[15] Bangham, A. D., "A correlation between surface charge and coagulant action of phospholipids," *Nature*, **192**, 1197 (1961).

[16] Wood, L. A., F. E. Horan, E. Sheppard, and I. S. Wright, "Zeta potential measurements as a tool for studying certain aspects of blood coagulation," in *Transactions of the Third Conference on Blood Clotting and Allied Problems* (New York: Josiah Macy, Jr., Foundation Publications, 1950), pp. 89–126.

[17] Macfarlane, R. G., "An enzyme in the blood clotting mechanism and its function as a biochemical amplifier," *Nature*, **202**, 498 (1964).

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*BIOSYNTHESIS OF BACTERIAL LIPOPOLYSACCHARIDE, IV. ENZYMATIC INCORPORATION OF MANNOSE, RHAMNOSE, AND GALACTOSE IN A MUTANT STRAIN OF SALMONELLA TYPHIMURIUM**,†

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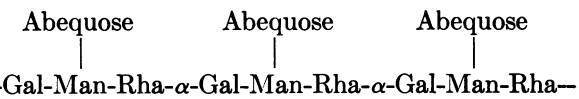
Communicated November 25, 1964

The cell-wall lipopolysaccharides which form the 0-antigens of Salmonella consist of immunologically specific heteropolysaccharides in covalent linkage to a specific lipid (lipid A). Recent work in the laboratories of Westphal[2–4] and others[5–8] has provided evidence that the polysaccharide portion of the lipopolysaccharide is composed of two distinct regions which differ chemically and immunologically. The outer region is composed of side chains which carry the specific determinants of the surface 0-antigens. These 0-antigenic chains are linked to a smaller, internal core region, whose structure may be similar in all Salmonellae. Previous work from this laboratory on the biosynthesis of the lipopolysaccharide of *S. typhimurium* has been concerned with the enzymatic incorporation of glucose,[9, 10] galactose,[11, 12] and *N*-acetylglucosamine[10] from appropriate nucleotide sugars into the core structure of the lipopolysaccharide. These enzyme systems were studied in mutant strains of *S. typhimurium* which form incomplete lipopolysaccharides as a result of failure to synthesize UDP-glucose or UDP-galactose.[7]

We have recently undertaken a study of the biosynthesis of the 0-antigen portion of the lipopolysaccharide. Staub and her colleagues[13, 14] have shown that the 0-antigen of *S. typhimurium* contains a galactosyl-mannosyl-rhamnosyl repeating unit. Abequose (3,6-dideoxy-D-galactose) is linked to this trisaccharide repeating unit in nonreducing terminal branches. The structure postulated by Staub is shown schematically as follows:



For investigation of the biosynthesis of this structure, we have employed a mutant of *S. typhimurium*, recently isolated in this laboratory, which is deficient in the synthesis of GDP-mannose. This organism (M_2) lacks the enzyme phosphomannose isomerase, which catalyzes the conversion of fructose 6-phosphate to mannose

6-phosphate; in the absence of exogenous mannose, the mutant is, therefore, unable to synthesize GDP-mannose. The isolation and characterization of the organism are reported elsewhere.[8] When cells of strain M_2 are grown in the absence of mannose, they produce an incomplete lipopolysaccharide which is deficient in mannose, rhamnose, and abequose, and which is similar in composition to the lipopolysaccharide found in certain rough mutants (rough II of Beckmann *et al.*[3, 15]). The polysaccharide of this mutant strain contains glucose, galactose, heptose, and glucosamine in molar ratios of 1:1:1:0.5, respectively. Trace amounts of rhamnose are also present (*ca.* 0.05 moles/mole glucose), but neither mannose nor abequose have been detected. When the mutant is grown in the presence of mannose, it produces the wild-type polysaccharide in which mannose, rhamnose, and abequose are major constituents.

In the present communication, we wish to report the enzymatic incorporation of mannose, rhamnose, and galactose into the incomplete lipopolysaccharide of this mutant. The enzyme activities are localized in the cell envelope fraction, which also contains endogenous acceptor lipopolysaccharide. Evidence for the biosynthesis of the postulated galactosyl-mannosyl-rhamnosyl sequence is provided by isolation of a trisaccharide, α-galactosyl-mannosyl-rhamnose, as well as a disaccharide, mannosyl-rhamnose, from partial acid hydrolysates of the enzymatic products.

