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72. I am grateful to all those who have kept me informed of the progress of their work and have permitted me to quote from their observations prior to publication. A number of scientists, both at Argonne and elsewhere, have reviewed this article at various stages of preparation. I am grateful for their assistance, but I am reluctant to involve anyone by name. I cannot dilute my responsibility for errors of commission or omission.

Lipopolysaccharide of the Gram-Negative Cell Wall

Biosynthesis of a complex heteropolysaccharide occurs by successive addition of specific sugar residues.

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L. D. Zeleznick, B. L. Horecker

In recent years there have been rapid advances in our knowledge of the chemical composition and structure of the bacterial cell wall. Gram-negative as well as Gram-positive forms have been shown to contain a rigid polymer built up of units of *N*-acetylglucosamine and a muramic acid peptide (*1*). In addition to this basic structural unit, many Gram-positive species also contain teichoic acids, which are polymers of ribitol phosphate or glycerol phosphate carrying a variety of side chains (*2*). In contrast, Gram-negative organisms appear to contain little or no teichoic acid. The walls of most Gram-negative forms contain a complex lipopolysaccharide in addition to large amounts of protein and phospholipid. These components appear to overlie the rigid mucopeptide layer and may account for the difference in sensitivity of Gram-negative and Gram-positive species to attack by lysozyme. The lipopolysaccharide possesses many interesting biological properties and has, therefore, attracted the attention of large numbers of investigators. It is highly antigenic and contains the specific surface antigens, or O-antigens, which provide the basis for the Kauffmann-White scheme for the serological classification of the Enterobacteriaceae (*3*). The lipopolysaccharide is also an integral component of the endotoxin, a protein-lipid-lipopolysaccharide complex which is responsible for the physiological effects produced by injection of heat-killed bacteria. These effects include fever, shock, diarrhea, edema, and internal hemorrhage. As a result of the early classic work from the laboratories of Boivin (*4*), Raistrick (*5*), and Morgan (*6*), it became clear that antigenic and toxic properties of the endotoxin were both associated with the lipopolysaccharide and that the polysaccharide portion, which can be liberated from the complex by mild acid hydrolysis, carried the complete somatic or O-antigen specificity of the microorganism. The isolated polysaccharide is nontoxic, and the nature of the chemical structure or structures responsible for toxicity of the intact lipopolysaccharide is still uncertain. The lipid moiety of the lipopolysaccharide, lipid A, is a complex, phosphorylated lipid of unusual composition, which contains glucosamine instead of glycerol and is rich in β-hydroxymyristic acid (*7, 8*). Although toxicity can be recovered in isolated lipid A preparations (*9, 10*), it is not clear whether the lipid, as isolated, can account entirely for the toxicity of the original endotoxin.

The chemical basis of O-antigen specificity was firmly established by the monumental work of Kauffmann on the immunological classification of *Salmonella* and *Escherichia* (*3*) and by more recent structural and immunochemical studies in the laboratories of Westphal (*9–12*), Staub (*13, 14*), and Robbins (*15*). It is clear that the determinants for all O-antigen specificities of a given organism are located in a single polysaccharide molecule, and it has been possible in many cases to identify the monosaccharide or oligosaccharide groupings which determine individual specificities. It was early recognized (*16*) that mutation could result in complete loss of O-antigen specificity, and that the altered surface structure in these so-called "rough" mutants was accompanied by loss of virulence. Recent investigations of the chemistry (*12*), immunology (*17*), and genetics (*18*) of rough mutants have been of particular value in unraveling the structure of the lipopolysaccharide.

Our interest in biosynthesis of the lipopolysaccharide was stimulated by the potential value of the lipopolysaccharide as a model system for study of mechanisms of biosynthesis and control of structurally specific heteropolysaccharides. *Salmonella* lipopolysaccharides may contain as many as eight different sugars, and the high degree of antigenic specificity argues for an equivalent specificity in sugar sequence and linkage. In addition, the occurrence of

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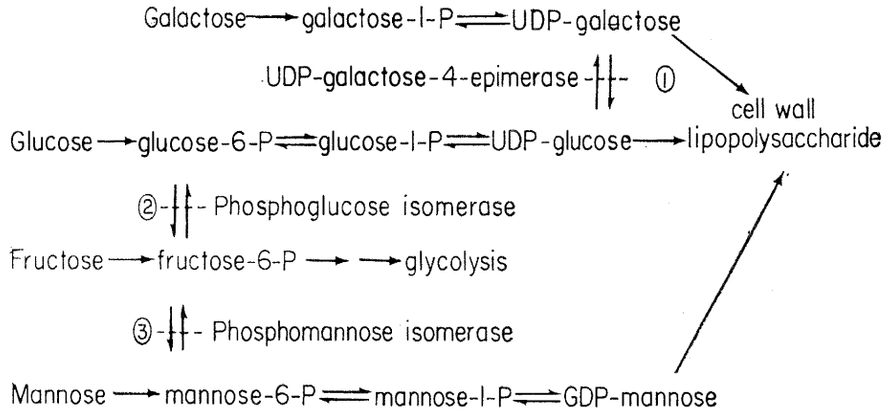

