*BIOSYNTHESIS OF BACTERIAL LIPOPOLYSACCHARIDE, V. LIPID-LINKED INTERMEDIATES IN THE BIOSYNTHESIS OF THE 0-ANTIGEN GROUPS OF SALMONELLA TYPHIMURIUM**

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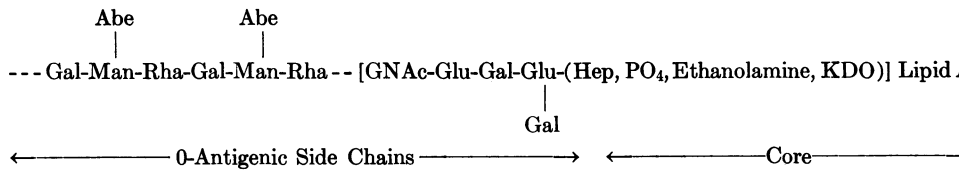
The cell envelope of *Salmonella* and related organisms contains a complex lipopolysaccharide composed of an inner core and an outer region containing the specific surface 0-antigens. The following structure has been postulated for the polysaccharide of *S. typhimurium*:[1]



Biosynthesis of the core proceeds by successive transfer of monosaccharide residues from nucleotide sugars to the incomplete lipopolysaccharide.[1] The origin of the repeating oligosaccharide units of the 0-antigenic side chains is less well understood. We previously described the incorporation of galactose, mannose, and rhamnose into 0-antigen repeating units in a mutant of *S. typhimurium* deficient in GDP-mannose.[2] Similar results have also been described by Nikaido and Nikaido[3] with a mutant of *S. typhimurium* lacking TDP-rhamnose and by Robbins[4] with wild-type *S. anatum*. However, in these experiments the nature of the intermediate reactions was not established.

Anderson *et al.*[5] have presented evidence for a lipid-linked disaccharide intermediate in the biosynthesis of cell-wall glycopeptide in *Staphylococcus aureus* and *Micrococcus lysodeikticus*, and our data suggest that analogous intermediates are involved in synthesis and polymerization of the 0-antigen of *S. typhimurium*. We have isolated lipid-linked derivatives of two oligosaccharides, rhamnosyl-galactosyl-1-phosphate and mannosyl-rhamnosyl-galactosyl-1-phosphate, related to the 0-antigen repeating units. Our evidence suggests that the trisaccharide-lipid is an intermediate in formation of polysaccharide chains containing the trisaccharide repeating units.

These experiments have been carried out with a galactose-negative strain which contains an incomplete core polysaccharide. Under the conditions used, the enzymically synthesized polysaccharide is not transferred to lipopolysaccharide but apparently remains attached through a galactose-1-phosphate reducing terminus to the lipid acceptor. It is postulated that this lipid-linked polysaccharide is a precursor of the 0-antigenic chains.

Materials and Methods.—These were as previously described,[2, 6] including the galactose-negative strain of *Salmonella tryphimurium* deficient in UDP-galactose-4-epimerase, the conditions of growth,[2] and the preparation of the cell-envelope enzyme fraction. This fraction was routinely sedimented by centrifugation at 40,000

TABLE 1

INCORPORATION OF MANNOSE, RHAMNOSE, AND GALACTOSE INTO THE CELL ENVELOPE OF THE MUTANT DEFICIENT IN UDP-GALACTOSE

Radioactive nucleotide sugar added	Nonradioactive nucleotide sugar added	Incorporation (mμmoles)
GDP-mannose-C^{14}	None	0.15
"	UDP-galactose	0.17
"	TDP-rhamnose	0.19
"	UDP-galactose + TDP-rhamnose	2.7
TDP-rhamnose-C^{14}	None	0.4
"	GDP-mannose	0.4
"	UDP-galactose	1.5
"	GDP-mannose + UDP-galactose	3.4
UDP-galactose-C^{14}	None	5.3
"	GDP-mannose	5.6
"	TDP-rhamnose	8.7
"	GDP-mannose + TDP-rhamnose	8.0

Each tube contained 0.1 *M* Tris·HCl, pH 8.4, 0.01 *M* $MgCl_2$, 0.001 *M* EDTA, and 1.0 mg cell-envelope protein in a total volume of 0.25 ml. The sugar nucleotides were present in the following concentrations: GDP-mannose, 0.1 mM; TDP-rhamnose, 0.1 mM; UDP-galactose, 0.12 mM. The specific activity of the radioactive substrates were: GDP-mannose, 875 cpm/mμmole; TDP-rhamnose, 500 cpm/mμmole; UDP-galactose, 450 cpm/mμmole. After incubation at 37° for 15 min, the reaction was stopped by the addition of 10 vol of cold 0.1 *M* acetic acid. The resulting precipitate was collected and washed twice with 0.1 *M* acetic acid, suspended in 50% ethanol containing 1% NH_3, plated, dried, and counted in a windowless gas-flow counter.

