calculated from the sugar composition of the repeating unit of the polymer.

^{1}H-N.m.r. spectra were recorded at 85° with a Varian CFT20 spectrometer operated at 80 MHz in the pulsed, Fourier-transform mode, with a 30° pulse-angle and an acquisition time of 1.5 s. High-resolution, ^{1}H-n.m.r. spectra were recorded at 85° with a Bruker WH-400 instrument (Regional N.m.r. Facility, University of Montreal, Montreal, Quebec) in the pulsed, Fourier-transform mode. The polysaccharides were twice lyophilized from 99.7% D_2O, and examined in the same solvent. The apparent, first-order coupling-constants (Hz) were measured directly, and the chemical shifts (δ) are expressed relative to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

^{13}C-N.m.r. spectra were recorded for solutions in 10-mm tubes, at 25°, with a Varian CFT20 spectrometer operated at 20 MHz in the pulsed, Fourier-transform mode with complete proton-decoupling. Chemical shifts are reported in p.p.m. downfield from external tetramethylsilane, and the ^{2}H resonance of deuterium oxide was used as a field-frequency lock-signal. The native and derivatized LP1092 polysaccharide were examined as solutions of 25-50 mg/mL of deuterium oxide.

Immunization procedures. — The *E. coli* LP1092 cells were grown as previously described, and were obtained in their lyophilized form following dialysis of the growth medium. New Zealand white rabbits (weight, ~2 to 3 kg) were immunized by means of six ear-vein injections, given over a period of three weeks, using 100 μg of cells in 1 mL of phosphate-buffered, physiological saline for the first injection, and doubling the amount of cells in each subsequent injection. The rabbits were test-bled after 7 days, and a further single booster-immunization, using the previously given sixth injection (containing 3.2 mg of cells) was given in the fifth week. The rabbits were bled 10 days after the final injection.

Immunodiffusion. — Double-radial immunodiffusion was performed in 1.0% agarose gels in phosphate-buffered, physiological saline containing 2% of poly(ethylene glycol) (M.W. 6000).

Reduction of LP1092 polysaccharide. — The polysaccharide (100 mg) in aqueous solution (10 mL) was treated with DEC (400 mg; obtained from Aldrich, Milwaukee, WI) as described by Taylor and Conrad[15,16]. Sodium borohydride (1 g) in water (25 mL) was then added while maintaining the pH of the solution at 8.5. Finally, the pH of the solution was adjusted to 6.5, using acetic acid (33%), and dialyzed. Lyophilization yielded the reduced LP1092 polysaccharide (100 mg), which still contained KDO (50%)[25].

Treatment of the O*-deacetylated LP1092 polysaccharide with DEC.* — The *O*-deacetylated polysaccharide (60 mg) was dissolved in water (10 mL), and treated with an excess of DEC (480 mg) while the pH of the mixture was kept at 4.75. This pH was maintained for 6 h at room temperature by the addition of 0.1M hydrochloric acid (at first) and then 0.1M sodium hydroxide. The solution was now dia-

lyzed and lyophilized, to yield the first DEC derivative of the polysaccharide (57 mg).

By repeating the procedure on the first DEC derivative, a second, more highly esterified, DEC derivative (47 mg) of the polysaccharide was obtained.

Ozonolysis of the O*-deacetylated LP1092 polysaccharide.* — The *O*-deacetylated polysaccharide (60 mg) was treated with ozone in aqueous solution (3 mg/mL), in a Welsbach model T-408 ozonizer, for 15 min at room temperature, and the solution was then lyophilized, to yield the ozonide (52 mg). The ozonide was dissolved in 0.1M sodium hydroxide (20 mL), sodium borohydride (50 mg) was added, and the solution was heated for 3 h at 60°. The pH was adjusted to 4.0 with acetic acid (33%), and the solution was dialyzed, and lyophilized, to yield 8 mg of the ozonized polysaccharide. The ^{1}H-n.m.r. spectrum of the ozonized polysaccharide was identical to that of the *O*-deacetylated polysaccharide, and exhibited no u.v. absorption at 264 nm.

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