Fig. 1. Pathways of sugar nucleotide synthesis and cell wall synthesis in *Salmonella typhimurium*.

widely differing structures in closely related species may prove of considerable interest from the point of view of biochemical evolution. In growing cells, biosynthesis of new cell wall material is coordinated with other processes of cell growth and cell division, and the synthesis of cell wall components must be under the control of complex regulatory mechanisms. Understanding of the problems of genetic and metabolic control of lipopolysaccharide synthesis will require detailed information concerning the structure and mechanism of biosynthesis of these complex polymers. During the past 3 years a fruitful approach to the problems of biosynthesis and structure has been provided by the discovery of mutant strains in which synthesis of specific lipopolysaccharide precursors is blocked.

Composition of the Lipopolysaccharide

The *Salmonella* lipopolysaccharide can be isolated either from whole cells or from purified cell walls and freed of protein by extraction with hot phenol (*19*). As isolated, the polysaccharide is linked covalently to the glucosamine-containing lipid, lipid A, from which it can be split by very mild acid hydrolysis (*7*). The soluble polysaccharide obtained in this manner may contain from five to eight different sugars (*10*). Sugars found in *Salmonella* polysaccharides are listed in Table 1. The polysaccharides of all salmonellas (and almost all strains of *Escherichia coli*) contain glucose, galactose, glucosamine, and an aldoheptose, as well as substantial amounts of organic phosphate (*10, 11*). In addition, it is probable that 2-keto-3-deoxyoctonate (KDO), first identified by Heath and Ghalambor (*20*) as a constituent of the lipopolysaccharide of *E. coli* O111, is present in the polysaccharides of all members of the *Escherichia–Salmonella* group. In addition to these basic components, the polysaccharide of wild type organisms may contain, in various combinations, mannose, galactosamine, rhamnose or fucose, and one of the 3,6-dideoxyhexoses (abequose, paratose, colitose, or tyvelose). Mutation to the "rough" state results in loss of these additional components; mutants of this type form incomplete polysaccharides containing only the five basic constituents found in all salmonellas. (For a complete review of the composition of the various strains of *Salmonella* and related organisms, see *9–12.*)

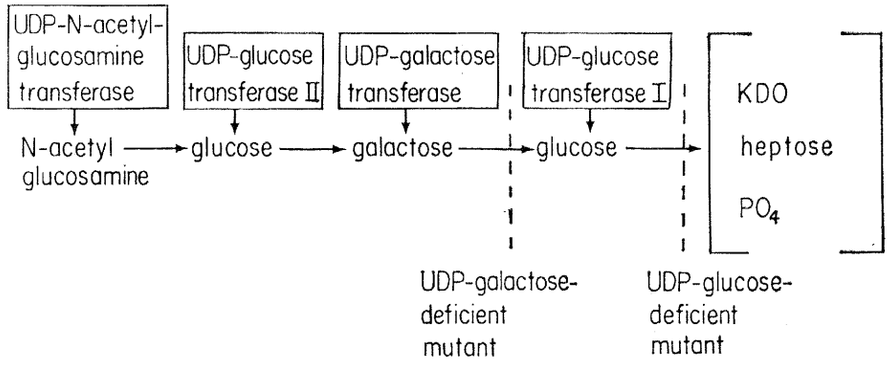

Fig. 2. Enzymatic synthesis of the core polysaccharide.