× *g* for 20 min in 0.05 *M* Tris buffer, pH 9.0. Descending paper chromatography was performed with (*a*) butanol:pyridine:water (6:4:3), and (*b*) ethylacetate: acetic acid:water (3:1:3).

E. coli alkaline phosphomonesterase (Worthington, chromatographically purified) was dialyzed to remove ammonium sulfate. This preparation was free of hydrolytic activity toward UDP-galactose.

Results.—Enzymic incorporation of 0-antigenic side chain sugars into the cell envelope fraction: Mannose, rhamnose, and galactose were transferred to the particulate cell-envelope fraction when all three sugar nucleotides were present (Table 1). Significant incorporation of mannose required the presence of all three sugar nucleotides. The incorporation of rhamnose required the presence of UDP-galactose and was enhanced by GDP-mannose. The transfer of galactose from UDP-galactose proceeded in the absence of the other sugar nucleotides; most of this activity represented incorporation into the incomplete lipopolysaccharide core but a significant fraction was shown to be related to the incorporation of mannose and rhamnose into the 0-antigen structure.

Relationship of the product to 0-antigen: In our previous studies with the mutant strain deficient in GDP-mannose,[2] the relationship to 0-antigen was established by isolation of a trisaccharide, α-galactosyl-mannosyl-rhamnitol, after partial acid hydrolysis of the enzymic product. In the present work, the polysaccharide product was characterized by essentially the same methods and partial hydrolysis yielded the same di- and trisaccharide, mannosyl-rhamnitol, and galactosyl-mannosyl-rhamnitol. Thus, with both mutant strains, products were synthesized containing identical repeating units.

Nature of the reaction product formed with all three nucleotide sugars: The properties of the isolated product indicated clearly that it was not linked to lipopolysaccharide. The radioactive product, formed by incubation of the cell-envelope fraction with GDP-mannose-C^{14} in the presence of unlabeled TDP-rhamnose and UDP-galactose, was present together with lipopolysaccharide in the aqueous phase after extraction of the cell-envelope material with phenol.[6] Gel filtration showed it

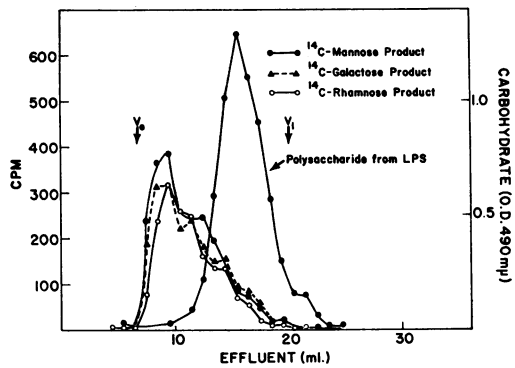


FIG. 1.— Gel filtration on Sephadex G-50 of enzymic products produced in the presence of all three nucleotide sugars. Incubation mixtures similar to those in Table 1, but 20 times larger in scale, were incubated for 2 hr. Each tube contained one sugar nucleotide labeled with C^{14} and the other two unlabeled. The products were isolated by phenol extraction and separated from lipopolysaccharide by centrifugation at 105,000 × g for 3 hr. The supernatant fractions were employed for gel filtration. Endogenous polysaccharide liberated from lipopolysaccharide by hydrolysis at pH 3.4[7] was included as a marker. The column was 1 × 20 cm and was equilibrated with 0.1 M acetic acid. The compounds were eluted with 0.1 M acetic acid at a flow rate of 0.5 ml/min. V_i and V_0 indicate the positions of included and excluded markers, respectively.

to be macromolecular and polydisperse (Fig. 1). The product differed from endogenous lipopolysaccharide in that it was not precipitated by the addition of 0.025 M $MgCl_2$[6] and was not sedimented by centrifugation for 3 hr at 105,000 × g.[7]