Materials and Methods.—Growth of cells: Bacteria were grown in proteose peptone-beef extract medium (Difco), and harvested in exponential growth.[8, 11] Preparation of the particulate cell wall-membrane (cell-envelope) fraction, sedimenting at 12,000 $\times$ *g*, has been described previously.[9, 11]

Substrates: UDP-Galactose-C^{14} was prepared according to the method of Osborn *et al.*[10, 11] GDP-Mannose-C^{14} was prepared according to the method of Rosen *et al.*[8] TDP-Rhamnose-C^{14} was prepared according to the method of Glaser *et al.*,[16] using extracts of *Streptococcus pyogenes.*

Analytical techniques: Descending chromatography was performed on Whatman no. 1 paper, using (*a*) butanol:pyridine:water (6:4:3), or (*b*) ethyl acetate:acetic acid:water (3:1:3). Sugars were detected with silver nitrate and alcoholic sodium

TABLE 1

INCORPORATION OF MANNOSE, RHAMNOSE, AND GALACTOSE INTO THE CELL-ENVELOPE FRACTION

	Radioactive nucleotide sugar added	Nonradioactive nucleotide sugar added	Incorporation (mμmoles)
1	GDP-mannose-C^{14}	None	5.7
2	"	UDP-galactose	5.6
3	"	TDP-rhamnose	6.3
4	"	UDP-galactose + TDP-rhamnose	7.8
5	TDP-rhamnose-C^{14}	None	0.06
6	"	GDP-mannose	0.06
7	"	UDP-galactose	0.76
8	"	GDP-mannose + UDP-galactose	1.34
9	UDP-galactose-C^{14}	None	0.18
10	"	GDP-mannose	0.23
11	"	TDP-rhamnose	0.63
12	"	GDP-mannose + TDP-rhamnose	0.99

Incubation mixtures contained 26 μmoles of Tris buffer, pH 8.5, 1.2 μmoles of EDTA, 4 μmoles of $MgCl_2$, 0.015 μmoles of nucleotide sugar-C^{14} (1×10^6 cpm/μmole) and 0.3 mg of enzyme protein (particulate cell-envelope fraction) in a volume of 0.25 ml. Nonradioactive nucleotide sugars (0.015 μmole) were added as indicated. After incubation for 20 min at 37°, 1.5 ml of cold 5% trichloroacetic acid in 0.05 *M* sodium pyrophosphate were added. The precipitate was washed, plated, and counted as previously described.[11]

hydroxide.[17] Radioactivity on paper was detected using a Baird-Atomic 4π scanogram.

Electrophoresis was carried out on Whatman no. 1 paper in pyridine:acetic acid:water (1:10:69), pH 3.5, at 55 v/cm for 1 hr.

α-Galactosidase was prepared from coffee beans by the method of Robbins and Uchida[18]; a trace of residual β-galactosidase activity was removed by passage through Sephadex G-100.

Results.—Incorporation of mannose, rhamnose, and galactose into the mannose-deficient cell wall: As shown in Table 1 (line 1), the particulate cell-envelope fraction of the mutant catalyzed the transfer of mannose from GDP-mannose-C^{14} into cell-wall material in the absence of other nucleotide sugars. In contrast, little or no incorporation of rhamnose or galactose occurred when TDP-rhamnose-C^{14} and UDP-galactose-C^{14} were the sole nucleotide sugars present (Table 1, lines 5 and 9). These results are similar to those obtained[9-12] with the mutants previously studied, which are unable to form UDP-glucose and UDP-galactose. In each case, the first sugar to be transferred into lipopolysaccharide *in vitro* was that which the organism could not synthesize from its growth substrate.