Studies with Mutant Organisms

Experimental approach to the biosynthesis of these complex molecules has been greatly facilitated by the availability of mutant organisms in which the biosynthesis of specific polysaccharide precursors is blocked. Such mutants were first employed by Smith *et al.* (*21*) in their studies of the biosynthesis of the pneumococcal capsular polysaccharides. Application of this technique to lipopolysaccharide biosynthesis was made possible as a result of the work of Nikaido and Fukusawa (*22, 23*) on certain galactose-negative mutants of *Escherichia coli* and *Salmonella* which were unable to synthesize uridine diphosphate galactose (UDP-galactose). These workers observed that mutants lacking the enzyme UDP-galactose-4-epimerase (Fig. 1, enzyme 1), which catalyzes the interconversion of UDP-glucose and UDP-galactose, formed incomplete cell wall polysaccharides. These polysaccharides lacked not only galactose but also certain other normal sugar components. The defect in cell wall synthesis was shown to be a result of the absence of the epimerase, which is essential for the formation of UDP-galactose in the absence of exogenous galactose (Fig. 1). The block can be by-passed by the addition of galactose to the growth medium; under these conditions UDP-galactose accumulates, galactose is transferred into the cell wall, and the polysaccharide composition rapidly returns to that of the wild type. Nikaido (*23*) was also able to show for the first time that not only intact cells but also cell-free extracts of the epimeraseless mutants were able to carry out the incorporation of galactose from UDP-galactose into the galactose-deficient material of the cell wall.

We were able to confirm Nikaido's original observation with an epimeraseless mutant of *Salmonella typhimurium* (*24*), and more recently we have used several analogous mutants for studying reactions involved in biosynthesis of the lipopolysaccharide (*25, 26*). The mutants used in this work, and the sugar composition of the polysaccharides which they contain, are summarized in Table 2. The analyses were carried out

Table 1. Sugar components of *Salmonella* polysaccharides.

Aldoheptose	Galactosamine
Glucose	Mannose
Galactose	Rhamnose, fucose
Glucosamine	3,6-Dideoxyhexoses:
2-Keto-3-deoxy-octonate (KDO)	abequose, paratose tyvelose, colitose

on the lipid-free soluble polysaccharide obtained after mild acid hydrolysis of the lipopolysaccharide. The polysaccharide of the wild type contains 2-keto-3-deoxyoctonate (*27*), L-glycero-D-mannoheptose (*24, 28*), glucose, galactose, and a small amount of glucosamine, in addition to mannose, rhamnose, and abequose. The first class of mutants is represented by two types: (i) a rough strain in which the enzyme defect is not yet known, and (ii) a mutant, isolated in our laboratory (*26*), which is unable to synthesize guanosine diphosphate mannose (GDP-mannose). Both these organisms form a polysaccharide which entirely lacks mannose and abequose and contains only a trace of rhamnose. These three sugars, along with galactose, have been shown by Staub (*13*) to be components of the O-antigen determinants. All the other components of the wild type polysaccharide are present in these strains. In contrast, the polysaccharide formed by the mutant which is unable to synthesize UDP-galactose is deficient not only in mannose, rhamnose, and abequose but also in *N*-acetylglucosamine and galactose. Finally, mutants in a third class are unable to synthesize UDP-glucose, and form a polysaccharide which contains only 2-keto-3-deoxyoctonate and heptose (*29*).

The pattern which emerges from these analytic data, together with immunochemical information already available, gives some insight into the overall structural organization of the complete wild type lipopolysaccharide. As originally proposed by Lüderitz *et al.* (*30*), the polysaccharide component appears to consist of two regions, which can be distinguished both structurally and immunologically. The outer region, whose composition and structure is group- or species-specific, carries the specific determinants of the surface O-antigens. These O-antigenic side chains must be linked to a smaller, internal core region, whose structure may be similar in all salmonellas, as well as in *Escherichia coli* and related genera. Thus, the polysaccharides of rough mutants derived from a wide variety of smooth types are closely related, if not identical, in composition and immunological specificity (*12*). In addition, the polysaccharides of UDP-galactose-deficient mutants of several strains of *Salmonella* and *E. coli* are similar in composition (*22–24, 31*), as are UDP-glucose-deficient mutants of the two genera (*29, 32*). If there is a block in the biosynthesis of the O-antigenic side chains, as in the mutant lacking GDP-mannose, lipopolysaccharide synthesis continues, but it progresses only as far as the inner core region of the polysaccharide. The composition of the mutants deficient in UDP-glucose and UDP-galactose provides further information about the structure of this core region. In particular, the fact that the polysaccharide of the mutant deficient in UDP-glucose contains only heptose, phosphate, and KDO suggests that these are components of the innermost polysaccharide backbone.

Information about the sequence of sugars in the core polysaccharide has come primarily from studies of its biosynthesis, in which enzyme preparations derived from mutants deficient in UDP-glucose and UDP-galactose were used. Four enzyme systems have been obtained which catalyze sequential addition of monosaccharide units onto the backbone containing heptose, phosphate, and KDO. These reactions are summarized in Fig. 2.

