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Isolation and Chemical Characterization of the Enterobacterial Common Antigen

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The isolation of the soluble form of the Enterobacteriaceae common antigen from *Salmonella montevideo* was achieved by a combination of the phenol/water method and the phenol/chloroform/petroleum ether extraction procedure. A phenol-soluble fraction highly enriched in the antigen was obtained which was further purified by DEAE-cellulose chromatography. A fraction eluted with 0.9 M ammonium acetate/methanol was highly active in coating erythrocytes for passive hemagglutination with antisera specific for enterobacterial common antigen and was an active inhibitor of the hemagglutinating system for this antigen.

Chemical analyses showed enterobacterial common antigen to be a linear polymer of 1,4-linked *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosaminuronic acid units esterified to a small extent by palmitic and acetic acids. These amino sugar and fatty acid components represent about 65 – 70% of the material. The presence of additional still unknown lipid components which might account for the missing 30% and for the low solubility of the isolated antigen in water is discussed. The molecular weight of the readily soluble part of the inhomogeneous isolated enterobacterial common antigen was determined by sedimentation studies in an analytical ultracentrifuge using methanol as solvent; it was found to be 2700.

Enterobacterial common antigen, sometimes abbreviated as ECA [1], is an antigen shared by almost all wild-type strains of Enterobacteriaceae [2,3]. It was first detected and defined by Kunin et al. [4] by its reactivity with antisera against *Escherichia coli* 014 in passive hemagglutination. Kunin, and later other investigators, tried different methods for isolation of this antigen and its chemical characterization [5 – 10]. A comparison of the reported data was recently made, showing that its chemical identity is not yet established [1]. It is difficult to draw any conclusion as to its chemical nature from these data, since each group developed individual extraction procedures and employed different bacterial strains as a source for their material.

A property of this antigen which has also hampered its characterization is its occurrence in two different forms. One is the ubiquitously occurring haptenic or free form, while the other is the immunogenic form which is restricted to a few R mutants of defined genetic background. Early observations by Kunin, later extended and confirmed by Neter et al. [11] and Suzuki et al. [6], indicated that in the immunogenic *E. coli* 014 enterobacterial common antigen is present in two forms one being soluble in 85% aqueous ethanol and thus being separable from lipopolysaccharide and the other one insoluble in ethanol and not separable from O-antigen [6]. More recent investigations showed that enterobacterial common antigen is immunogenic in R mutants of *E. coli* and *Shigella* which have the complete R core structure of the *coli* R1 or R4 core types and possess a functional *rfaL* gene [12 – 14]. The dependence of this immunogenicity on a defined R-core structure and a functional translocase system is a strong indication for an enzymatic transfer of haptenic enterobacterial common antigen onto the R lipopolysaccharide thus rendering it immunogenic.

Recently it was shown by Mäkelä et al. [15] and Schmidt et al. [16] that defects in either of two *ilv*-linked genes (*rfe* and *rff*) result in the formation of ECA-negative mutants. An *rfe* defect also blocks

Abbreviations. ECA, enterobacterial common antigen; GlcN, D-glucosamine; ManN, D-mannosamine; ManNUA, D-mannosaminuronic acid; carbodiimide reagent, *N*-cyclohexyl-*N*-[2-(4-morpholinyl)-ethyl]-carbodiimide methyl *p*-toluene sulfonate.

Enzyme. Hexokinase or ATP:D-hexose-6-phosphotransferase (EC 2.7.1.1).

0-chain synthesis [17], therefore *rfe*$^-$ mutants are also R mutants, whereas *rff*$^-$ mutants are S and ECA-negative [1].

Since ECA$^-$ mutants were available from *Salmonella montevideo* which could be used as negative controls during its isolation, the *S. montevideo* wild-type strain SH94 was used as source of enterobacterial common antigen.

In this communication the isolation and chemical characterization of enterobacterial common antigen will be discussed. The antigenic and immunogenic properties of the isolated material will be described in the following paper. Its state in a number of defined mutants of *Salmonella minnesota* and *S. typhimurium* will be described elsewhere.

MATERIALS AND METHODS

Chemicals

Crystalline α-benzyl-*N*-carbobenzoxy-D-mannosaminuronic acid was kindly provided by Dr P. H. Gross (Stockton, Ca.) and was used as source of the standard of ManNUA. As water-soluble carbodiimide *N*-cyclohexyl-*N*-[2-(4-morpholinyl)-ethyl]-carbodiimide methyl *p*-toluene sulfonate was purchased from Fluka (Buchs, Switzerland). All other chemicals were commercially available and were of pA quality.

Bacterial Strains

S. montevideo wild-type strain SH94 was used for extraction of enterobacterial common antigen. It was obtained from the Central Public Health Laboratory, Helsinki, Finland. As source of enterobacterial common antigen as indicator antigen, *E. coli* K-12 mutant D21e8 (*galU* mutant) [18] was always used. For production of antisera to enterobacterial common antigen the ECA-immunogenic strains *E. coli* 014:K7 (F2387), *Shigella boydii* type 3$^-$ (F3140) and *E. coli* 08$^-$:K27$^-$ (F470) were used as with previous studies [19].

Bacteria were grown in a fermentor at 37 °C at a constant pH of 7.2. The detailed conditions for growth and the medium used have been described previously [20]. For labeling of the GlcN and mannosaminuronic acid residues of enterobacterial common antigen the method of Kiss [21] was adopted: 10 ml of standard I medium (Merck, Darmstadt) containing 0.4% D-glucose was inoculated with 1 ml of an overnight culture of *S. montevideo* and with 50 μCi [1-^{3}H]-GlcNAc. At the end of the logarithmic growth phase the cells were harvested by centrifugation (10 min at 800 × *g*) and twice washed with saline.