Since the postulated structure of the O-antigen contains repeating trisaccharide units composed of galactose, rhamnose, and mannose, incorporation of one sugar should be enhanced by the presence of the other two sugar nucleotides. This was indeed found to be the case. Thus, mannose-C^{14} incorporation was significantly

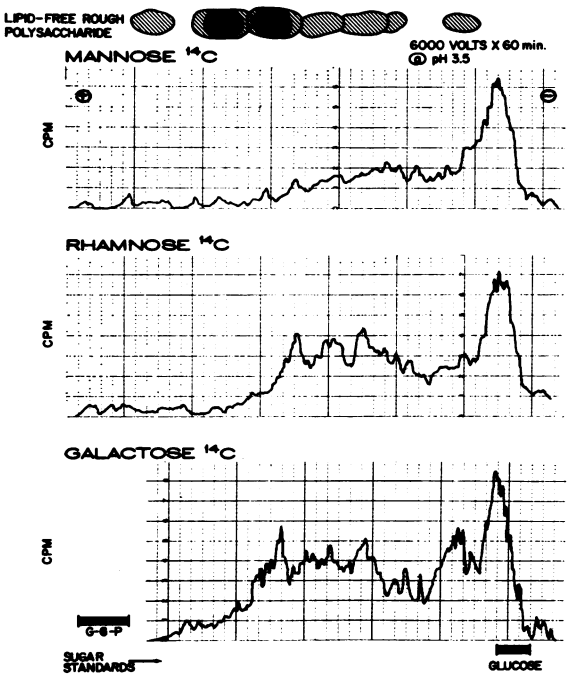


FIG. 1.—Paper electrophoresis of radioactive enzymatic products. Three reaction mixtures, each containing GDP-mannose, TDP-rhamnose, and UDP-galactose with one of the three sugars labeled with C^{14}, were incubated for 35 min. Incubation conditions were as described in Table 1, except that the scale was tenfold greater. The reaction was terminated by addition of cold 5% TCA in 0.01 *M* sodium pyrophosphate. The precipitates were washed once with cold 5% TCA, then twice with water. The polysaccharide portion of the lipopolysaccharide was released by hydrolysis in 3 ml of 0.5 *N* acetic acid at 100° for 30 min. Hydrolysis with acetic acid was repeated twice more; at this point essentially all of the radioactivity incorporated had been released from the residues. The combined hydrolysates were concentrated under reduced pressure prior to electrophoresis. Samples of lipid-free polysaccharide (100–200 μg) derived from the unreacted mutant lipopolysaccharide were run on the same strip for reference. The results with this material are shown by the shaded areas at the top of the figure. This strip was developed with the silver nitrate reagent; the others were scanned for radioactivity. The electropherograms are labeled according to the labeled sugar used in the experiment.

increased when both TDP-rhamnose and UDP-galactose were present (Table 1, line 4), and the small incorporation of galactose was increased fivefold by the addition of the other two nucleotide sugars (Table 1, line 12). Similarly, rhamnose was extensively incorporated in the complete system (Table 1, line 8), although essentially no incorporation of this sugar occurred in the absence of other nucleotide sugars (line 5). Although the presence of GDP-mannose was required for maximal incorporation of rhamnose and galactose, it should be noted that appreciable incorporation of these sugars into acid-precipitable cell-envelope material occurred in the absence of GDP-mannose (lines 7 and 11, Table 1). This incorporation of rhamnose occurred only in the presence of UDP-galactose and, similarly, galactose incorporation required TDP-rhamnose. The significance of these mannose-independent reactions and their relationship to lipopolysaccharide biosynthesis is not yet clear. It is possible that additional, as yet undefined, products containing rhamnose and galactose are present in the crude cell-envelope fraction.

Characterization of the radioactive products: A distinguishing characteristic of the cell wall polysaccharide is its anionic migration upon paper electrophoresis at pH 3.5;[6] this property is related to the presence of phosphate in the polymer. The