Transfer of Glucose to the Polysaccharide Backbone

The first reaction, the transfer of glucose onto the heptose-phosphate backbone, is catalyzed by enzyme systems derived from the mutant deficient in UDP-glucose. This organism was originally isolated by Sylvia Smith of the Lister Institute as a glucose- and galactose-negative mutant which appeared to form an incomplete cell wall lipopolysaccharide (*33*). The biochemical defect was established, in our laboratory, by D. Fraenkel (*29*), who showed the organism to be deficient in the enzyme phosphoglucose isomerase (Fig. 1, enzyme 2). In the absence of exogenous glucose, this mutant is unable to form glucose 6-phosphate and hence is unable to synthesize UDP-glucose. As indicated above, the polysaccharide of the glucose-deficient cells contains only heptose, phosphate, and KDO. The metabolic block can be by-passed by the addition of glucose to the medium; under these circumstances, the composition of the polysaccharide returns to that of the wild type.

Cell-free extracts of this mutant are able to catalyze the transfer of glucose-C^{14} from UDP-glucose-C^{14} into the endogenous glucose-deficient cell wall material (*25*). The particulate cell wall–membrane fraction, isolated from sonicates by differential centrifugation, contains both the enzyme protein and the endogenous incomplete lipopolysaccharide, which acts as acceptor for the glucose transferred. The reaction is highly specific for UDP-glucose, which cannot be replaced by UDP-galactose or thymidine diphosphate glucose (TDP-glucose). Characterization of the radioactive product as lipopolysaccharide is summarized in Table 3. The lipopolysaccharide is isolated, after precipitation of cell wall material with trichloroacetic acid, by extraction of the acid-insoluble material with hot phenol, according to the method of Westphal *et al.* (*19*). The crude product, which is contaminated by nucleic acid as well as by other polysaccharides, can be obtained in a high state of purity by precipitation with magnesium ions. This purified lipopolysaccharide fraction contains approximately 75 percent of the radioactivity originally incorporated into

Table 2. Polysaccharide composition in *Salmonella typhimurium* and in mutants with deficiencies in cell wall components.

Strain	Deficiency	KDO	Heptose-P	Glucose	Galactose	*N*-Acetyl glucosamine	Mannose	Rhamnose	Abequose
Wild type	None	+	+	+	+	+	+	+	+
Rough	Unknown	+	+	+	+	+	0	Tr*	0
Phosphomannose isomeraseless	GDP-mannose	+	+	+	+	+	0	Tr*	0
UDP-galactose-4-epimeraseless	UDP-galactose	+	+	+	0	0	0	0	0
Phosphoglucose isomeraseless	UDP-glucose	+	+	0	0	0	0	0	0

* Trace.

Table 3. Isolation of UDP-glucose transferase product.

Fraction	Total radioactivity (count/min)	Percentage recovered
Precipitated cell wall material	20,700	100
Crude lipopolysaccharide (phenol extract)	19,800	93
Purified lipopolysaccharide (Mg^{++} precipitate)	15,000	73
Polysaccharide (*p*H 2 hydrolyzate)	16,100	78

the cell wall material. After release of the polysaccharide from the lipid by mild acid hydrolysis, the recovery of radioactivity in the soluble polysaccharide fraction is quantitative.

Further characterization of the polysaccharide is based on its electrophoretic migration. Since it contains large amounts of phosphate in addition to heptose and KDO, it migrates as an anion, and all of the radioactivity is recovered in a fraction corresponding to one of the major fractions of the authentic mutant polysaccharide. Complete hydrolysis of this fraction shows that all the radioactivity is present as glucose. However, it has not yet been possible to determine the site of linkage of glucose to the heptose-phosphate-KDO backbone. Although heptose is perhaps the most logical site of linkage, neither KDO nor phosphate has been entirely eliminated as an acceptor site for glucose.

Galactose Incorporation into the Galactose-Deficient Cell Wall

The mutant lacking UDP-galactose-4-epimerase was used for a study of the incorporation of galactose into the lipopolysaccharide (*24, 34*). A second transferase system, specifically catalyzing the transfer of galactose from UDP-galactose into the endogenous, galactose-deficient lipopolysaccharide, was demonstrated in the particulate cell wall fraction of this organism. The properties of this enzyme were generally similar to those of the UDP-glucose transferase. At least 90 percent of the incorporated galactose could be recovered in the isolated lipid-free polysaccharide, and the electrophoretic migration of the radioactive product coincided with major fractions of the authentic mutant polysaccharide.