Antisera

Antisera were prepared as described previously [19]. Briefly, rabbits (New Zealand White) were intravenously immunized at 4-days intervals with increasing amounts (0.25, 0.5 and 1.0 ml) of bacterial suspensions (10^{10} cells/ml), which had previously been heated to 100 °C for 1 h and washed with saline.

Isolation and Purification of Enterobacterial Common Antigen

Bacterial cells were extracted by a combination of the hot phenol/water and phenol/chloroform/petroleum ether extraction methods. Phenol-killed bacteria were first extracted by 45% aqueous phenol at 68 °C as described by Westphal et al. [22]. The resulting aqueous phase, after dialysis and lyophilisation, was further treated with a mixture of phenol/chloroform/petroleum ether (2/5/8) according to Galanos et al. [23]. After removal of the volatile chloroform and petroleum ether under reduced pressure, lipopolysaccharide was precipitated by adding some drops of water to the resulting phenol phase; enterobacterial common antigen remains in solution and can be recovered from the phenol phase after extensive dialysis and lyophilisation. The resulting material, being highly enriched for this substance, was resuspended in water and centrifuged at 105000 × *g* for 4 h. The supernatant was taken for further purification by DEAE-cellulose chromatography. A column (20 × 2 cm) of DEAE-cellulose (DE-32, Whatman), previously equilibrated with 0.5 M ammonium acetate/methanol buffer of pH 6.2 [24] was used. The material from the supernatant fraction was applied to the column dissolved in 0.5 M ammonium acetate/methanol buffer and was stepwise eluted with a buffer of increasing concentration of ammonium acetate (0.5, 1.0, and 1.5 M) in methanol. The resulting fractions were combined, dialyzed and/or electrodialyzed [25] and freeze-dried. Rechromatography of the middle fraction (eluate with 1.0 M ammonium acetate/methanol) with a buffer of 0.9 M ammonium acetate/methanol was monitored by differential refractometry (Lamidur Refractometer, Winopal Forschung, Hannover). The eluates were checked for enterobacterial common antigen content by Ouchterlony gel precipitation using a high-titered antiserum to this antigen.

Analytical Methods

Hydrolysis of enterobacterial common antigen was done with HCl using various conditions; for the non-reduced polymer a maximum release of sugar constituents was observed with 4 M HCl at 100 °C for 2 h. Amino sugars were separated and identified by high-voltage paper electrophoresis using a vertical chamber system at field strengths of 45 V/cm and the following buffers: (A) pyridine/formic acid/acetic acid/water (1:1.5:10:90, v/v) of pH 2.8, (B) pyridine/

acetic acid/water (10:4:86, v/v) of pH 5.3 and (C) molybdate buffer of pH 5.0 [26].

Thin-layer chromatography was carried out on cellulose plates (DC cellulose on aluminium foil, Merck) with the following solvent: butan-1-ol/acetic acid/water (4:1:5). Reducing sugars were identified by alkaline silver nitrate (Trevelyan reagent) and amino sugars also by ninhydrin. Lactones of uronic acids were detected by the hydroxamate reagent [27].

Analysis of neutral sugars was done by converting the sugars of the hydrolysate into the volatile alditol acetates and passing them over an ECNSS-M column. Amino sugars and amino acids were separated and quantified on an amino acid analyzer (Beckman model 120B). Ester-linked and amide-linked fatty acids were characterized and quantified by gas-liquid chromatography according to Rietschel et al. [28]. Total *O*-acetyl and *N*-acetyl groups were determined according to the method of Fromme and Beiharz [29].

Permethylation of the isolated fraction of enterobacterial common antigen was done by the methylsulfinylcarbanion/methyl iodide reactant of Stellner et al. [30]. Gas-liquid chromatography/mass spectrometry was performed on a Finnigan quadrupole model 3200 system coupled to a model 6000 data and graphic output system. A U-shaped glass column (0.2×152 cm) filled with ECNSS-M (3% on Gaschrom Q) was used with a helium flow of 20 ml/min. The conditions were: 160 °C, 4°/min to a final temperature of 200 °C, electron energy 70 eV, mass range of $m/e = 40-400$, integration time 7 ms per 1 m/e. 3-Deoxy-D-*manno*-octulosonic acid was assayed by the thiobarbituric acid method [31], phosphorus according to Lowry et al. [32] and nitrogen by the Kjeldahl test.

Calculation of the number of carboxylic groups in the polymer was obtained by titrating the electrodialyzed preparation of enterobacterial common antigen with 5 mM NaOH and recording the titration curve by an automatic titrator (Radiometer Copenhagen, Type TTT 1b). The sedimentation coefficient was determined by the method of Yphantis [33] and the partial specific volume calculated according to Kratny et al. [34] using a Spinco analytical ultracentrifuge (model E with schlieren optics).