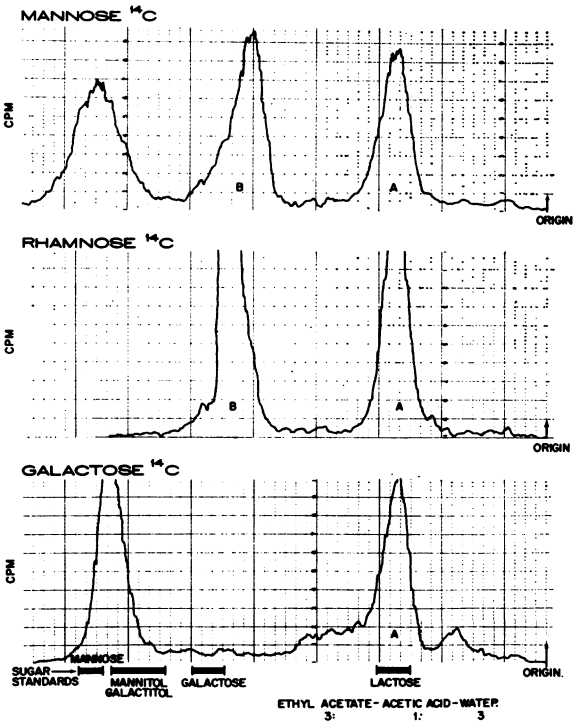


FIG. 2.—Purification of the radioactive oligosaccharides. Products containing mannose-C^{14}, galactose-C^{14}, and rhamnose-C^{14} were prepared as described in the legend to Fig. 1. The acetic acid hydrolysates (*ca.* 15,000 cpm in each) were taken to dryness and dissolved in 1 ml of 60% HCOOH for hydrolysis. After removal of HCOOH by vacuum distillation, the hydrolysates were taken up in 1 ml of H_2O and treated with 10 mg of $NaBH_4$. The solutions were kept overnight at 4°. Excess borohydride was destroyed by acidification with acetic acid, the samples were passed through Dowex-50 H^+ columns (1-ml resin bed volume), and H_3BO_3 was removed by repeated evaporation to dryness from methanol. After descending chromatography in solvent A, the radioactive oligosaccharide areas were eluted with 0.1 *N* acetic acid and rechromatographed in solvent B for 48 hr. Rhamnitol had moved off the paper in this time.

radioactive products were isolated from three parallel incubation mixtures, each containing one C^{14}-labeled nucleotide sugar in the presence of the other two C^{12}-nucleotide sugars. When each labeled product was subjected separately to paper electrophoresis at pH 3.5, three similar electropherograms were obtained (Fig. 1). In each case, the bulk of the radioactivity migrated anionically, and the patterns indicated that incorporation of the three sugars into similar fractions had occurred.

In addition, each of the labeled polysaccharides showed identical elution patterns when chromatographed on Sephadex G-50, and a similar pattern was obtained on elution of authentic rough polysaccharide from the same column.

Isolation and structure of the trisaccharide units: In order to establish that the three sugars were incorporated into trisaccharide units, radioactive polysaccharide produced in separate experiments with each of the three radioactive sugar nucleotides was subjected to partial acid hydrolysis. This was accomplished by heating with 60 per cent formic acid at 100° for 90 min. Formic acid was removed by repeated evaporation under vacuum, and the mixture of oligosaccharides and monosaccharides was reduced with sodium borohydride. Reduction served several purposes: (1) it converted the reducing end groups of any di- or trisaccharides to the corresponding alcohol, permitting identification of the reducing end groups; (2) it destroyed formyl esters which might have formed during hydrolysis; and (3) it allowed the chromatographic separation of a reduced disaccharide containing mannose and rhamnitol (peak *B*, Fig. 2) from free galactose; the unreduced di-

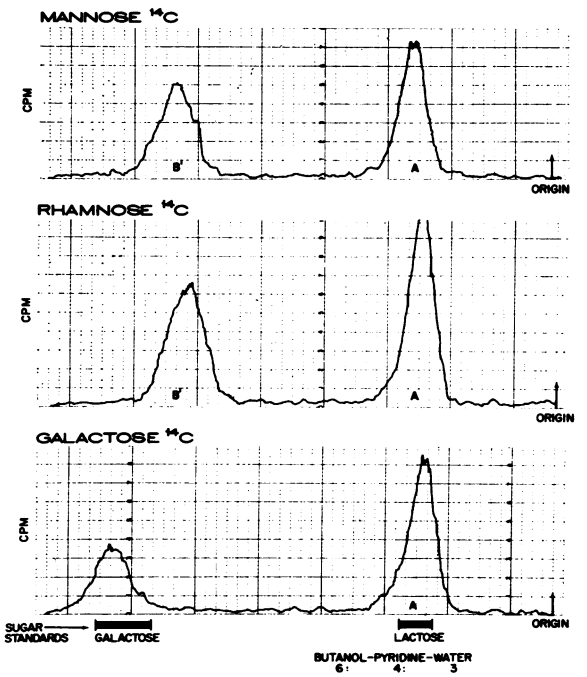


FIG. 3.—Products of hydrolysis of compound A with α-galactosidase. Three parallel samples of compound A, containing mannose-C^{14}, galactose-C^{14}, and rhamnose-C^{14}, respectively (approximately 3000 cpm in each sample), were incubated with 120 μg of α-galactosidase (specific activity, 3 μmoles mg/hr with melibiose as substrate) in 0.02 *M* sodium acetate buffer, pH 4.5, in final volume of 0.050 ml. After 4 hr at 37°, the total incubation mixture was spotted on paper and chromatographed in solvent A for 24 hr.