Our initial attempts to determine the site of attachment of galactose to the polysaccharide were hampered by the relatively high acid lability of the newly formed galactosyl linkage. The difficulty was overcome by oxidizing the galactosyl linkage to a more stable galactosyluronic acid linkage without disrupting the polysaccharide. This was accomplished by successive treatment with galactose oxidase and bromine. The reactions involved in this treatment are summarized in Fig. 3, where *-X-Y-* represents the polysaccharide chain to which galactosyl residues are linked. Galactose oxidase, derived from *Dactylium dendroides* (*35*) specifically oxidizes galactosyl residues at the 6-position to yield the 6-aldehyde, and is active with galactose in polysaccharides (*36*). The aldehyde intermediate obtained was quantitatively converted by oxidation with bromine or hypoiodite to the uronic acid derivative. After partial acid hydrolysis of the oxidized radioactive polysaccharide, a radioactive, uronic acid-containing oligosaccharide could be isolated, in 70 percent yield, by paper electrophoresis. This material proved to be a disaccharide containing galacturonic acid and glucose, with glucose as the reducing component. Periodate oxidation and methylation studies established the structure shown in Fig. 4, 3-*O*-D-galactosyluronic acid-D-glucopyranose. The configuration of the galactosyl linkage was established by treating the original labeled polysaccharide with α- and β-galactosidases, a procedure which established it as an α-galactoside. The UDP-galactose lipopolysaccharide transferase, therefore, appears to transfer galactose in α-configuration specifically to the 3-position of polysaccharide-linked glucose. No evidence has been obtained for the formation of other linkages or for the linkage of galactose to any other position in the polysaccharide.

Addition of Glucose to the Galactose Product

The product of the UDP-galactose transferase reaction serves, in turn, as a substrate for the next reaction in the sequence, the addition of a second residue of glucose (*37*). No significant incorporation of glucose into lipopolysaccharide was detected when the particulate enzyme-acceptor complex of the galactose-deficient mutant was incubated with UDP-glucose alone. However, if the cell wall fraction was first incubated with nonradioactive UDP-galactose, reisolated by centrifugation, and then exposed to UDP-glucose-C^{14}, rapid incorporation of glucose into lipopolysaccharide was observed (Fig. 5). Again, the radioactive glucose was recovered in the lipid-free polysaccharide fraction, and the electrophoretic migration of the C^{14}-labeled product corresponded to that of fractions of the authentic mutant polysaccharide. The expected glucosyl-galactose disaccharide has been isolated from partial acid hydrolyzates of the labeled product, and determination of the structure of this disaccharide is in progress. These results indicate that the enzymatically incorporated galactose provides acceptor sites for further addition of glucose residues, and further, that the two transferase systems catalyze sequential addition of monosaccharide units to the growing chain. This glucosyl transferase has been called UDP-glucose lipopolysaccharide transferase II (see Fig. 2) in order to distinguish it from the system described earlier (UDP-glucose transferase I), which transfers glucose onto the glucose- and galactose-deficient backbone.

Table 4. Acceptor specificities of soluble UDP-glucose I and UDP-galactose transferase systems.

Strain from which acceptor was prepared	Incorporation of C^{14} ($m\mu$ mole/10 min) from	
	UDP-glucose	UDP-galactose
Glucose-deficient	0.23	0.14
Galactose-deficient	0.02	1.07
Wild type	$<$ 0.01	0.15
None added	0.01	0.07

Soluble Lipopolysaccharide Transferase Activities

While most of these enzyme activities in cell-free extracts are associated with the particulate cell wall–membrane fraction, significant quantities of enzyme are present in a soluble form which is not sedimented by centrifugation at 104,000*g* (*25*). No activity is observed with this soluble enzyme preparation, however, unless a suitable acceptor is added. The cell wall fraction, heated to destroy its endogenous enzymatic activity, has been used as acceptor in these studies. As shown in Table 4, the heat-inactivated cell wall fraction from the glucose-deficient mutant served as acceptor for the soluble UDP-glucose lipopolysaccharide transferase I,

whereas acceptor prepared from the galactose-deficient mutant or the wild type was entirely inactive. Similarly, the heated cell wall fraction from the galactose-deficient mutant acted as specific acceptor for the soluble UDP-galactose transferase. In this case, acceptor prepared from the glucose-deficient mutant or the wild type showed no activity. The glucose-deficient acceptor lacks the glucosyl residues which form the specific attachment sites for galactose, while in the wild type, all of the galactose acceptor sites are already filled and are unavailable for the attachment of additional galactosyl residues in vitro. It is of considerable interest that neither the purified mutant lipopolysaccharides nor the soluble polysaccharide moiety show acceptor activity in the soluble transferase systems.