In high-voltage paper electrophoresis the radioactive product migrated anionically (Fig. 2A) in contrast to the intact high molecular weight lipopolysaccharide which remained at the origin. On the other hand, it migrated more slowly than the polysaccharide liberated by hydrolysis[8] at pH 3.4 from the endogenous lipopolysaccharide. This polysaccharide appeared at 40 cm from the origin under these conditions. The mobility and behavior on gel filtration of the radioactive product were unaltered by hydrolysis at pH 3.4. It was thus clear that the radioactive

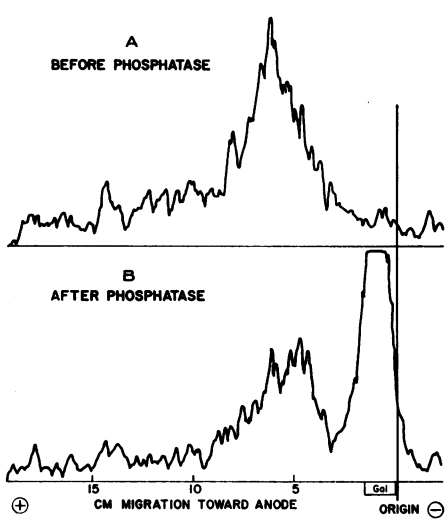


FIG. 2.—(A) Electrophoresis of enzymically produced polysaccharide. The product labeled with mannose-C^{14}, prepared as described in the legend to Fig. 1, was subjected to electrophoresis at pH 3.5 [pyridine: acetic acid: water, (1:10:69)] for 90 min at 96 v/cm on Whatman no. 1 paper. The paper was scanned on a Baird-Atomic 4π-strip scanner. The positions of standards are noted on the abscissa. (B) Electrophoresis of a portion of the same product treated with alkaline phosphatase. Product corresponding to 0.8 mμmoles of C^{14}-mannose was treated with 1 μg of alkaline phosphatase for 1 hr at 37° in 0.1 ml of 0.01 M Tris buffer, pH 8, containing 0.001 M $MgCl_2$.

product, isolated by phenol extraction, was not linked to lipopolysaccharide. Products labeled with galactose-C^{14} and rhamnose-C^{14} showed identical properties.

After treatment with alkaline phosphatase, most of the radioactive product was electrophoretically neutral (Fig. 2*B*). The sensitivity to alkaline phosphatase suggested the presence of a terminal phosphate ester linkage and this was confirmed by the identification of galactose-1-phosphate at the reducing terminus. A product obtained as described in Table 1, with C^{14}-galactose as the labeled sugar, was treated with alkaline phosphatase and then subjected to hypoiodite oxidation and hydrolysis in 2 *N* HCl for 3 hr at 100°. Galactonic acid was recovered by electrophoresis of the hydrolysis mixture at pH 6 and accounted for approximately 6 per cent of the total radioactivity. No C^{14}-galactonic acid was detected when hypoiodite oxidation was carried out prior to treatment with alkaline phosphatase. No rhamnose or mannose-reducing end groups were detected in parallel experiments with dephosphorylated products labeled with C^{14}-mannose and C^{14}-rhamnose.

Formation of a disaccharide-lipid intermediate: When the particulate enzyme fraction was incubated with a mixture of UDP-galactose and TDP-rhamnose, incorporation of both sugars into the particulate fraction was observed (Table 2, incubation I). Three tubes were run in parallel; tube 1 contained UDP-galactose-C^{14} and nonradioactive TDP-rhamnose, tube 2 contained nonradioactive UDP-galactose and TDP-rhamnose-C^{14}, and tube 3 contained both unlabeled nucleotide sugars; this was used to study the incorporation of mannose-C^{14} (see below). Product labeled with C^{14}-rhamnose was quantitatively extracted into chloroform-methanol (3:1); most of the C^{14}-galactose was incorporated into the lipopolysaccharide core but a significant fraction was also extracted into chloroform-methanol. The ratio of galactose-C^{14} to rhamnose-C^{14} in the chloroform-methanol extractable fraction was very close to 1. Chromatography of the lipid-linked fractions in several systems on paper and thin-layer plates demonstrated that all of the radioactivity migrated as a single substance.

