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The Synthesis of Exopolysaccharide by *Klebsiella aerogenes* Membrane Preparations and the Involvement of Lipid Intermediates

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1. Membrane preparations from *Klebsiella aerogenes* type 8 were shown to transfer glucose and galactose from their uridine diphosphate derivatives to a lipid and to polymer. The ratio of glucose to galactose transfer in both cases was 1:2. This is the same ratio in which these sugars occur in native polysaccharide. Galactose transfer was dependent on prior glucosylation of the lipid. Mutants were obtained lacking (*a*) glucosyltransferase and (*b*) galactosyltransferase. The transferase activities in a number of non-mucoid mutants was examined. 2. Glucose transfer was partially inhibited by uridine monophosphate, and incorporation of either glucose or galactose into lipid was decreased in the presence of uridine diphosphate. The sugars are thought to be linked to a lipid through a pyrophosphate bond, and treatment of the lipid intermediates with phenol yielded water-soluble compounds. These could be dephosphorylated with alkaline phosphatase. Transfer of glucuronic acid to lipid or polymer from uridine diphosphate glucuronic acid was much lower than that of the other two sugars. 3. The fate of sugars incorporated into polymer was also followed. Some conversion of glucose into galactose and glucuronic acid occurred. Mutants unable to transfer glucose or galactose to lipid were unable to form polymer. Other mutants capable of lipid glycosylation were in some cases unable to form polymer. A model for capsular polysaccharide synthesis is proposed and its similarity to the formation of other polymers outside the cell membrane is discussed.

Two polysaccharides common to most Gram-negative bacteria, mucopeptide and lipopolysaccharide, both contain repeating units in their structure. The synthesis of each of these compounds, reviewed by Ghuysen, Tipper & Strominger (1969) and by Nikaido (1968) respectively, occurs by membrane-bound enzymes. Sugars are added sequentially from sugar nucleotide precursors to a lipid carrier. The lipid involved is apparently very similar in each system and has been identified as a phosphorylated C_{55}-isoprenoid alcohol of the general structure: $-[CH_2-C(CH_3)=CH-CH_2]-$ (Wright, Dankert, Fennessey & Robbins, 1967; Higashi, Strominger & Sweeley, 1967). The Gram-positive species *Micrococcus lysodeikticus* similarly uses a lipid carrier during synthesis of an extracellular mannan (Scher, Lennarz & Sweeley, 1968).

A third type of extracellular polysaccharide synthesized by Gram-negative bacteria, and found also in some Gram-positive species, is capsule or slime material. The synthesis of this has received much less attention than that of other polymers. Studies with *Diplococcus pneumoniae* showed the requirement for particulate enzyme preparations and the incorporation of radioactively labelled sugars from appropriate sugar nucleotides into polymer (Smith, Mills & Bernheimer, 1961). More recent work on the structure of a number of capsular or slime polysaccharides from Gram-negative bacteria showed that they are generally composed of fairly simple repeating units composed of three to six sugar residues (e.g. Hungerer, Jann, Jann, Ørskov & Ørskov, 1967; Conrad, Bamburg, Epley & Kindt, 1966; Sutherland, 1969). There is thus some similarity between the O-antigen component of lipopolysaccharides and the exopolysaccharides synthesized by such species as *Escherichia coli* or *Klebsiella aerogenes*. Both are extracellular polymers and both contain sugar repeating units of grossly similar type. The only indication of similarities in the biosynthesis of these two polysaccharides was the report of involvement of a lipid carrier stage in the synthesis of capsular polysaccharide by a *K. aerogenes* strain (Troy & Heath, 1968). The component sugars of the polymer were galactose, mannose and glucuronic acid, but no details of the structure were given.

The strain A4 (serotype 8) of *K. aerogenes* has a simple tetrasaccharide repeating unit (Fig. 1) (Sutherland, 1970). It does not contain acetyl or

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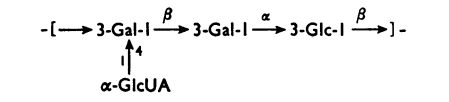


Fig. 1. Tetrasaccharide repeating unit of *K. aerogenes* strain A4 capsular polysaccharide.

pyruvyl groups and was chosen for its simple structure and the ready availability of the presumed glycosyl donors: UDP-glucose, UDP-galactose and UDP-glucuronic acid. An examination of several of the enzymes thought to be involved in the formation of the sugar nucleotide glycosyl donors required for exopolysaccharide synthesis has already been made (M. Norval & I. W. Sutherland, unpublished results) with the wild-type strain and a number of mutants. The present study reports the use of membrane preparations in a cell-free system for the transfer of sugars from sugar nucleotides to lipid-soluble material and to polysaccharide-resembling capsular material.

MATERIALS AND METHODS

Cultures. The wild-type strain of *K. aerogenes* strain A4 (serotype 8) was studied by Dudman & Wilkinson (1956) and the structure of its exopolysaccharide capsule has been determined (Sutherland, 1970). The isolation of mutants from it and the contents of several of the enzymes involved in sugar nucleotide synthesis have been examined (M. Norval & I. W. Sutherland, unpublished work). Bacteria were normally grown in nutrient broth or in trypticase soy broth (Baltimore Biologicals Laboratory, Baltimore, Md., U.S.A.) supplemented by 1% (w/v) glucose. A number of mutants were also grown in nutrient broth containing 1% (w/v) galactose. Cultures were grown in 1 litre quantities in 2-litre Erlenmeyer flasks shaken at 35°C for 15h at 200rev./min.