saccharide migrated identically with galactose. The reduced hydrolysis products were chromatographed in solvent A. Radioactive areas were eluted and rechromatographed in solvent B (Fig. 2). In addition to the free alditols, two labeled oligosaccharides were detected; a fast-moving material, which contained radioactive mannose, and rhamnose (compound B), and a slower-moving component, which contained all three labeled sugars (compound A). At least 30 per cent of the total radioactivity of each hydrolysate was recovered in the two purified oligosaccharide fractions.

Evidence that compound A was a trisaccharide containing a nonreducing terminal α-galactosyl residue was obtained by hydrolysis with coffee bean α-galactosidase (Fig. 3). Following this treatment, chromatography in solvent A showed the presence of free galactose-C^{14} and a component (compound B′), which contained only mannose-C^{14} and rhamnose-C^{14}, and which appeared to be identical to the previously isolated compound B. As expected, trisaccharide labeled with C^{14}-galactose did not yield a radioactive disaccharide, but only C^{14}-galactose. Some unreacted compound A remained in each case. The presumed disaccharides, B and B′, were hydrolyzed to the component monosaccharides in 2 *N* HCl for 2 hr at 100°. The hydrolysates were chromatographed on borate-impregnated Whatman no. 1 paper in solvent A, and showed the presence of mannose-C^{14} and rhamnitol-C^{14}. It was thus established that rhamnose was the reducing end of the di- and trisaccharides, and that the disaccharide was mannosyl-rhamnose.

To determine whether the galactose was linked to mannose in a straight chain trisaccharide, or to rhamnose in a branched trisaccharide, the galactosyl linkage was converted to a galactosyluronic acid linkage, so that a disaccharide containing this residue might be obtained after acid hydrolysis. It is well known that aldobiuronic acids are readily isolated from oligosaccharides containing uronic acid residues be-

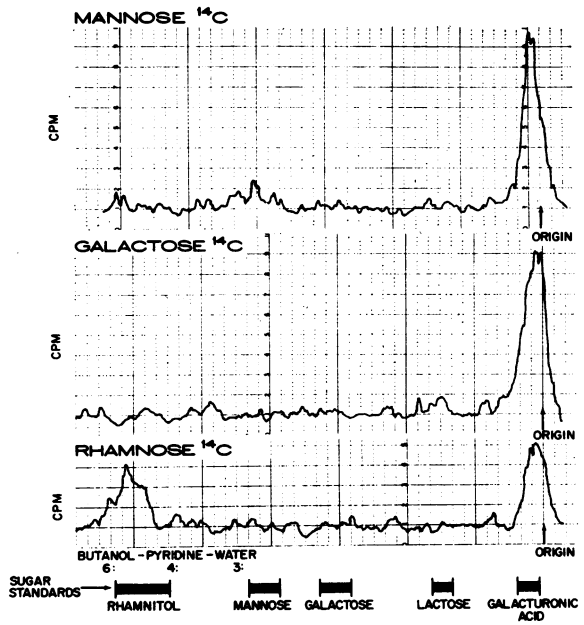


FIG. 4.—Chromatography of the galactosyluronic acid trisaccharide after hydrolysis. Samples of trisaccharide A, eluted from the chromatograms shown in Fig. 3 (each containing about 3000 cpm of labeled sugar), were taken up in 0.025 ml of 0.01 *M* phosphate buffer, pH 7.5, and treated with 250 units of galactose oxidase (total volume = 0.05 ml) for 18 hr at 25°. At this time, 0.1 ml of 0.1 *M* Na_2CO_3 and 0.025 ml of 0.1 *M* $KI-I_2$ were added; after 30 min at 25°, the solutions were passed over Dowex-50-H^+ (1-ml resin bed volume), concentrated, and the oxidized trisaccharides isolated by chromatography in solvent A. The galactosyluronic acid trisaccharides were hydrolyzed at 100° in 0.25 ml of 1 *N* HCl for 1 hr, HCl was removed by extraction with dioctyl methylamine in $CHCl_3$, and the hydrolysates were chromatographed in solvent A.