Complete Structure of the Core Polysaccharide

Evidence that the lipopolysaccharide transferase systems catalyze the formation of products corresponding to structures normally existing in the core polysaccharide, as well as additional insight into the complete structure of the core, has come from analysis of rough strains and strains deficient in GDP-mannose. Mutants of the latter type, which have not previously been described, were obtained in this laboratory (*26*) by selection of mannose-negative organisms which were resistant to bacteriophage P22. This bacteriophage appears to require the complete wild type lipopolysaccharide for adsorption to the cell; all known classes of mutants having incomplete polysaccharides are resistant to it. The mutant deficient in GDP-mannose lacks the enzyme phosphomannose isomerase, which catalyzes the reversible interconversion of fructose 6-phosphate and mannose 6-phosphate (Fig. 1, enzyme 3). The organism is unable to utilize mannose 6-phosphate by the usual pathways of carbohydrate metabolism and is, therefore, mannose-negative. Fructose 6-phosphate cannot be converted to mannose phosphate, and the mutant is therefore unable to form GDP-mannose in the absence of an exogenous supply of mannose. Analysis of the lipopolysaccharide of the mutant lacking phosphomannose isomerase is shown in Table 5. Neither mannose nor abequose can be detected in the polysaccharide of this mutant. Traces of rhamnose have been detected, by chromatographic and enzymatic methods. However, the rhamnose content is less than 1 percent that of the wild type, and the significance of these trace amounts of rhamnose is not clear. It is of interest that the polysaccharide of the mutant deficient in GDP-mannose has a composition identical to that of a rough strain. Preliminary results of structural studies now in progress suggest that the polysaccharides of the two mutants also have similar structures and represent the normal core region of the wild type polysaccharide. The proposed structure of this portion of the polysaccharide is shown in Fig. 6. This structure is consistent both with the analytical data and with the products resulting from activity of the enzymatic lipopolysaccharide transferase systems.

Table 5. Polysaccharide composition of mannose-deficient and rough mutants.

Component	Molar ratios relative to heptose	
	Mannose-deficient	Rough
Heptose	1.0	1.0
Glucose	0.93	0.85
Galactose	1.0	0.84
Glucosamine	0.53	0.47
Rhamnose	0.04–0.07	0.04–0.08
Mannose	< 0.01	< 0.01
Abequose	< 0.01	< 0.01

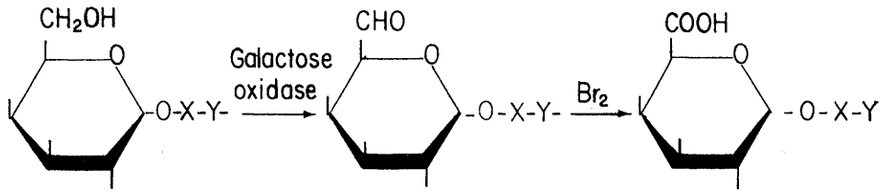

Fig. 3. Reactions involved in treatment of the galactosyl linkage with galactose oxidase and bromide.

Analytical data show the presence of two equivalents of galactose in the polysaccharides of both the GDP-mannose-deficient mutant and the rough strain. The second galactose residue is released by treatment of the polysaccharide with α-galactosidase and must, therefore, be in a nonreducing terminal position. The site of attachment of this galactosyl residue is not yet known.

Incorporation of N-Acetylglucosamine

The position assigned to the *N*-acetylglucosamine residue was initially suggested by isolation of an *N*-acetylglucosaminyl-glucosyl-galactose trisaccharide from partial acid hydrolyzates of the rough-strain polysaccharide. In confirmation of this structure, we have detected the predicted UDP-*N*-acetylglucosamine transferase system in the cell wall–membrane fraction of the mutant deficient in UDP-galactose. Since the polysaccharide of this mutant contains only one equivalent of glucose, and no galactose, it would be expected that incorporation of *N*-acetylglucosamine into the mutant polysaccharide would occur only after prior incorporation of both glucose and galactose. The particulate enzyme-acceptor complex was incubated with a mixture of nonradioactive UDP-galactose and UDP-glucose, reisolated by centrifugation and washed free of nucleotide sugars, and then incubated with UDP-*N*-acetylglucosamine-C^{14} in the absence of the other nucleotide sugars.

As shown in Table 6, substantial incorporation of *N*-acetylglucosamine was observed under these conditions; no incorporation could be detected when either UDP-galactose or UDP-glucose was omitted from the first incubation mixture. Measurement of the incorporation of each sugar in the three sequential steps yielded ratios for (i) galactose and glucose and (ii) galactose and *N*-acetylglucosamine of 10:2.2 and 10:1.2, respectively. The product of the UDP-*N*-acetylglucosamine reaction was identified as lipopolysaccharide by the previously established techniques of isolation and electrophoresis of the polysaccharide.