Analytical methods. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Glucose, galactose and glucuronic acid were determined as described by Sutherland (1970).

Chemicals. Unlabelled sugar nucleotides were purchased from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.) and from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). ^{14}C-labelled sugar nucleotides (randomly labelled in the sugar moieties) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Other chemicals and biochemicals were of the highest grade commercially available.

Chromatography and electrophoresis. Descending paper chromatography was performed at room temperature in the following solvent systems with Whatman no. 1 paper: solvent A, butan-1-ol–pyridine–water (6:4:3, by vol.) (Whistler & Conrad, 1954); solvent B, ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.) (Feather & Whistler, 1962); solvent C, ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.) (Fischer & Doerfel, 1955); solvent D, ethanol–1.0M-ammonium acetate (pH7.5) (7:3, v/v) (Paladini & Leloir, 1952). T.l.c. on silica gel G plates was carried out in solvent E [chloroform–methanol–water (12:6:1, by vol.)]. Paper electrophoresis on Whatman no. 1 paper was performed in pyridine–acetic acid buffer, pH5.3. Locarte paper-electrophoresis equipment was used with a current of 90mA applied for 3h at 40V/cm. Development of chromatograms or electrophoresis strips was carried out with alkaline $AgNO_3$ reagent or with the phosphate reagent of Hanes & Isherwood (1949).

Measurement of radioactivity. Radioactivity on paper strips was located with a Tracerlab 4 pi Scanner. Because of the low efficiency of counting, results were usually checked by cutting out 1cm segments and placing them in vials with 10ml of toluene containing 0.5% of 2,5-diphenyloxazole for scintillation counting in a Beckmann ambient-temperature scintillation spectrometer (Lennarz & Talamo, 1966). Lipid extracts were evaporated to dryness at 60°C and their radioactivities counted in 10ml of Triton scintillation fluid (Lennarz & Talamo, 1966). Radioactivities of material from t.l.c. plates were also counted in this fluid. For aqueous samples a dioxan-based scintillation fluid was used [Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K.]. The efficiency of counting for ^{14}C was approx. 50% for liquid samples and 30% for paper samples determined by scintillation counting.

Preparation of membrane material. Cells from 2 litres of culture were harvested by centrifugation at 5000*g* and washed twice in 0.9% NaCl buffered to pH8.0 with 10mM-tris. They were then resuspended in 50ml of 10mM-tris–HCl buffer, pH8.0, and 10ml portions were treated for 2min in MSE ultrasonic equipment with 100W output. The container was cooled with an ethanol–ice mixture and the unbroken cells were removed by centrifugation at 5000*g* for 20min. The supernatant fluid from this preparation was then centrifuged at 20000*g* for 60min and washed by resuspension in tris buffer. The reddish gel of membrane material was carefully removed and resuspended in 5ml of water at 0°C.

Assay for lipid material. Samples (50μl) from incubation mixtures were added to 4ml of chloroform–methanol (2:1, v/v) and homogenized in a vortex mixer. The tubes were then placed in a water bath at 60°C for 5min and again mixed. Then 1ml of 0.9% NaCl was added and the mixture was again homogenized. Phase separation was achieved by centrifugation for 15min in a bench centrifuge. The aqueous layer and interface were carefully removed by using a long Pasteur pipette and gentle suction. The chloroform layer was carefully transferred to scintillation vials with fresh Pasteur pipettes. Zero-time controls consistently showed radioactivities of 20–25c.p.m. This was also true of samples of the different labelled sugar nucleotides included to check that there was no breakdown or carry-over of sugars with this technique.

Assay for polymer material. Samples (25 or 50μl) from incubation mixtures were applied to Whatman no. 1 paper as 2cm strips. The sheets of paper were irrigated for 20h in solvent D to remove low-molecular-weight material. The areas at the origin forming strips 2cm×1cm were cut out and their radioactivities determined.

RESULTS

Transfer of sugars to lipid material. The ability of osmotically shocked cells of *K. aerogenes* to

transfer sugars from sugar nucleotides to lipid material was observed by Troy & Heath (1968). There are, however, considerable disadvantages in using whole cells. In an attempt to overcome these, membrane material was prepared as described in the Materials and Methods section. This was then used as a potential source of enzymes for exopolysaccharide synthesis. Incubation mixtures were prepared as follows: 0.25M-tris–HCl buffer, pH 8.0, 100μl; 50mM-magnesium chloride, 50μl; membrane suspension, 200μl; water, 100μl. Sugar nucleotides were added in the same proportions as the sugars were found in the polysaccharide, namely 400nmol of UDP-glucose or UDP-glucuronic acid and 800nmol of UDP-galactose. Labelled nucleotides were added as follows: UDP-glucose, 3.7×10^5 c.p.m.; UDP-galactose, 1.1×10^5 c.p.m.; UDP-glucuronic acid, 5.5×10^5 c.p.m. The total volume was adjusted to 550μl with water. The mixtures were allowed to equilibrate to room temperature (20°C) before addition of the sugar nucleotides and finally the membrane.