TABLE 2

SEQUENTIAL INCORPORATION OF NUCLEOTIDE SUGARS INTO THE CELL-ENVELOPE FRACTION

	Tube 1 UDP-galactose-C^{14} (during incubation I)		Tube 2 TDP-rhamnose-C^{14} (during incubation I)		Tube 3 GDP-mannose-C^{14} (during incubation II & III)	
Incubation	Total (mμmoles)	$CHCl_3$-MeOH soluble (mμmoles)	Total (mμmoles)	$CHCl_3$-MeOH soluble (mμmoles)	Total (mμmoles)	$CHCl_3$-MeOH soluble (mμmoles)
I	144	36.9(26)*	24.1	24.1(23)	—	—
II	152	24.4(16)	24.0	18.3(17)	15.2	11.3(11)
III	155	17.3 —†	22.8	4.5 —	20.0	3.8 —

* The figures in parentheses are corrected for the small amount of lipopolysaccharide entrained in the organic phases. To correct for this, a portion of the extract was analyzed chromatographically on paper with chloroform:methanol:water (65:25:4). A correction factor for lipopolysaccharide (which remains at the origin) was derived by scanning the chromatogram and integrating the areas under the peaks. Only in the case of the galactose-labeled product was this correction quantitatively significant.

† The quantity of nonlipopolysaccharide radioactivity was too small to be estimated.

First incubation: Each tube contained 0.1 *M* Tris·HCl, pH 8.4, 0.01 *M* $MgCl_2$, 0.001 *M* EDTA, 41 mg cell-envelope protein, 0.16 mM UDP-galactose, and 0.08 mM TDP-rhamnose in a total volume of 10 ml. After incubation at 37° for 20 min, the reaction mixtures were diluted with 25 ml cold 0.05 *M* Tris·HCl, pH 9.0, the particulate was collected by centrifugation at 40,000 × *g*, and washed once with the same buffer. The final pellets were suspended in 10 ml of the Tris, $MgCl_2$, EDTA mixture and brought to 10°. A 4.0-ml aliquot of each was taken into 25 ml of cold 0.2 *M* acetic acid for subsequent washing and analysis.

Second incubation: 6.0 ml of each residue were added to tubes containing GDP-mannose or GDP-mannose-C^{14} to give a final concentration of 0.03 mM. After 5 min at 10°, a 4-ml aliquot was removed for analysis.

Third incubation: The reaction mixtures were placed in a 37° bath for 15 min after which 1.5-ml aliquots were removed for analysis (all values shown were corrected to 4.0 ml).

Analysis: The pellets were collected by centrifugation and washed twice with 0.1 *M* acetic acid. They were suspended in 3.0 ml of water by sonication, a small portion removed for counting, and the remainder extracted 4 times with 25 ml chloroform-methanol (3:1), water being added to maintain the same volume of aqueous phase. The combined organic phases were filtered and portions dried and counted. The specific activities were: UDP-galactose, 3550 cpm/mμmole; TDP-rhamnose, 5800 cpm/mμmole; GDP-mannose, 5590 cpm/mμmole.

The component extracted by chloroform-methanol was converted by treatment with hot phenol to rhamnosyl-galactosyl-1-phosphate, which is characterized below.

Conversion of the disaccharide-lipid intermediate to trisaccharide-lipid: A portion of each of the particulate fractions described in the preceding section, after incorporation of galactose and rhamnose, was washed free of nucleotides and incubated with nonradioactive GDP-mannose (tubes 1 and 2) or GDP-mannose-C^{14} (tube 3). Rapid incorporation of mannose into the particulate fraction was observed (Table 2, incubation II). This incorporation was entirely dependent on prior or simultaneous incubation of the particulate fraction with both TDP-rhamnose and UDP-galactose (see Table 1). Under the conditions employed (5 min at 10°), about 70 per cent of the mannose-C^{14} was incorporated into a product soluble in chloroform-methanol. The ratio of mannose:rhamnose:galactose in the lipid extract was approximately 0.6:1:1; in other experiments the ratio of mannose to rhamnose ranged from 0.7 to 1.0. As described below, both residual rhamnosyl-galactosyl-1-phosphate and a new product identified as mannosyl-rhamnosyl-galactosyl-1-phosphate could be isolated from this extract after treatment with phenol.