Reduction of Carboxyl Groups in the Polymer

Reduction of the carboxylic groups of the mannosaminuronic acid residues in the polymer was achieved by the method of Taylor and Conrad [35]. Prior to reduction, the carboxylic groups were substituted by reaction with a water-soluble carbodiimide reagent, then the reduction was carried out with $NaBH_4$. Briefly, the uronic-acid-containing fraction was desalted by treatment with Dowex $50/H^+$ and then dissolved in a small volume of water. The pH was brought to 4.75 and was maintained close to this value during the esterification by dropwise addition of 0.1 M HCl (Radiometer Copenhagen, type TTT 1b). For carboxyl group substitution, 212 mg of the carbodiimide reagent were added as aqueous solution to 5 mg of the polymer-containing fraction. The mixture was allowed to react for 1 h at room temperature. For reduction, the substituted polymer was dissolved in water, a drop of octan-1-ol was added and then an aqueous solution of 2 M $NaBH_4$ was added dropwise. The reaction mixture was kept at pH 7 for 1 h by automatic addition of 4 M HCl (Radiometer Copenhagen, type TTT 1b). The reaction product was then dialyzed and lyophilized.

Alkali Treatment of Lipopolysaccharide

For sensitizing red blood cells, lipopolysaccharide was treated with alkali (0.25 M NaOH, 56 °C, 1 h) as described by Neter [36].

Serological Methods

Passive hemagglutination was performed with fresh human erythrocytes (blood group A), although glutardialdehyde-preserved human red blood cells gave comparable results. They were thoroughly washed with saline and suspended to a concentration of 0.5% in saline or phosphate-buffered saline (NaCl/P_i) before addition of antigen. For sensitizing red blood cells with the determinant for enterobacterial common antigen, supernatants of heated cultures (1 h, 100 °C) or 50 µg of purified antigen were added to 5 ml of red blood cell suspension. Incubation was performed at 37 °C for 30 min, then the excess of antigen was washed off and the sensitized cells were resuspended in saline or NaCl/P_i to give a 0.5% suspension. The blood suspension was added to a series of antisera dilutions as described in detail [37]. For measuring the inhibitory capacity of a substance in the hemagglutination inhibition test the inhibiting substance was dissolved in normal rabbit serum (1:50 diluted with NaCl/P_i) and serial dilutions (25 µl) of the inhibitor substance ranging from $250-0.25$ µg/ml were prepared. 25 µl of the appropriate serum dilutions ($2-3$ hemagglutination units) were added. After incubation at 37 °C for 1 h, 50 µl of the sensitized red cells were added and the plates again incubated for the same length of time. The lowest inhibitor concentration giving total inhibition of the hemagglutination was measured after 1 h at room temperature.

Agar Gel Precipitations

These were performed according to Ouchterlony [38] and immunoelectrophoresis in sodium barbiturate buffer of pH 8.6 by the microtechnique of Scheidegger et al. [39].

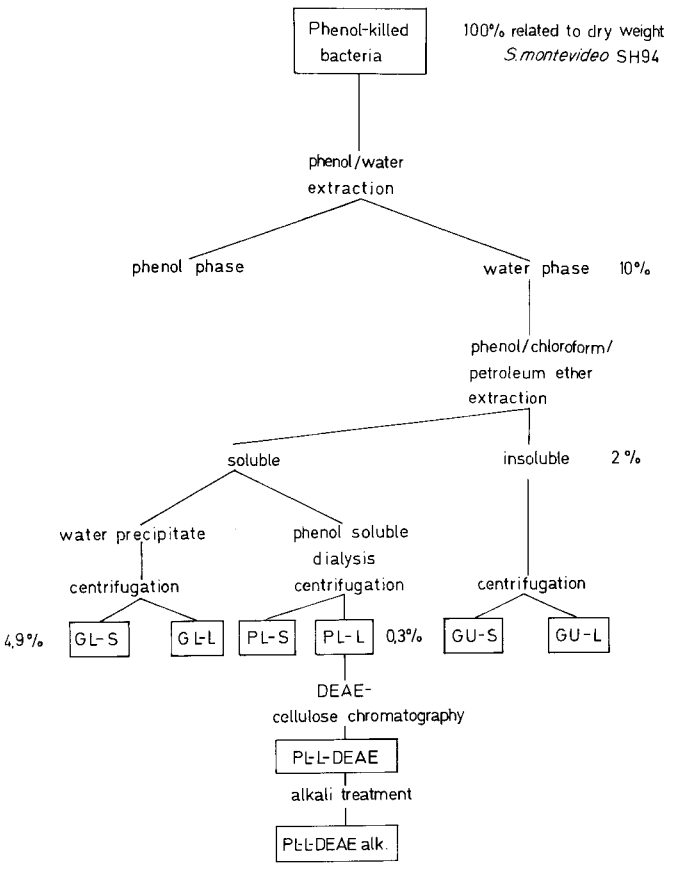

Fig. 1. *Scheme of extraction and purification procedure of enterobacterial common antigen obtained from* S. montevideo *SH94*. Fraction GU-S = sediment and fraction GU-L = supernatant of the fraction insoluble in phenol/chloroform/petroleum ether; fraction GL-S = sediment and fraction GL-L = supernatant of the water precipitate; fraction PL-S = sediment and fraction PL-L = supernatant of the phenol-soluble fraction; all these fractions were obtained after centrifugation at 105000 × *g* for 4 h

Phosphorylation by ATP and Hexokinase

Determination of the optical configurations of GlcN and ManN in hydrolysates of the carboxyl-reduced enterobacterial common antigen was performed with ATP and hexokinase as described recently [40].