When single nucleotides were added, with membrane from wild-type cells, there was rapid transfer of glucose or galactose from their respective sugar nucleotides to lipid material but no transfer of glucuronic acid. The rate of incorporation of either sugar was maximal over the first 10min but continued at a decreased rate for 30–40min. Typical results are shown in Fig. 2. After 30min the ratio of glucose incorporation to galactose incorporation was approx. 3.5:1, and no glucuronic acid was transferred.

On the addition of all three sugar nucleotides, each in turn being labelled, a different pattern of incorporation was observed. Typical results (Fig. 3) show that the amount of galactose incorporated into lipid is approximately twice that of glucose. Much less glucuronic acid than glucose or galactose was transferred. As the cells contain an active UDP-galactose 4-epimerase in the membrane fraction (M. Norval & I. W. Sutherland, unpublished work) it was to be expected that some interconversion of the sugars would occur. Such interconversion would only be absent from epimerase-less mutants and attempts to obtain these were unsuccessful. The presence of a particulate UDP-glucose dehydrogenase would also permit some incorporation of glucose into UDP-glucuronic acid and hence into lipid, but the very small extent of transfer of labelled glucuronic acid from UDP-glucuronic acid to lipid was unexpected. The absence of such transfer activity might be due to: (i) large quantities of membrane-bound UDP-glucuronic acid; (ii) the addition of glucuronic acid from a precursor other than UDP-glucuronic acid; (iii) a soluble glucuronyltransferase; or (iv) the addition of glucuronic acid at a relatively late stage in biosynthesis to a neutral sugar 'core'. The first three of these possibilities can be excluded. Extraction of membrane preparations with ethanol yielded only trace amounts of UDP-glucuronic acid, identified and determined as described by Grant, Sutherland & Wilkinson (1970). Nor was any other glucuronic acid-containing nucleotide detected. The addition to the assay mixture of the 20000*g* supernatant obtained during membrane preparation caused no increase in glucuronic acid transfer to lipid.

Transfer activity. Attempts to obtain transfer of the neutral sugars to lipid material were made with

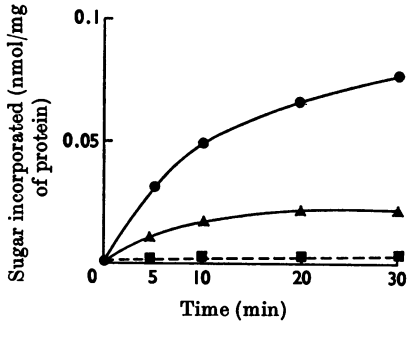


Fig. 2. Transfer of sugar from sugar nucleotides to lipid. The standard incubation mixture was used containing a single sugar nucleotide together with ^{14}C-labelled material. Samples (50μl) were withdrawn at intervals and extracted (400nmol of UDP-glucose or UDP-glucuronic acid or 800nmol of UDP-galactose was used) with chloroform–methanol (2:1, v/v). ●, UDP-glucose; ▲, UDP-galactose; ■, UDP-glucuronic acid.

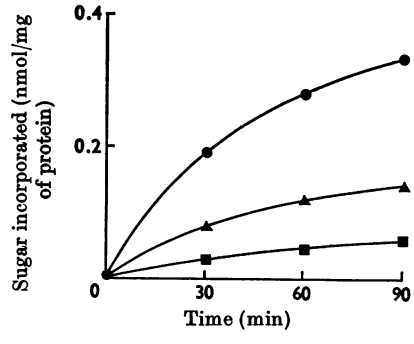


Fig. 3. Transfer of sugars from mixtures of sugar nucleotides to lipid. The standard incubation mixture contained, in each case, all three sugar nucleotides, one of which was labelled with ^{14}C in the sugar moiety. Samples were taken periodically for chloroform–methanol extraction. ●, UDP-galactose; ▲, UDP-glucose; ■, UDP-glucuronic acid.

a number of cell fractions. Only a complete ultrasonic lysate and the material deposited by centrifugation at 20000*g* were active in the transfer of glucose or galactose. On the basis of protein content the membrane preparations were two to three times as effective as an equivalent amount of whole-cell lysate in the transfer of either sugar from the corresponding sugar nucleotide to lipid material. The supernatant fluid from the 20000*g* centrifugation and material deposited from it by ultracentrifugation at 100000*g* were completely inactive.

Normally membrane preparations were used immediately after preparation, all stages during the process being performed in the cold. However, preparations held in vials completely surrounded by ice at 0°C for up to 4 days retained 90% of their activity. Preparations that were frozen and then rapidly thawed at room temperature lost all transferase activity. When thawing was carried out slowly over a period of 4–5h frozen samples retained about 80% of their activity with respect to glucose or galactose transfer. This was true of material kept for up to 28 days at −20°C.

A requirement for Mg^{2+} has been shown for glycosyltransferase activity in other polysaccharide-synthesizing systems, and magnesium chloride was accordingly included in the present assay system. Omission of Mg^{2+} resulted in decrease of glucose transfer by about 25%. It is, however, difficult to wash membrane preparations thoroughly with water, as the presence of mucoid material from wild-type cells leads to a loosely packed sediment that probably carries over ions from the original culture. Consequently the validity of this result is difficult to assess. Replacement of Mg^{2+} with other bivalent cations was also tested. The presence of Co^{2+} or Ca^{2+} ions resulted in the same extent of glucose transfer but Mn^{2+} caused a 30% decrease. This could be due to competition for sites normally using Mg^{2+}.