cause of the strong resistance of the biuronic linkage to acid hydrolysis. This method had previously been employed to determine the linkage of galactose in the core polysaccharide.[12] Oxidation of the three preparations of trisaccharide A, each containing a different C^{14}-labeled sugar, was accomplished with galactose oxidase[19] which converts galactosyl residues to the corresponding 6-aldehydes. The C-6 aldehyde derivatives were then converted to the galactosyluronic acid derivatives by treatment with KI-I_2 in sodium carbonate. The reaction mixtures were then purified by chromatography in solvent A; in this solvent the oxidized trisaccharide remained near the origin and was well separated from the original trisaccharide. Hydrolysis of the oxidized trisaccharide in 1 *N* HCl at 100° for 1 hr yielded rhamnitol-C^{14} as the only monosaccharide (Fig. 4). All of the radioactivity from the samples labeled with mannose-C^{14} and galactose-C^{14} remained associated with the acidic fraction at the origin. Approximately half of the rhamnitol-C^{14} remained in the acidic fraction, and was recovered in unhydrolyzed trisaccharide (see Fig. 5). The material which remained at the origin in solvent A was subjected to paper electrophoresis at pH 3.5 in order to separate galacturonic acid-containing components (Fig. 5). In addition to unreacted trisaccharide (see major peak in lowest strip) and some free C^{14}-galacturonic acid (see middle strip), the only radioactive product detected was a new acidic disaccharide which migrated between the free uronic acid and the trisaccharide; this product contained both radioactive galacturonic acid and radioactive mannose. Isolation of this galactosyluronic acid-mannose disaccharide provided proof that the galactose was linked to mannose to form a straight-chain trisaccharide.

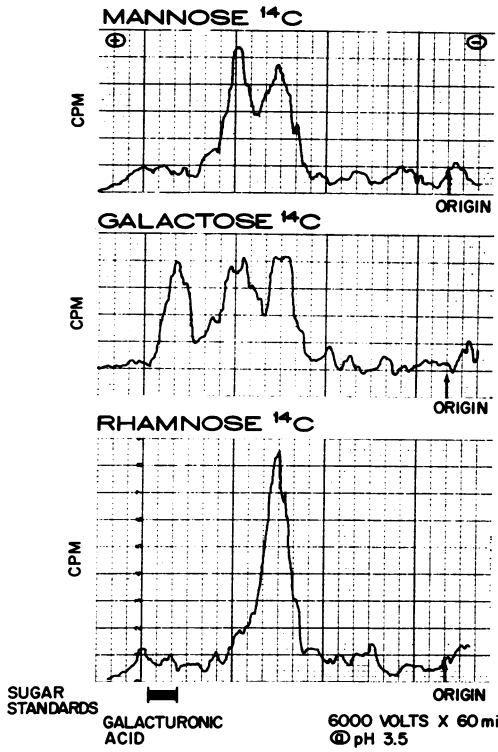


FIG. 5.—Paper electrophoresis of the acidic fraction after hydrolysis of the galactosyluronic acid trisaccharide. The radioactive zones near the origin were eluted from the paper with 0.1 *N* acetic acid. The eluates were then separated by paper electrophoresis at pH 3.5.

Discussion.—The present studies show that the particulate cell-envelope fraction of the mutant deficient in GDP-mannose contains an enzyme system which catalyzes the incorporation of mannose, rhamnose, and galactose into an acid-insoluble product. Isolation of the trisaccharide, α-galactosyl-mannosyl-rhamnose, after partial acid hydrolysis of the enzymatic product, provides strong evidence that these reactions are related to the normal pathway for biosynthesis of the 0-antigenic side chains. Although we have not studied the incorporation of abequose,

the other major component of the polysaccharide, Nikaido and Nikaido[20] have reported incorporation of abequose, dependent on the nucleotides of mannose, rhamnose, and galactose, in extracts of a TDP-rhamnose-deficient mutant.