RESULTS

Isolation of Enterobacterial Common Antigen

It has previously been observed that lipopolysaccharide extracted from strains containing enterobacterial common antigen by the phenol/water procedure always contained the determinant of this antigen in addition to endotoxic lipopolysaccharide. Lipopolysaccharide of R mutants containing enterobacterial common antigen and isolated by the phenol/chloroform/petroleum ether method was, however, found to be free of this antigen [12]. Attempts were therefore made to combine these two extraction procedures for the isolation of enterobacterial common antigen. Similar techniques have also been used by Galanos et al. [41] for the purification of lipopolysaccharide of enterobacterial S forms. With this method (shown in Fig. 1) phenol-killed bacteria of *Salmonella montevideo* SH 94 were used for the extraction. Purified lipopolysaccharide, free of any detectable specificity for enterobacterial common antigen, was obtained in a yield of 5% (of bacterial dry weight) in the GL-S fraction (Table 1). From the phenol phase a fraction PL-L was obtained as the supernatant fraction after extensive dialysis, lyophilisation and ultracentrifugation (105000 × *g*, 4 h). The material

Table 1. *Distribution of serological O and enterobacterial-common-antigen activities in different fractions obtained from* S. montevideo *SH94 (see Fig. 1)*
Titers in the passive hemagglutination test and hemagglutination inhibition test with antisera specific for anterobacterial common antigen (ECA) and O-antigen are shown. ECA-antiserum = antiserum directed against *Shigella boydii* type 3$^-$; O-antiserum = antiserum directed against *S. montevideo* SH94; ECA-antiserum 1:640 and ECA-containing lipopolysaccharide derived from *E. coli* K12 D21e8 were used for the inhibition test and the latter also as indicata antigen for the passive hemagglutination

Fraction	Passive test				Inhibition test
	ECA-antiserum			O-antiserum	ECA-antiserum 1:640
	100 µg	10 µg	1 µg	100 µg	100 µg
					µg/ml
GL-L	5120	2560	<10	1280	3.9
GL-S	< 10	< 10	<10	160	≥ 250
GU-L	5120	2560	<10	80	0.25
GU-S	≥ 10240	5120	<10	320	15.6
PL-L	≥ 10240	≥ 10240	2560	40	< 0.25
PL-S	≥ 10240	5120	2560	40	< 0.25

amounted to about 0.3% of bacterial dry weight, and was found by serological tests (hemagglutination inhibition) to be highly enriched for enterobacterial common antigen (Table 1). It did, however, still show a slight O-titer when tested against the homologous *S. montevideo* SH 94 antiserum. Examination of the PL-L fraction in immunoelectrophoresis with a high-titered antiserum to enterobacterial common antigen revealed a precipitation line towards the anode indicating that the antigen was highly negatively charged. Although a high inhibition of an hemagglutinating system for enterobacterial common antigen was also obtained with the sediment fraction after ultracentrifugation (fraction PL-S in Fig. 1), most of the further work was performed with the PL-L fraction.

Purification of Enterobacterial Common Antigen

Ion-exchange chromatography on DEAE-cellulose was used as a further step in purification of the negatively charged antigen. With the knowledge that this antigen contains a high percentage of amino sugars, the PL-L fraction was extracted from bacteria labeled by growth in [1-^{3}H]GlcNAc-containing medium [21]. The elution profile of the material with ammonium acetate/methanol buffer of increasing concentration (0.1 – 1.6 M) is shown in Fig. 2. The peak of the eluted ^{3}H content coincided with the precipitation pattern obtained by agar double diffusion of the individual fractions. The bulk of the antigen elutes with 0.8 – 0.9 M ammonium acetate in methanol. For preparative purposes a stepwise elution with ammonium acetate of increasing concentration (0.5, 1.0 and 1.6 M) was carried out, yielding the entire enterobacterial common antigen material in the middle fraction. The first fraction contained some lipophilic material without serological specificity for enterobacterial common antigen and was not further examined.

Rechromatography of the middle fraction with 0.8 M ammonium acetate buffer eluted enterobacterial common antigen as a symmetrical peak monitored by differential refractometry. This purified fraction (PL-L-DEAE) contained all the serological properties of the native antigen, i.e. erythrocyte-coating capacity, hemagglutination with its antisera, inhibition of its hemagglutinating system, as well as agar gel precipitation with high-titered antisera to enterobacterial common antigen. This material was used for the analytical and structural studies described below.

Chemical Analyses

Qualitative chemical studies showed that enterobacterial common antigen consists primarily of D-glucosamine (GlcN) and D-mannosaminuronic acid (ManNUA) both being *N*-acetylated. A direct quantification of the aminuronic acid was not possible due to its rapid degradation under the strong acidic conditions used for its liberation [42]. After hydrolysis with hydrochloric acid the majority of liberated aminuronic acid is present as lactone [40] which is easily demonstrable by high-voltage paper electrophoresis, by staining with either Trevelyan reagent, ninhydrin or the hydroxamate reagent (Fig. 3). After *N*-acetylation of the hydrolysate a negatively charged component in buffer A was detectable with $m_{GlcN} = 0.27$. The migration data in buffer systems A, B and C were the same as described recently for mannosaminuronic acid. Buffer system C readily separates glucosaminuronic, galactosaminuronic and mannosaminuronic acids [26]. Final proof of the chemical identity of the aminuronic acid of enterobacterial common antigen with mannosaminuronic acid was obtained by co-electrophoresis with an authentic standard of D-mannosaminuronic acid and by reducing the aminuronic acid in the polymer to D-mannosamine. For quantitative determination of mannosaminuronic acid the polymer was reduced with $NaBH_4$ after reaction with the carbodiimide reagent (repeated four times) and hydrolysis of the reduced polymer with 4 M HCl for various periods of time. Fig. 4 shows that a maximal release of ManN was obtained after 6 h, and that GlcN and ManN are present in approximately a 1:1 ratio.