Transferase activity in mutants. A number of mutants unable to synthesize capsular polysaccharide were isolated. Examination of 23 of these non-mucoid mutants showed that when membrane preparations were incubated with the three sugar nucleotides transfer of glucose or galactose to lipid was lower than in wild-type preparations. Results are exemplified by strains 030, 032, 034 and 036 shown in Table 1. In all of these mutants tested, the ratio of glucose to galactose incorporation into lipid resembled that of wild-type material in having a value of almost 1:2. In only two mutants (040 and 042) of this group were deletions known to occur before the sugar nucleotide stage. These two mutants were defective in phosphoglucomutase and UDP-glucose pyrophosphorylase respectively (M. Norval & I. W. Sutherland, unpublished work). The other 21 strains had normal profiles for the enzymes leading to sugar nucleotide formation and modification. Four further mutants (K119, K121, 029 and 038) incorporated glucose but not galactose. Repeated experiments confirmed this result. Two mutants (K138 and 037) showed no incorporation of either glucose or galactose into lipid. When equal quantities of membrane preparations from strain 037 and either strain 029 or strain 038 were combined, incorporation of glucose was detected together with some incorporation of galactose. This indicated that some galactose transferase activity was present in the strain-037 preparation and that galactose transfer was dependent on prior glucose transfer. The results for several of these strains together with those for a normal *K. aerogenes* A4 preparation for comparison are shown in Table 1. Incorporation of glucuronic acid from UDP-glucuronic acid was too low to permit accurate assay.

Table 1. *Comparison of sugar incorporation into lipid material by wild-type and mutant strains*

The standard incubation mixture containing UDP-glucose, UDP-galactose and UDP-glucuronic acid was used with either UDP-[^{14}C]glucose or UDP-[^{14}C]galactose. Samples were withdrawn at 30min intervals and extracted with chloroform–methanol (2:1, v/v). The resultant incorporation is based on the values obtained at 60min after the start of incubation.

	Sugar incorporation (nmol/h per mg of membrane protein)	
Strain	Glucose	Galactose
Wild-type	0.124	0.284
030	0.010	0.019
032	0.046	0.068
034	0.053	0.090
036	0.053	0.124
037	0	0
029	0.013	0
038	0.020	0
037 + 038	0.009*	0.004

* Results expressed relative to total protein.

The generally lower extent of sugar transfer in the mutant strains could be due to lowered production of the enzymes involved, perhaps through specialized control mechanisms. To test this membrane preparations from the phosphoglucomutase-less strain were used. The cells were grown (*a*) in the trypticase soy medium supplemented with glucose and (*b*) in the same medium supplemented with galactose. The results for sugar transfer obtained with the two preparations are shown in Table 2. In this strain at least there is much lower transferase activity with membranes prepared from cells in the glucose medium, i.e. conditions under which no exopolysaccharide is synthesized.

Effects of potential inhibitors. In other systems in which transfer of sugars to lipid occurs a number of inhibitors are known. Thus the detergent Triton X-100 selectively prevents mannan synthesis from mannolipid in *M. lysodeikticus* (Scher & Lennarz, 1969) and bacitracin stops phosphate release in the final stage of mucopeptide synthesis (Siewert & Strominger, 1967). If the process of exopolysaccharide synthesis is similar to these other systems such inhibitors might also be effective. A membrane preparation from wild-type cells was used and the results of Triton and bacitracin on sugar transfer to lipid are shown in Table 3. Unlike the effect on the mannan system, Triton inhibits transfer of sugars to lipid. Bacitracin also affects sugar transfer to some extent, thus resembling the effect on mucopeptide system but differing from that on lipopolysaccharide biosynthesis.

The mechanism of transfer of the initial sugar in O-antigen synthesis involves transfer of the sugar 1-phosphate together with release of the nucleotide phosphate. Thus, if the first step in capsule polysaccharide formation is the transfer of glucose 1-phosphate from UDP-glucose, UMP should be released and it should also inhibit the reaction. Similarly subsequent sugar transfer might be inhibited by UDP. The standard assay system for glucose incorporation was first used with UDP-glucose as the only sugar nucleotide present. Similarly the effect on galactose transfer from a mixture of UDP-glucose and UDP-galactose was also tested. The results for these mixtures are shown in Figs. 4(*a*) and 4(*b*) respectively. Clearly the transfer of glucose to lipid is strongly inhibited by the presence of UMP. The effect of UDP is probably in stopping transfer of galactose from UDP-galactose, formed by epimerization of the UDP-glucose. The almost complete inhibition of galactosyl transfer by UDP

Table 2. *Activity of membrane preparations from a phosphoglucomutase-less mutant (strain 040) grown in media supplemented with glucose or galactose*

The standard incubation mixture with all sugar nucleotides was used. Samples were withdrawn at 30min intervals and extracted with chloroform–methanol (2:1, v/v).