As yet, we have no information on the site of attachment of the newly synthesized galactosyl-mannosyl-rhamnosyl units to the core polysaccharide which acts as acceptor in these reactions, nor do the data allow conclusions as to the detailed mechanism of biosynthesis of the O-antigenic side chains. Previous work in this laboratory[7, 10] and that of Heath[21] on the biosynthesis of the inner core region of the polysaccharide has shown that this region of the polymer is formed by sequential addition of single sugar residues to the growing chain. Biosynthesis of the repeating units of O-antigenic side chains may occur by a similar mechanism, but the possibility of nucleotide oligosaccharide intermediates has not been excluded.

* Communication no. 23 from the Joan and Lester Avnet Institute of Molecular Biology. This work was supported by grants from NIH and the National Science Foundation. Preliminary reports of portions of this work have been presented previously.[1]

† While this manuscript was in preparation, a description of the biosynthesis of the O-antigenic side chains of *S. anatum* was published by P. W. Robbins, A. Wright, and J. L. Bellows [these PROCEEDINGS, **52**, 1302 (1964)].

‡ Postdoctoral research fellow of the USPHS, grant no. 5-F2-AI-6460-02 from the Institute of Allergy and Infectious Diseases.

§ Research Career Development Awardee of the USPHS grant no. 7-K3-GM-6450-04.

[1] Rosen, S., L. D. Zeleznick, D. Fraenkel, M. J. Osborn, and B. L. Horecker, *Federation Proc.*, **23**, 380 (1964); Zeleznick, L. D., S. Rosen, M. Saltmarsh-Andrew, M. J. Osborn, and B. L. Horecker, *Abstracts*, Sixth International Congress of Biochemistry, New York, 1964, p. 592.

[2] Lüderitz, O., and O. Westphal, *Mosbacher Kolloquium 1964* (Heidelberg: Springer-Verlag), in press.

[3] Beckmann, I., O. Lüderitz, and O. Westphal, *Biochem. Z.*, **339**, 401 (1964).

[4] Kauffmann, F., L. Kruger, O. Lüderitz, and O. Westphal, *Zentr. Bakteriol. Parisitenk. Abt. I, Orig.*, **182**, 57 (1961).

[5] Nikaido, H., these PROCEEDINGS, **48**, 1337, 1542 (1962).

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

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

[8] Rosen, S. M., L. D. Zeleznick, D. Fraenkel, I. M. Wiener, M. J. Osborn, and B. L. Horecker, *J. Biol. Chem.*, in press.

[9] Rothfield, L., M. J. Osborn, and B. L. Horecker, *J. Biol. Chem.*, **239**, 2788 (1964).

[10] Osborn, M. J., and L. D'Ari, *Biochem. Biophys. Res. Commun.*, **16**, 568 (1964).

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

[12] Rosen, S. M., M. J. Osborn, and B. L. Horecker, *J. Biol. Chem.*, **239**, 3196 (1964).

[13] Staub, A. M., and R. Tinelli, *Bull. Soc. Chim. Biol.*, **42**, 1637 (1960).

[14] Stocker, B. A. D., A. M. Staub, R. Tinelli, and B. Kopacka, *Ann. Inst. Pasteur*, **98**, 505 (1960).

[15] Beckmann, I., T. V. Subbaiah, and B. A. D. Stocker, *Nature*, **201**, 1299 (1964).

[16] Glaser, L., and S. Kornfeld, *J. Biol. Chem.*, **236**, 1795 (1961).

[17] Block, R. I., E. L. Durrum, and G. Zweig, *A Manual of Paper Chromatography and Paper Electrophoresis* (New York: Academic Press, 1958), pp. 179–180.

[18] Robbins, P. W., and T. Uchida, *Biochemistry*, **1**, 323 (1962). The authors are grateful to Dr. Robbins for communicating details of the purification.

[19] Avigad, G., D. Amaral, C. Asensio, and B. L. Horecker, *J. Biol. Chem.*, **237**, 2736 (1962).

[20] Nikaido, H., and K. Nikaido, *Abstracts*, Sixth International Congress of Biochemistry, New York, 1964, p. 520.

[21] Edstrom, R. D., and E. C. Heath, *Biochem. Biophys. Res. Commun.*, **16**, 576 (1964).