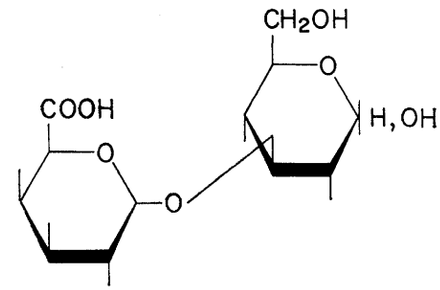

Fig. 4. Structure of 3-*O*-D-galactosyluronic acid-D-glucopyranose.

The studies of Heath and his co-workers (*38, 39*) on lipopolysaccharide biosynthesis in a UDP-galactose-4-epimeraseless mutant of *Escherichia coli* O111 have provided evidence for a similar core structure in this organism. They have demonstrated three lipopolysaccharide transferase reactions which carry out the sequential addition of galactose, glucose, and *N*-acetylglucosamine to the incomplete mutant polysaccharide. The similarity in sequence in the core polysaccharides of *E. coli* O111 and *Salmonella typhimurium* is particularly interesting, since the O-antigen specificities of the two organisms are unrelated. These observations lend further support to the view that all the lipopolysaccharides of the *Salmonella–Escherichia* group have the same basic core structure, to which widely varying O-antigenic side chains can be added.

Biosynthesis of the Antigenic Side Chains

Availability of the mutant lacking GDP-mannose has allowed us to extend the mutant approach to a study of biosynthesis of the portion of the polysaccharide responsible for O-antigen specificity (*26, 40*). The evidence summarized above strongly supports the hypothesis that the core polysaccharide is synthesized by sequential addition of monosaccharide units to the growing polysaccharide chain. Biosynthesis of the antigenic side chains may occur by a similar mechanism. However, there is strong evidence that the side chains carrying the O-antigen determinants of *Salmonella typhimurium* contain a repeating galactosyl-mannosyl-rhamnosyl-trisaccharide unit (*13*) (see Fig. 6), and considerable interest has been expressed in the possibility that the repeating unit might be synthesized as a nucleotide oligosaccharide and transferred into the growing polysaccharide as a unit. Nucleotide oligosaccharide derivatives have been isolated from several tissues (*41*), but their function is as yet unknown. Preliminary experiments with the cell wall–membrane fraction of the mutant deficient in GDP-mannose are summarized in Table 7. Incorporation of mannose from GDP-mannose into cell wall material in the absence of other sugar nucleotides was observed; however, mannose incorporation was markedly stimulated by the presence of TDP-rhamnose and UDP-galactose. Similarly, incorporation of galactose and rhamnose was stimulated when all three sugar nucleotides were present. After isolation of the radioactive products, 50 to 70 percent of the radioactivity incorporated was recovered in anionic products which are electrophoretically indistinguishable from the authentic lipid-free polysaccharide. Characterization of labeled oligosaccharides obtained by partial acid hydrolysis of these products is in progress. Preliminary results are consistent with biosynthesis of the expected galactosyl-mannosyl-rhamnosyl sequence, and further enzymatic studies should make it possible to choose between the two postulated mechanisms of synthesis.

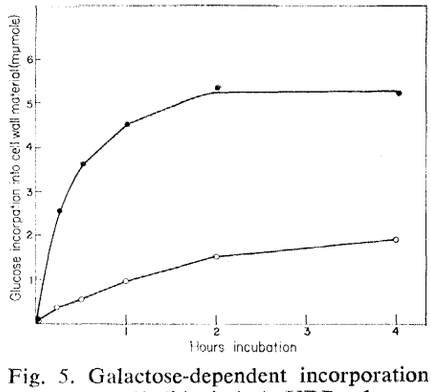

Fig. 5. Galactose-dependent incorporation of glucose. (Solid circles) UDP-galactose present in first incubation; (open circles) UDP-galactose lacking in first incubation.

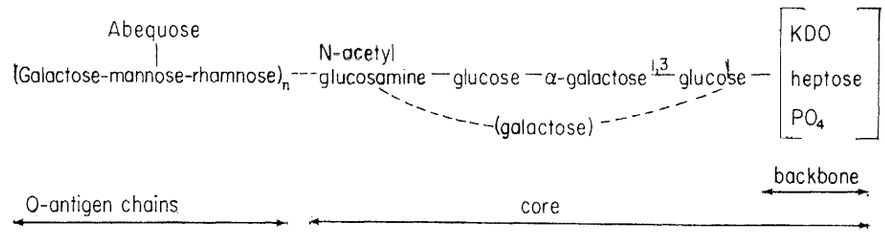

Fig. 6. Postulated structure of the polysaccharide of *Salmonella typhimurium*.