	Sugar incorporation (nmol/h per mg of protein)	
Medium containing	Glucose	Galactose
Glucose	0.006	0.006
Galactose	0.031	0.090

Table 3. *Effect of bacitracin or Triton X-100 on sugar transfer to lipid*

The incubation mixture in all experiments contained all three sugar nucleotides together with either UDP-[^{14}C]-glucose or UDP-[^{14}C]galactose. Samples were extracted with chloroform–methanol (2:1, v/v) at 30min intervals and the values for sugar incorporation are based on the 60min value.

	Sugar incorporation (nmol/h per mg of protein)	
Incubation mixture	Glucose	Galactose
Complete	0.246	0.405
Complete+250μg of bacitracin	0.170	0.311
Complete+0.25% Triton X-100 (final concn.)	0.150	0.141

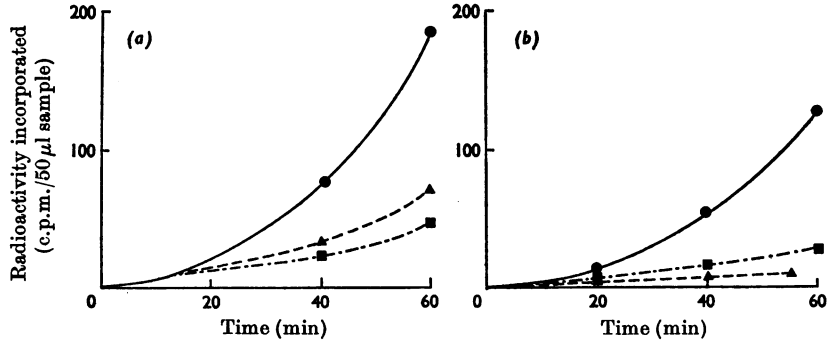


Fig. 4. Effect of UDP and UMP on sugar transfer to lipid. The incubation mixture with UDP-glucose only was used in (*a*) to show the effect on glucose transfer of the addition of UDP (200nmol) or UMP (200nmol). Samples were taken at intervals and extracted with chloroform–methanol. In (*b*) the effect of the same concentrations of UDP or UMP on galactose transfer from a mixture of UDP-glucose and UDP-galactose is shown. ●, Complete system; ▲, +UDP; ■, +UMP.

is shown in Fig. 4(*b*), whereas the extent of inhibition by UMP corresponds to that expected from the results for UDP-glucose. As a further check on the initial reaction, involving glucose transfer, a series of ten standard incubation mixtures containing UDP-glucose only was prepared. After 30min of incubation at 20°C with *K. aerogenes* A4 membrane the reactions were terminated by pouring the suspensions into boiling ethanol. Extraction of nucleotides was performed as described by Grant *et al.* (1970). The water-soluble nucleotides were separated by paper electrophoresis for 4h and identified by staining with molybdate reagent (Hanes & Isherwood, 1949). Only two major spots were detected, corresponding in their electrophoretic mobilities to UDP-glucose and UMP respectively. A very small amount of material moving ahead of UDP-glucose was possibly UDP. A control experiment in which no membrane was added to the incubation mixture showed UDP-glucose only.

Characterization of lipid-soluble material. Attempts to scale up the incubation mixture to obtain sufficient glycosyl-lipid were not satisfactory. Better results were obtained by pooling material from a number of experiments and using the standard incubation mixture. The chloroform–methanol-soluble material was obtained in the normal manner except that extraction was at room temperature for 15min. The chloroform layers were then pooled, back-washed with water and evaporated under reduced pressure. The residual material was extracted into a small volume of methanol. The recovery of radioactive material was approx. 90% of that originally present in the chloroform layers. Such material was prepared from incubation mixtures with either UDP-glucose as the sole sugar nucleotide or complete mixtures with each sugar in turn labelled. On electrophoresis in pyridine–acetic acid buffer, pH 5.3, all radioactivity remained at the origin. Chromatography in solvent D or t.l.c. on silica gel yielded some separation. In solvent D chromatograms of lipid labelled from either UDP-glucose or UDP-galactose showed two main areas of radioactivity. A broad band with R_{Gal} 1.26–1.34 contained 70% of the radioactivity detected. A further 15% moved with R_{Gal} 1.09, and three other peaks at R_{Gal} 0.65, 0.48 and 0.22 accounted for the remaining radioactivity. On the basis of comparison with UDP-glucose or UDP-galactose run under the same conditions, the peaks with R_{Gal} 0.65 and 0.48 are probably cyclic phosphates, which occur as artifacts under the chromatographic conditions used (Tovey & Roberts, 1970). When UDP-[^{14}C]glucuronic acid was used two areas of radioactivity with R_{Gal} 1.26 and 0.13 were obtained. These possibly correspond to lipid-bound tetrasaccharide and tetrasaccharide phosphate respectively. On t.l.c. in solvent E material labelled with glucose or galactose showed two peaks with R_F 0.63 and 0.25 respectively, about 80% of the label being in the slower-moving peak. This is thus similar in its mobility to the mannolipid described by Caccam, Jackson & Eylar (1969). Insufficient glucuronic acid-labelled material was available to allow accurate identification of peaks.