Both sugars in the reduced polymer were found to be substrates for enzymatic phosphorylation by hexokinase/ATP [43] showing the D-configuration of

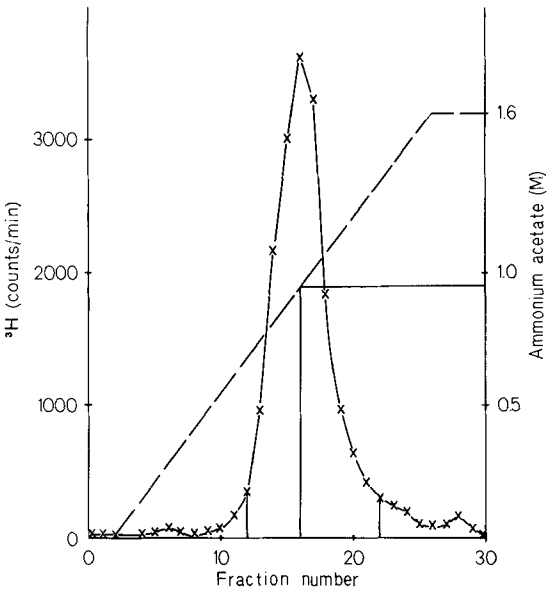

Fig. 2. *Column chromatography on DEAE-cellulose of fraction PL-L containing enterobacterial common antigen from* S. montevideo *SH94 labelled with* ^3H. The antigen was eluted from the column (2 × 20 cm, DE 32, Whatman) at room temperature with a linear gradient (——) ranging from 0.1 – 1.6 M ammonium acetate. (× —— ×) *N*-Acetyl-[1-^{3}H]glucosamine incorporated

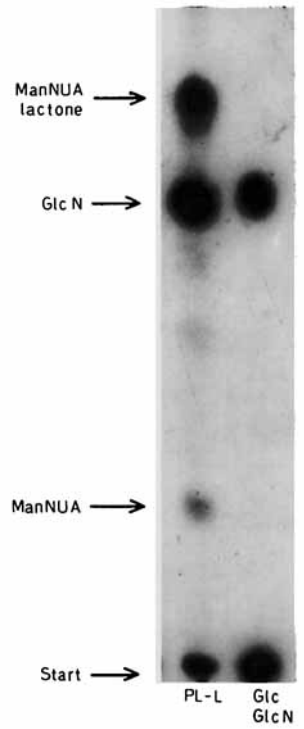

Fig. 3. *High-voltage electropherogram of fraction PL-L from the wild-type* S. montevideo *SH94*. 50 µg PL-L material was hydrolyzed with 4 M HCl at 100 °C for 2 h. After electrophoresis (3 kV) in buffer system (A) at pH 2.8 the electropherogram was stained with alkaline silver nitrate

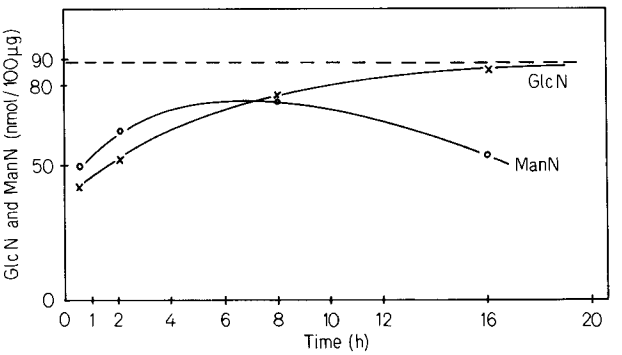

Fig. 4. *Kinetics of the liberation of GlcN and ManN from the carboxyl-reduced enterobacterial common antigen from* S. montevideo *SH94 by 4 M HCl at 100 °C*. Reduction was performed according to the method described by Taylor and Conrad [35]

Table 2. *Chemical composition of enterobacterial common antigen derived from* S. montevideo *SH94*
Amounts are given as percentages of the material dry weight. n.d. = not detectable

Constituent	Amount	Remarks
	%	
Amino sugars	45.6–50	D-GlcN, D-ManNUA (in a 1:1 ratio)
Acetyl groups	13	11.7% *N*-acetyl, 1.3% *O*-acetyl
Total fatty acids	2.5	mostly $C_{16:0}$ ester-bound (2.0%)
Phosphate	0.47	
Neutral sugars	n.d.	
Amino acids	n.d.	

GlcN and ManN and also of mannosaminuronic acid in enterobacterial common antigen.

The amount of amino sugar found by these analyses was 33% of the dried PL-L-DEAE fraction. This value is surely the minimal content because neither the reduction of ManNUA to ManN nor the liberation of the amino sugars by acid hydrolysis can be complete.

Investigation of the PL-L-DEAE fraction for neutral sugars (by gas-liquid chromatography) or for 3-deoxy-D-*manno*-octulosonic acid (thiobarbituric acid reaction) was completely negative. Amino acids were found only in trace amounts, no amino acid being found preferentially. Fatty acids were present in a rather small percentage (Table 2); the main (ester-linked) fatty acid was palmitic acid.

Total nitrogen was determined as 3.58%. Assuming that the amino sugars are the only nitrogen-containing constituents of enterobacterial common antigen and that they occurred in a 1:1 ratio, the total percentage of amino sugars can be calculated as 48.8%. This value is the upper limit of the amino sugar content and it agrees with a calculation based on the *N*-acetyl determination. Assuming all amino sugars are *N*-acetylated, the *N*-acetyl content of 11.7% is then equivalent to 45.6% of the amino sugar mixture.