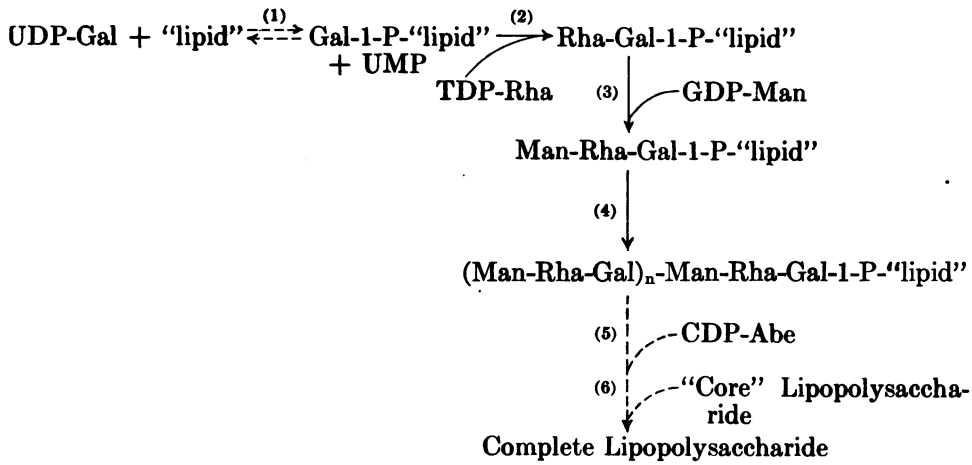
Polymerization of the trisaccharide intermediate: When the temperature of the incubation mixtures was raised to 37° (Table 2, incubation III), there was little increase in total mannose incorporation; however, the solubility of the product in chloroform-methanol was greatly diminished and it was now identical with the macromolecular product described in an earlier section. The data support the hypothesis that mannose was first transferred to the disaccharide phospholipid and that the resulting trisaccharide derivative was subsequently polymerized to form the final macromolecular product.

Characterization of the oligosaccharide components of the chloroform-methanol-soluble intermediates: (1) *Disaccharide phosphate:* Samples of particulate preparations corresponding to the first part of the experiment in Table 2, containing label from UDP-galactose-C^{14} or from TDP-rhamnose-C^{14}, were subjected to extraction with hot phenol. The material in the aqueous phase migrated as a single major anionic peak (compound A) on paper electrophoresis (Fig. 3*A*); a similar product was obtained from the galactose-C^{14}-labeled product. Compound A was converted quantitatively to an electrophoretically neutral product when it was treated with *E. coli* alkaline phosphomonoesterase (Fig. 3*B*). The dephosphorylated product behaved as a single component on chromatography in solvent (*a*) ($R_{galactose}$ = 0.9) and contained radioactivity from both rhamnose and galactose. The dephosphorylated product was oxidized with hypoiodite and hydrolyzed; C^{14}-galactonate and C^{14}-rhamnose were the only radioactive products obtained. The phosphorylated disaccharide was insensitive to oxidation by hypoiodite, suggesting that it was esterified in the 1 position. This is consistent with the acid lability of the phosphate group. Compound A lost its electrophoretic mobility completely when treated with 0.1 *N* HCl at 100° for 10 min. It is thus established that compound A is rhamnosyl-galactosyl-1-phosphate.

(2) *Trisaccharide phosphate:* The chloroform-methanol extract obtained from the second incubation (Table 2), in which mannose-C^{14} had been incorporated at 10° was dried, extracted with phenol, and the material in the aqueous phase subjected to electrophoresis (Fig. 4). This yielded two anionic radioactive components (*B* and *C*), which migrated somewhat behind rhamnosyl-glactose-1-phosphate;

the same components were also detected when the label was present as rhamnose or galactose. Fraction *B* was identified as mannosyl-rhamnosyl-galactosyl-1-phosphate; fraction *C* has not been identified. Treatment of *B* with alkaline phosphatase yielded a single neutral compound which, on chromatography in solvent (*a*), migrated with $R_{galactose} = 0.5$. The corresponding galactose- and rhamnose-labeled products behaved similarly. All of the galactose-C^{14} was recovered as galactonate after phosphatase treatment, hypoiodite oxidation, and hydrolysis. The sequence was established by partial acid hydrolysis (60% HCOOH, 30 min, 100°), and reduction with $NaBH_4$. Chromatography in solvents (*a*) and (*b*) showed, in addition to free alditols, a single disaccharide containing mannose-C^{14} and rhamnitol-C^{14}. These results established the structure of compound B as mannosyl-rhamnosyl-galactosyl-1-phosphate.