Phenol treatment. The pyrophosphate bridge of sugar diphosphate–lipid complexes can be broken by treatment with 45% (v/v) phenol at 60°C for 5min (Kent & Osborn, 1968). This method was used to examine the sugar diphosphate–lipid complexes formed by incubation of the sugar nucleotides with *K. aerogenes* membrane preparations. The lipid material was dissolved in 50μl of methanol and added to 450μl of water, and 500μl of aq. 90% (v/v) phenol was added. After 5min at 60°C the mixture was cooled and extracted twice with 2ml portions of ether. Aqueous layers were freeze-dried. Samples of both organic and aqueous layers were checked for radioactivity, which was all found in the aqueous layers. Samples from the aqueous layers were submitted to paper electrophoresis. Little difference was detected in the products obtained with UDP-[^{14}C]glucose or UDP-[^{14}C]galactose in a complete mixture of nucleotides. In each case the only radioactive material detected moved in a broad band with M_{GlcUA} 0.93–0.95 (cf. glucose 6-phosphate, M_{GlcUA} 0.65). After treatment with alkaline phosphate for 1h at 37°C under conditions where 1mmol of glucose 1-phosphate was completely dephosphorylated, the preparations were again subjected to paper electrophoresis. All radioactivity was now detected in three areas, M_{GlcUA} 0.60 and 0.45 and at the origin. The electrophoretic mobilities correspond to those obtained for trisaccharides and a tetrasaccharide obtained by partial acid hydrolysis of *K. aerogenes* A4 polysaccharide and included on the paper for comparison (Sutherland, 1970). When UDP-glucose was the sole sugar nucleotide present and the reaction was stopped after 30min at room temperature, the product of phenol treatment was slightly different. Two areas of radioactivity were detected after electrophoresis (M_{GlcUA} 0.65 and 0.60). After phosphatase treatment all radioactivity remained at the origin.

When the products of phenol treatment were incubated with alkaline phosphatase and applied to chromatograms in solvent B, the results from mixtures containing all nucleotides and labelled with UDP-[^{14}C]glucose or UDP-[^{14}C]galactose were similar. Each showed large amounts of radioactivity with R_{Glc} 0.05 and 0.10, equidistant with a trisaccharide and a tetrasaccharide isolated previously from *K. aerogenes* A4 polysaccharide by partial acid hydrolysis (Sutherland, 1970). A small amount of material equidistant with glucose was observed. Another major peak of radioactivity had R_{Glc} 0.35.

This is probably the disaccharide *O*-α-D-galactosyl-(1→3)-D-glucose, as lactose and melibiose, the only galactosides available for comparison, had R_{Glc} values 0.32 and 0.30 respectively in this solvent. The product obtained when UDP-glucose was the sole sugar nucleotide present in the reaction mixture revealed only radioactivity equidistant with glucose and with the presumed disaccharide. These results strongly suggested that the sugars were being incorporated into the lipid in the same configuration in which they are present in the polysaccharide. The products from the UDP-glucose incorporation would thus be glucose 1-phosphate and galactosylglucose 1-phosphate. Insufficient glucuronyl-labelled material was available to permit comparison with the other products.

Polymeric material. No specific enzymes hydrolysing *K. aerogenes* serotype 8 exopolysaccharide are known. Consequently assay methods for the polymer are very limited. That described in the Materials and Methods section was used as a routine for convenience, but it is non-specific. A second, specific, method was used to confirm these results. This utilized the observation (Sutherland, 1970) that mild acid hydrolysis (0.25M-sulphuric acid at 100°C for 30min) of the serotype 8 polysaccharide yielded a number of oligosaccharides that could be separated by paper electrophoresis or by chromatography in solvent B. In all cases where polymer synthesis was shown by the first method it was confirmed by scanning after paper electrophoresis of partial acid hydrolysates. All radioactivity was found to coincide with the oligosaccharides or with neutral material.

When wild-type membrane preparations were incubated with UDP-glucose a small amount of sugar was incorporated into polymer. This was to be expected as the UDP-glucose could be converted into UDP-galactose and UDP-glucuronic acid. However, incubation with UDP-galactose or UDP-glucuronic acid failed to yield polymer. When a complete mixture of nucleotides was incubated with the membrane, polymer, as determined by both assay methods, was formed. Typical results for polymer formation, as determined by the non-specific assay with each sugar nucleotide in turn in the mixture labelled, are shown in Fig. 5. It is clear that both glucose and galactose are incorporated to a considerable extent and that the ratio of glucose to galactose incorporation is approx. 1:2. Much less glucuronic acid was incorporated. The pattern is thus very similar to that obtained for transfer of sugar to lipid.

Examination of the various mutants showed that in most cases little if any polymer was formed. This was to be expected in those mutants from which the enzymes responsible for lipid intermediate formation were known to be absent or in those which were not known to have defects in nucleotide synthesis or in lipid transferases. In these it was inferred that failure to synthesize capsule must be due either to lack of glucuronic acid transfer or to some later stage in synthesis. However, when the mutants lacking UDP-glucose pyrophosphorylase and phosphoglucomutase were examined a rather surprising result was obtained. The pyrophosphorylase-less mutant grown either on glucose or galactose media formed polymer to much the same extent as the wild-type strain. This was also true for the phosphoglucomutase-less strain when grown on media containing galactose. After growth on glucose sugar transfer to lipid occurred but no polymer was formed. The reason for this remains unclear. It is possible that under these conditions some acceptor required for polymer formation is absent and synthesis thus stops at the lipid intermediate stage. The mutant was obtained by using ethyl methanesulphonate as mutagen and as far as can be determined it is not a double mutant.