Titration of the carboxylic groups of mannosaminuronic acid in the PL-L-DEAE fraction (after electrodialysis) gave a value corresponding to 23–27% mannosaminuronic acid (three independent measurements). This is approximately half the amount of total amino sugar as calculated by nitrogen or *N*-acetyl determination (see above), a value which supports the sugar analysis of the reduced polymer which gave an approximate ratio of 1:1 for GlcN and ManNUA.

Analysis of the Amino Sugar Linkages

To determine the linkages of the two amino sugar constituents, a methylation analysis according to Hakomori was carried out with the carboxyl-reduced polymer. The partially methylated amino sugars in the hydrolysate were converted to alditol acetates and separated on an ECNSS-M column. Two peaks,

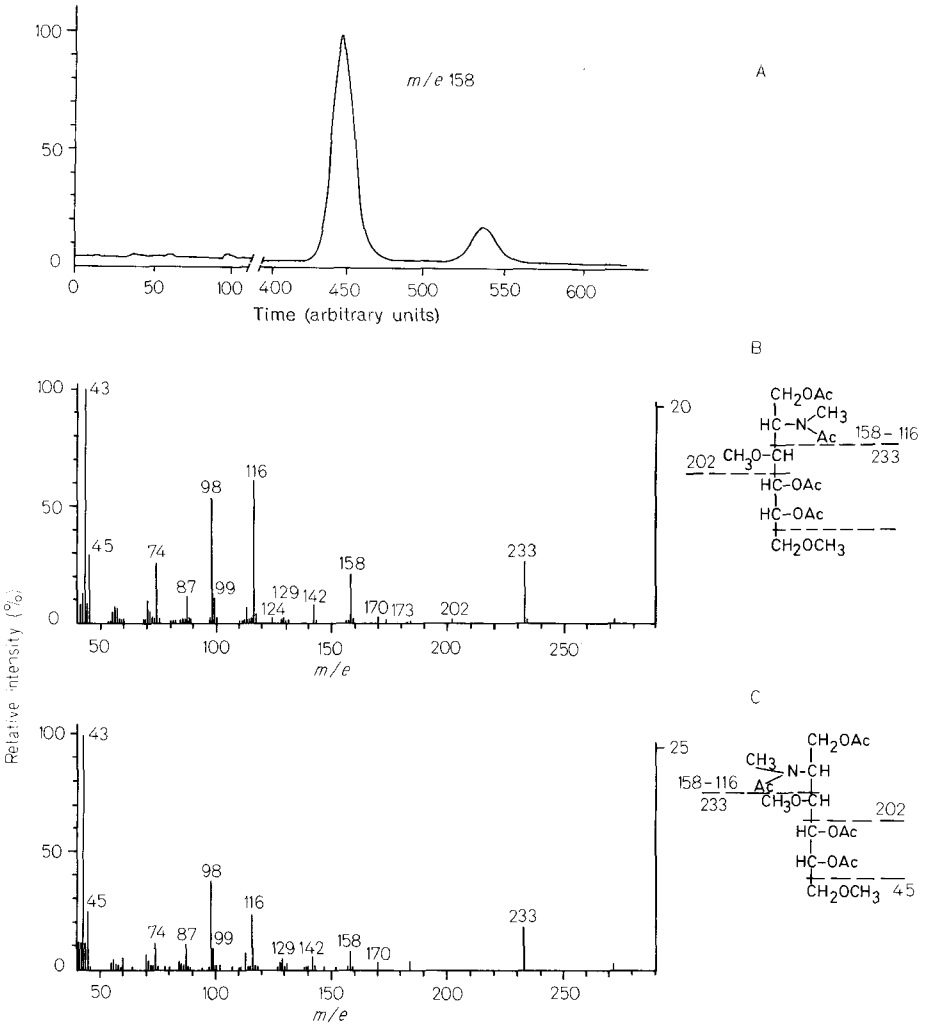

Fig. 5. *Separation of partially methylated alditol acetates according to the method of Stellner et al. [30] on gas-liquid chromatography (ECNSS-M column) at 160 °C (program 4 °C/min to 200 °C).* (A) Mass fragmentography of mass *m/e* 158; (B, C) mass spectra and fragmentation schemes of 3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol (B) and 3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamido-mannitol (C). The mass spectra were taken at an ionizing energy of 70 eV with a Finnigan quadrupol model 3200 system

eluted at 33.3 and 40.4 min, were found by monitoring the characteristic mass *m/e* 158. The spectra of the two components were almost the same showing that both were derived from 3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-deoxy-2-*N*-acetamido-hexitols (Fig. 5). No permethylated amino sugar and no mono-*O*-methyl derivative of amino sugars could be detected, indicating that enterobacterial common antigen consists predominantly of a linear chain of 1,4-linked *N*-acetyl-amino sugars (Fig. 6)[1]. The lack of a permethylated (terminal) amino sugar is surprising considering the low molecular weight (about 2700) (see below). Periodate oxidation of the reduced polymer shows, however, that ManN is partly destroyed (27%), whereas GlcN remains unaffected. We were not able to render the reduced polymer completely periodate-oxidizable by a preceding hydrazinolysis. Periodate oxidation of the unreduced hydrazinolyzed polymer and a subsequent mild acid hydrolysis followed by $NaBH_4$ reduction, afforded some erythronic acid, but no glyceric acid (Table 3) again pointing to a 1,4-linkage of the aminuronic acid.