Discussion.—On the basis of the present results, we propose the following mechanism for biosynthesis of the 0-antigen side chains:



The postulated reaction (1) is analogous to the first step in biosynthesis of cell-wall glycopeptide; Struve and Neuhaus[9] and Anderson *et al.*[5] have identified UMP as a product of the reversible reaction between UDP-*N*-acetyl muramyl peptide and the lipid acceptor. We have observed the incorporation of UMP-C^{14} into UDP-galactose in the presence of the cell-envelope fraction of the epimeraseless mutant. The synthesis of the corresponding rhamnosyl-galactose derivative and its incorporation into 0-antigen in the presence of GDP-mannose has been observed in *S. anatum.*[10] The nature of the lipid remains unknown.

Reactions (5) and (6), addition of abequose to the trisaccharide repeating units and attachment to the core polysaccharide have not yet been studied in this organism. Although polymerization of the trisaccharide intermediate can occur in the absence of CDP-abequose (CDP-Abe), the possibility remains that addition of abequose normally occurs before polymerization.

The intact lipid-linked polysaccharide, postulated as the product of reaction (4), has not yet been isolated, but the demonstration that the polymeric product obtained by phenol extraction terminates in galactose-1-phosphate strongly supports this structure for the primary reaction product.

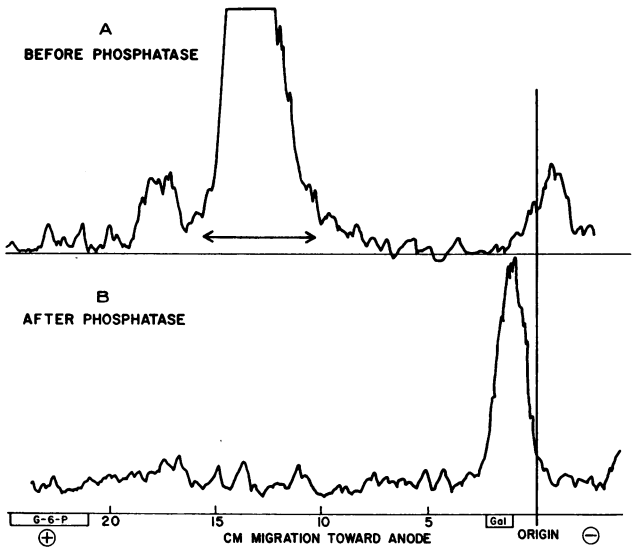


FIG. 3.—(*A*) Electrophoresis of the disaccharide phosphate produced from UDP-galactose and TDP-rhamnose-C^{14}. An enzymic pellet corresponding to the first incubation in Table 2 was subjected to phenol extraction and electrophoresis of the aqueous phase at pH 3.5, 60 v/cm, for 45 min. (*B*) Electrophoresis of the disaccharide fraction after treatment with alkaline phosphatase. A portion of compound A eluted from the electropherogram in (*A*) was treated with alkaline phosphatase (as described in Fig. 2*B*) before electrophoresis.

The enzymically synthesized polysaccharide is of particular interest because of its possible relation to the haptenic 0-antigens recently described in several laboratories. Beckmann *et al.*[11] have found that R_I mutants of *Salmonella*, which produced an incomplete lipopolysaccharide lacking both 0-antigenic side chains and parts of the core structure, contained a separate polysaccharide chemically and serologically related to 0-antigen. This material (the L_I fraction) contained all the sugar components of the 0-antigenic side chains but lacked heptose and long-chain fatty acid and was of lower molecular weight than lipopolysaccharide.

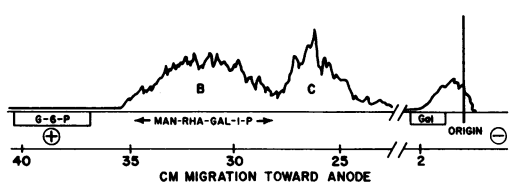


FIG. 4.—Electrophoresis of mannose-C^{14}-labeled trisaccharide phosphate. The chloroform-methanol extract of the enzymic pellet (Table 2, incubation II) was dried, extracted with warm phenol, and the aqueous phase analyzed by electrophoresis at pH 3.5, 60 v/cm for 90 min.