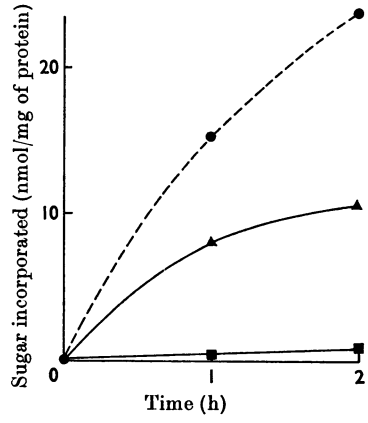


Fig. 5. Transfer of sugars from sugar nucleotides to polymer. The complete incubation mixture containing all three sugar nucleotides was used with one of them labelled in the sugar moiety in each case. Samples (25μl) were withdrawn at intervals and applied to a 2cm strip on a sheet of Whatman no. 1 paper. This was irrigated overnight with solvent D. The area of the origin was cut out, placed in scintillation fluid in a counting vial and residual radioactivity was determined. ●, UDP-galactose; ▲, UDP-glucose; ■, UDP-glucuronic acid.

Fate of label transferred to polymer. Polymer was prepared from pooled incorporation experiments in which each sugar nucleotide in turn was labelled. The starting material was the aqueous layer after chloroform–methanol extraction. Exopolysaccharide was isolated either by phenol extraction followed by dialysis and ultracentrifugation to remove sugar phosphates and lipopolysaccharide respectively, or by precipitation with 5 vol. of cold acetone.

Table 4. *Distribution of* ^{14}C *in polymer labelled with* ^{14}C*-labelled sugars from sugar nucleotides*

The polymeric material prepared by incubation of membrane with complex mixtures of sugar nucleotides, one being labelled in each case, was prepared as described in the text. Hydrolysis and chromatographic separation were then performed also as described in the text.

	Distribution of radioactivity (c.p.m.)		
Initial label	Glucose	Galactose	Glucuronic acid + lactone
Glucose	2221	193	79
Galactose	269	1239	45
Glucuronic acid	247	163	2127

In either case the product was redissolved in a small volume of water, exhaustively dialysed and freeze-dried. The crude products in each preparation were dissolved in water to give approx. 5000 c.p.m. in 200 μl. An equal volume of M-sulphuric acid was added and the samples were hydrolysed in sealed tubes for 16 h at 100°C. After neutralization with saturated barium hydroxide solution, salts and charged material were separated by paper electrophoresis, each preparation being applied to a 6 cm strip on the paper. From each paper 2 cm guide strips were cut out and checked for radioactivity. All radioactive material was located in each preparation equidistant with glucuronic acid and with the neutral material at the origin. These areas on the remaining 4 cm strips were eluted, pooled and re-run on paper chromatograms in solvent A. The areas corresponding to glucuronic acid and lactone, glucose and galactose were cut out and their radioactivities counted. The distribution of radioactivity is shown in Table 4.

If the polymer is identical with serotype 8 polysaccharide, the same products of partial acid hydrolysis should be obtained. This was performed as for the specific assay described above. After neutralization the samples were applied to Whatman no. 1 paper and subjected to paper electrophoresis. The material between glucuronic acid and the neutral material was eluted as one fraction and the neutral material as a second. They were applied to separate paper chromatograms run in solvents B and A respectively. In solvent B all material ran equidistant with the oligosaccharides isolated earlier from serotype 8 polysaccharide (Sutherland, 1970) and used as standards. No radioactivity equidistant with glucuronic acid was detected. This confirms that the conditions of hydrolysis were insufficient to hydrolyse the aldobiouronic acid *O*-α-D-glucuronosyl-D-galactose. The chromatogram of the neutral material showed that all the radioactivity ran equidistant with glucose and galactose. This is also similar to the pattern with authentic serotype 8 polysaccharide, which fails to yield any neutral oligosaccharides on partial acid hydrolysis.

DISCUSSION

The gross similarity in the structure of the repeating units of exopolysaccharide capsules of *K. aerogenes* and the O-antigenic components of lipopolysaccharides indicated a possible similarity in their synthetic processes. The fact that both polymers are located outside the cell membrane also points to possible similarities in precursor synthesis, organization, control and polymerization. The O-antigens are synthesized by particulate enzymes contained in the cell membrane. Sequential transfer of sugars from sugar nucleotide precursors to an isoprenoid alcohol phosphate acceptor occurs (Nikaido, 1968).

The results obtained in the present study showed that transfer of glucose from UDP-glucose to a lipid acceptor occurs in membrane preparations of *K. aerogenes* serotype 8. The transfer of galactose occurs similarly. The sequence is most probably glucosyl transfer followed by galactosyl transfer. This is indicated on the basis of two observations: (i) although some transfer of galactose occurred in the absence of sugar nucleotides other than UDP-galactose, the highest yields of galactose-labelled lipid were obtained when UDP-glucose was also present; (ii) mutants were obtained that transferred (*a*) neither glucose nor galactose and (*b*) glucose only; no mutants transferring galactose only were obtained. In the type of mutant unable to transfer glucose galactosyltransferase activity was present. Galactosyltransferase thus requires glucosyl transfer as a prerequisite.