Another example of a mutant in which synthesis of a specific O-antigen sugar is blocked has recently been described by Nikaido *et al.* (*42*). In this organism, which was isolated as a classical rough mutant of *Salmonella typhimurium* (*18*), the final step in the synthesis of TDP-rhamnose is blocked. Enzymatic incorporation of rhamnose into the mutant lipopolysaccharide has also been reported by these workers (*43*).

Although final confirmation is as yet lacking, indirect evidence suggests that the polysaccharide is glycosidically linked to lipid A through keto-deoxyoctonate residues (*27*). Thus, after liberation of the polysaccharide from lipid A by mild acid hydrolysis, keto-deoxyoctonate is the only detectable reducing end group of the polysaccharide chains. In addition, the lability of the polysaccharide-lipid linkage in response to acid hydrolysis is similar to that of KDO glycosidic bonds. The site at which this residue is attached to the lipid is unknown; hydroxyl groups are available both in β-hydroxymyristic acid and in glucosamine as possible sites of attachment. Direct evidence for linkage of KDO to a lipid component has recently been obtained by Heath and his co-workers (*39*), who have observed enzymatic transfer of KDO from cytidine monophosphate-KDO to a degraded lipid A fraction.

Conclusions and Summary

The use of mutants of *Salmonella typhimurium* in which biosynthesis of specific lipopolysaccharide precursors is blocked has made possible both biosynthetic studies and structural analyses which provide the basis for the structure of the core polysaccharide shown in Fig. 6. The simplest mutant, which is unable to synthesize UDP-glucose, forms only the backbone structure, containing heptose, phosphate, and keto-deoxyoctonate. To this backbone are attached side chains containing glucose, galactose, and *N*-acetylglucosamine. The

Table 6. Incorporation of *N*-acetylglucosamine in the cell wall–membrane fraction of a galactose-deficient mutant.

Nucleotide sugars added		*N*-acetylglucosamine incorporation (mμ mole)
First incubation	Second incubation	
UDP-glucose + UDP-galactose	UDP-*N*-acetyl-glucosamine-C^{14}	0.85
UDP-glucose	UDP-*N*-acetyl glucosamine-C^{14}	0.03
UDP-galactose	UDP-*N*-acetyl glucosamine-C^{14}	0.09
None	UDP-*N*-acetyl glucosamine-C^{14}	0.04

Table 7. Incorporation of mannose, galactose, and rhamnose into cell wall material in the mannose-deficient mutant.

Addition	Incorporation (mμ mole)
GDP-mannose-C^{14}	
UDP-galactose, TDP-rhamnose	1.0
TDP-rhamnose	0.37
None	0.25
UDP-galactose-C^{14}	
TDP-rhamnose, GDP-mannose	0.37
TDP-rhamnose	0.20
None	0.12
TDP-rhamnose-C^{14}	
UDP-galactose, GDP-mannose	0.61
UDP-galactose	0.34
None	< 0.02

resulting core structure is found in the lipopolysaccharide of the rough strain, as well as in that of the GDP-mannose-deficient mutant. In the wild type organism, long O-antigenic chains composed of repeating units containing galactose, mannose, rhamnose, and abequose are linked to the core, perhaps to the *N*-acetylglucosamine residue, as indicated in Fig. 6. The rough phenotype could presumably arise from mutation either at the level of nucleotide sugar synthesis or at some stage in assembly or attachment of the O-antigenic side chains. The pathways of nucleotide sugar synthesis appear to be normal in most rough strains of *S. typhimurium* (*42*), a finding which suggests loss of a lipopolysaccharide transferase reaction in these mutants. The site of the enzymatic defect has not yet been established in these cases, but two distinct genetic types of rough mutants have been detected (*18*).

It is interesting to speculate about the function of the lipopolysaccharide. The lipopolysaccharide can account for as much as 5 percent of the dry weight of the cell, and its synthesis clearly involves major expenditure both of energy and of material. Yet loss of the antigenic side chains, or even of a major part of the core structure, appears to have little or no effect on the ability of the organism to survive under laboratory conditions, since the rough and mutant strains grow as well as the wild type does. However, only the wild types, possessing the complete antigenic side chains, are pathogenic. It is possible that the lipopolysaccharide is an important factor in aiding the bacterium to evade host defense mechanisms, such as phagocytosis. Such a role is well established for the capsular polysaccharides of the pneumococci. No mutants have thus far been detected which lack the backbone or lipid portions of the lipopolysaccharide. It may be that these parts of the lipopolysaccharide play an essential role in the physiology of the organism.

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