[1] In the nuclear-magnetic-resonance spectrum of alkali-treated antigen (after H/^{2}H exchange) in ^2H_2O a broad signal with an intensity of 1 is found at $\delta = 5.33$ ppm. The signals of equatorial anomeric protons are always in this region, hence one of the two glycosidic linkages expected for the antigen is α and the other one must therefore be β. Further studies are needed for a more detailed determination of the anomeric structures.

Isolation of Oligosaccharides

From enterobacterial common antigen subjected to 4 M HCl hydrolysis at 100 °C, two charged oligosaccharides could be separated by preparative high-voltage electrophoresis. These oligosaccharides seem to be disaccharides. Both consist exclusively of GlcN

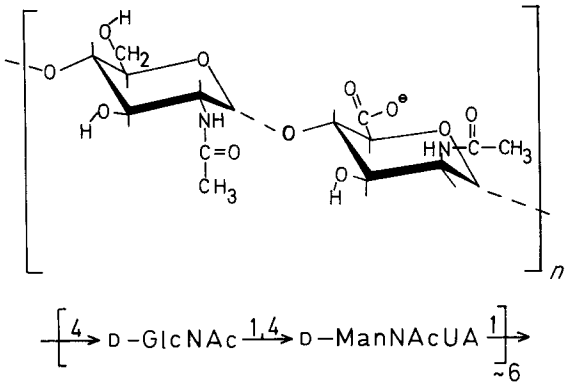

Fig. 6. *Proposed structure for the sugar moiety of enterobacterial common antigen obtained from* S. montevideo *SH94*

and ManNUA as shown by further hydrolysis of the eluted fractions. One oligosaccharides (a) is readily liberated after short hydrolysis (1 h) with 4 M HCl at 100 °C. It has a migration value of $m_{GlcN} = 1.35$ in high-voltage electrophoresis at pH 5.3 and contains ManNUA in the lactone form as judged from the positive hydroxamate test. Reduction of oligosaccharide (a) with $NaBH_4$ and a subsequent hydrolysis and peracetylation does not give glucosaminitol peracetate but glucosamine peracetate, proving that GlcN is not the reducing terminal of oligosaccharide (a). Treatment of oligosaccharide (a) with 0.5 M NaOH (10 min, 25 °C) easily liberates GlcN and a reaction product showing a significant absorption at 300 nm.

The second oligosaccharide (b) can be isolated after strong acid hydrolysis (4 M HCl, 100 °C, 6 h) by high-voltage electrophoresis in buffer A with m_{GlcN} being 0.72. It is very acid-stable and even after drastic conditions (6 M HCl, 100 °C, 32 h) it is not completely hydrolyzed. GlcN forms the reducing end as demonstrated by $NaBH_4$ reduction. This oligosaccharide is therefore similar to aldobiuronic acids which are known for their extreme stability to acid hydrolysis.

Physico-chemical Properties of Enterobacterial Common Antigen

The isolated material was not soluble in water at concentrations higher than 0.3%. After removal of the ester-linked fatty acids by alkali treatment the solubility was only slightly increased to 0.5%. The material is, however, soluble in methanol. For this reason, the sedimentation studies on the untreated enterobacterial common antigen in the analytical ultracentrifuge were carried out in methanol as solvent. The sedimentation coefficient in methanol determined by the method of Yphantis [34] is $s^{\circ}_{20} = 1.5$ S and that calculated in water is $s^{\circ}_{20} = 0.56$ S. The partial specific volume of the substance was measured [35] as 0.5759 ml/g at 20 °C. From these data a molecular weight of 2700 was calculated for the part of the PL-

Table 3. *Comparison of the electrophoretic and chromatographic behaviour of glyceric acid, erythronic acid and the fragment of enterobacterial common antigen obtained after periodate oxidation, mild hydrolysis and $NaBH_4$ reduction*
Migration relative to dOclA (3-deoxy-D-*manno*-octulosonic acid), m_{dOclA}, was measured by high-voltage electrophoresis (3 kV, 45 V/cm) in system A at pH 2.8 and in system B at pH 5.3. R_F values were measured by thin-layer chromatography on cellulose plates in butan-1-ol/acetic acid/water (4/1/5)

Substance	m_{dOclA} in system		R_F
	A (pH 2.8)	B (pH 5.3)	
dOclA	1	1	–
Glyceric acid	1.07	1.47	0.32
Erythronic acid	0.89	1.37	0.4
Antigen fragment	0.89	1.36	0.4

L-DEAE material which was readily soluble in methanol. It is obvious that enterobacterial common antigen in aqueous solutions forms micelles and co-micelles of much higher molecular weight.

DISCUSSION

The data presented in this paper show that the enterobacterial common antigen is a linear heteropolymer composed predominantly of alternating units of *N*-acetyl-D-glucosamine and *N*-acetyl-D-mannosaminuronic acid which are partly esterified by palmitic acid. The high negative charge is due to the content of the aminuronic acid. At least 50% of the isolated and purified material consists of these two *N*-acetyl-amino sugars which occur in a molar ratio of about 1:1. The amino sugars are arranged in a chain with 1,4-linkages. Oligosaccharides, probably disaccharides, consisting of both GlcN and ManNUA were isolated. Only one had GlcN as the reducing terminal, the other one had it as the non-reducing terminal. This suggests that these two sugars occur in an alternating sequence in the polymer chain. As expected for 1,4-linked *N*-acetyl-amino sugars, periodate oxidation had no effect. We have, however, no explanation so far as to why the amino sugars are not completely destroyed by periodate oxidation after preceding hydrazinolysis. Incomplete de-*N*-acetylation [44] or steric hindrance by the bulky carboxyl groups [45] might be responsible for the lack of reactivity. The observed partial destruction of ManN ($\approx$ 27%) in the reduced polymer could indicate that either some of the ManNUA residues in the antigen chain are in a terminal position or carry unsubstituted $-NH_2$ groups. The extent of the oxidation would be consistent with a structure having 8–10 amino sugars in a 1,4-linked chain, which is also indicated by the low molecular weight of 2700. On the other hand no permethylated mannosamine could be detect-