A similar low molecular weight 0-antigen hapten has been found in wild-type strains of *E. coli*.[12] The relationship between this hapten and the enzymically synthesized product is currently under investigation.

* Preceding publications of this series have appeared in these PROCEEDINGS,[2] and in *J. Biol. Chem.* This is communication no. 33 from the Joan and Lester Avnet Institute of Molecular Biology. This work was supported by grants from NIH (GM 11301), and the National Science Foundation (GB 1465 and GB 1161).

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‡ Career Investigator of the Health Research Council of the City of New York.

[1] Osborn, M. J., S. M. Rosen, L. Rothfield, L. D. Zeleznick, and B. L. Horecker, *Science*, **145**, 783 (1964).

[2] Zeleznick, L. D., S. M. Rosen, M. Saltmarsh-Andrew, M. J. Osborn, and B. L. Horecker, these PROCEEDINGS, **53**, 207 (1965).

[3] Nikaido, H., and K. Nikaido, *Biochem. Biophys. Res. Commun.*, **19**, 322 (1965).

[4] Robbins, P. W., A. Wright, and J. L. Bellows, these PROCEEDINGS, **52**, 1302 (1964).

[5] Anderson, J. S., M. Matsuhashi, M. A. Haskin, and J. L. Strominger, these PROCEEDINGS, **53**, 881 (1965).

[6] Osborn, M. J., S. M. Rosen, L. Rothfield, and B. L. Horecker, these PROCEEDINGS, **48**, 1831 (1962).

[7] Westphal, O., O. Lüderitz, and F. Bister, *Z. Naturforsch.*, **7b**, 148 (1952).

[8] Osborn, M. J., these PROCEEDINGS, **50**, 499 (1963).

[9] Struve, W. G., and F. C. Neuhaus, *Biochem. Biophys. Res. Commun.*, **18**, 6 (1965).

[10] Wright, A., M. Dankert, and P. W. Robbins, these PROCEEDINGS, **54**, 235 (1965).

[11] Beckmann, I., T. V. Subbaiah, and B. A. D. Stocker, *Nature*, **201**, 1302 (1964).

[12] Ribi, E., R. L. Anacker, K. Fukushi, W. T. Haskins, M. Landy, and K. C. Milner, in *Bacterial Endotoxins*, ed. M. Landy and W. Braun (New Brunswick: The Institute of Microbiology, Rutgers, 1964), p. 16.

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*EVIDENCE FOR AN INTERMEDIATE STAGE IN THE BIOSYNTHESIS OF THE SALMONELLA 0-ANTIGEN**

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The 0-antigen of E-group *Salmonella* strains such as *S. anatum* and *S. newington* consists primarily of long polysaccharide chains attached to a complex core or backbone structure.[1] The polysaccharide chains have a basic repeating glycosidic sequence that can be represented simply by the formula

$$(\text{-D-mannosyl-L-rhamnosyl-D-galactosyl-})_n.$$

We have recently reported the enzymatic synthesis of these 0-antigen chains from the nucleotide precursors UDP-D-galactose, TDP-L-rhamnose, and GDP-D-mannose.[2] In the present paper, evidence is presented for an intermediate stage in the biosynthesis of the *S. newington* 0-antigen in which sugars are transferred from the nucleotide sugar precursors to an acceptor prior to incorporation into lipopolysaccharide. Omission of GDPM from the *in vitro* 0-antigen synthesizing system leads to the accumulation of a compound which has been characterized as a rhamnosylgalactosylphosphate-derivative. On subsequent addition of GDPM to the system containing this disaccharide intermediate, oligosaccharide repeating units are incorporated into 0-antigen chains. The isolation and some properties of the disaccharide intermediate are described.

Particulate enzyme systems that catalyze the synthesis of the *S. typhimurium* 0-antigen have been described by Zeleznick *et al.*[3] and by Nikaido and Nikaido.[4] The demonstration of an intermediate stage in the *S. typhimurium* system similar to that described in the present paper appears in the accompanying paper by Weiner *et al.*[5]

Materials and Methods.—The bacterial strain used for this study was the lysogenic strain of *Salmonella anatum*, $A_1(\epsilon^{15})$ (= *Salmonella newington*).[1] $A_1(\epsilon^{15})$ cells grown as previously described