The first stage in glycosylation of the lipid is probably similar to the first stage occurring in the synthesis of other extracellular polymers. In this reaction glucose 1-phosphate is transferred from UDP-glucose to the lipid carrier together with release of UMP. This is based on the inhibition of glucose transfer by UMP and identification of UMP as the major nucleotide product of interaction between UDP-glucose and the membrane-bound enzyme system. The ratio of glucose to galactose transfer in both lipid and polymer was 1:2, the same ratio in which these

sugars occur in the native exopolysaccharide. Although not conclusive, this suggests that the system is in fact one concerned with exopolysaccharide synthesis and not with some other polymer such as lipopolysaccharide from which glucose is essentially absent (Sutherland & Wilkinson, 1966). Those mutants unable to transfer glucose or galactose to lipid did not form polysaccharide, indicating that the glycosylated lipids are intermediates in exopolysaccharide formation. Further, in following the fate of the labelled sugars, radioactive material corresponding in electrophoretic and chromatographic properties to characteristic oligosaccharide components of the exopolysaccharide (Sutherland, 1970) was shown.

The lipid acceptor has not yet been characterized. Initial experiments indicated that glycosyl-labelled lipid had several properties common to the isoprenoid intermediates known to occur in the synthesis of three polysaccharides discovered outwith the bacterial cell: lipopolysaccharide (Wright *et al.* 1967), mucopeptide (Higashi *et al.* 1967) and mannan (Scher *et al.* 1968). Chromatographic results also compared well with those for a similar exopolysaccharide system studied by Troy & Heath (1968; F. C. Troy, personal communication). If the lipid is indeed an isoprenoid alcohol such compounds appear to be involved generally in the synthesis of polysaccharides outside cell membranes. This activity has also been noted in kidney cells synthesizing mannose-containing glycoproteins (Caccam *et al.* 1969). They are thus not confined to procaryotic cells in this function.

Interchangeability of lipid acceptors between mannan and exopolysaccharide systems (F. Frerman, E. C. Heath, M. Lahav, T. C. Chiu & W. J. Lennarz, unpublished work, cited by Lahav, Chiu & Lennarz, 1969) has been reported. It is not clear whether the lipids are identical in these and similar systems or merely show sufficient similarity to function in either system. If the former, the cell must possess suitable control mechanisms whereby lipid is allocated to the biosynthetic processes requiring it. It is perhaps significant that in *K. aerogenes* both UDP-galactose 4-epimerase and UDP-glucose dehydrogenase are membrane-bound (M. Norval & I. W. Sutherland, unpublished work). Thus for synthesis of polysaccharides outside the cell membrane UDP-glucose formed intracellularly would be passed to the modifying enzymes within the membrane. From these it could be spatially passed to the particle-bound enzymes and lipid carriers for the systems requiring them: exopolysaccharide with UDP-glucuronic acid and both exopolysaccharide and lipopolysaccharide with UDP-galactose. This is shown diagrammatically in Scheme 1. This could explain the failure to incorporate significant amounts of glucuronic acid from UDP-glucuronic acid. The spatial configuration of the enzymes may only accept UDP-glucose, modify it and in this way lead to polymer synthesis. It may also be relevant that glucuronic acid forms short side chains in the polysaccharide and is not part of the linear chain (Sutherland, 1970).

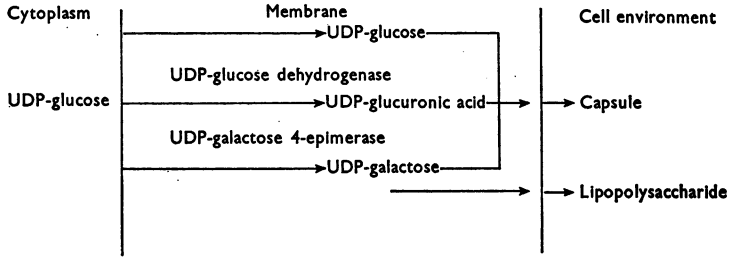
The formation of exopolysaccharides thus appears to be a complex process involving a series of reactions:

(i) formation of intermediates such as UDP-glucose;

(ii) modification of the sugar nucleotides to yield UDP-glucuronic acid;

(iii) sequential transfer of the sugars to a lipid carrier, which is probably an isoprenoid alcohol phosphate;

(iv) polymerization and extrusion into the extracellular environment. Associated with the final stage there must presumably be some mechanism of attachment to the cell surface, as it is known that stable mutants arise yielding exopolysaccharide slime but no discrete capsules (Wilkinson, Duguid & Edmunds, 1954; M. Norval & I. W. Sutherland, unpublished work). The entire process thus



Scheme 1. Postulated structural arrangement for polysaccharide synthesis. UDP-glucose passes through the cell membrane to reach the membrane-bound dehydrogenase and epimerase enzymes. After modification by these enzymes the sugar nucleotides so formed, UDP-glucuronic acid and UDP-galactose, are utilized by the transferases to form precursors for capsule or in the latter case also for lipopolysaccharide. Finally, polymer is excreted from the exterior surface of the membrane.

requires a considerable number of enzymes and a correspondingly large amount of genetic information for the synthesis of structures whose role remains largely unknown and that in the laboratory are not essential for cell viability.

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