ed in the methylated reduced sugar polymer. The material used for the methylation study was, however, only reduced once and it is possible that the mannosamine obtained by a single reduction step gives so little permethylated material that it is below the limit of detection. It should be pointed out that the M_r of 2700 is that of the readily soluble fraction of the inhomogeneous antigen isolated in methanol. In aqueous solutions it forms micelles of much higher molecular weight.

The partial esterification of enterobacterial common antigen by palmitic acid is (partly) responsible for its hydrophobic character and may be necessary for its incorporation into the outer membrane of Enterobacteriaceae. Its low solubility in water, even after removal of the ester-linked fatty acids, suggests the presence of a hitherto undetected lipid component. The addition of all components detected so far shows a deficit of about 30% on a weight basis. Marx et al. [10] described a cephaline-like L-phosphoglyceride as a constituent of their isolated material from *S. typhimurium*. A careful search for cephaline-like substances in our material was not successful.

Fig. 6 shows the structure proposed for the sugar moiety of enterobacterial common antigen. No information is available so far on the point of attachment of the palmitic acid. From the molecular weight and the percentage of palmitic acid present, it is obvious that not more than a single palmitic acid can be linked to one polymer molecule. Nevertheless splitting off this fatty acid leads to a complete loss of its erythrocyte-coating abilities [12]. The structure proposed for the sugar part (Fig. 6) shows that β-elimination is theoretically possible and is expected. Indeed, it was found that the one disaccharide isolated after short hydrolysis (4 M HCl, 1 h) immediately liberated the terminal GlcN under alkaline conditions and gave a second reaction product with an absorption in the 300-nm range [46,47]. It is therefore assumed that β-elimination can also occur during alkali treatment, when some of the carboxylic groups of the ManNUA residues are esterified. This may be the reason for the lower inhibitory capacity of alkali-treated enterobacterial common antigen observed in an hemagglutination system (discussed in the following paper).

The chemical composition described in this paper does not agree with earlier reports on the chemical nature of enterobacterial common antigen. Most investigators found, however, that the Kunin antigen was negatively charged [5,8,10,12] and that it contained considerable amounts of glucosamine [5,7,10]. A sample of material isolated, extracted and purified according to the method of Marx et al. [10] was kindly provided for us; we have demonstrated that it contained appreciable amounts of ManNUA and GlcN, indicating that part of the material may be identical to our PL-L-DEAE fraction. The high hexose content described by Johns et al. [8] for their serologically uniform preparation, is not in accord with our isolated material, which was essentially free of neutral sugars. Since mutants with enzymatic defects in glucose, galactose and mannose biosynthesis (UDPG-pyrophosphorylase-less mutant Gal 23 K^-, UDPGal-4-epimerase-less mutant PL-2 of *E. coli* K-12, and phosphomannose-isomerase-less mutant of *S. typhimurium*) all synthesize enterobacterial common antigen in normal amounts and contain the GlcN/ManNUA polymer (data not shown) to an appreciable extent, these neutral sugars are therefore not essential parts of the antigen.

From these facts it might be assumed that not only a single antigen is shared by several Enterobacteriaceae but the presence of at least two have to be considered. In a following paper we will show that the serological and immunological properties of our isolated material are identical to that of the common antigen first described and defined by Kunin. We hope to show further that mutants deficient in enterobacterial common antigen (of the *rfe*$^-$ and the *rff*$^-$ type) lack the GlcN/ManNUA polymer (ECA$^-$ mutants) even in the PL fractions (unpublished results).

The amphipathic character of enterobacterial common antigen makes it very surface-active. It readily attaches to lipopolysaccharide or cellular components, to erythrocytes and lymphocytes [4,48], thus providing the material with specificity for enterobacterial common antigen. This might be a reason for the conflicting data on its isolation and localisation.

ManNUA is an essential constituent of enterobacterial common antigen and modification of this constituent in the polymer by esterification or reduction of the carboxylic group leads to a dramatic change of its serological behaviour (precipiting line in immunoelectrophoresis, hemagglutination inhibition capacity). This aminuronic acid is a rare constituent in gram-negative bacteria. It was described as constituent of the K7 and the K56 antigen of *E. coli* [40,49] and of the K15 antigen of *Vibrio parahaemolyticus* [50]. In gram-positive bacteria ManNUA was described as part of the cell wall polysaccharide of *M. lysodeikticus* [42,51] and as a surface antigen of *Staphylococcus aureus* [52]. The biosynthesis of ManNUA in *E. coli* was investigated by Kawamura [53]. UDP-GlcNAc is first epimerized to UDP-ManNAc and then converted by a dehydrogenase to UDP-ManNAcUA, the activated form of ManNUA. This allows the labeling *in vivo* of both amino sugars (GlcN and ManNUA) by growing the bacteria in the presence of labeled GlcNAc as described previously by Kiss [21]. Labeled enterobacterial common antigen will certainly be of value for further studies on the biochemistry and the biology of this antigen.

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