*BIOSYNTHESIS OF BACTERIAL LIPOPOLYSACCHARIDE, I. ENZYMATIC INCORPORATION OF GALACTOSE IN A MUTANT STRAIN OF SALMONELLA**

By M. J. Osborn,† Samuel M. Rosen,‡ L. Rothfield,§ and B. L. Horecker

DEPARTMENT OF MICROBIOLOGY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

Communicated August 6, 1962

Although the mechanism of biosynthesis of homopolysaccharides, such as glycogen,[1] dextran,[2] and starch,[3] has been studied in some detail, relatively little attention has been given to the biosynthesis of the heteropolymeric mucopolysaccharides and lipopolysaccharides elaborated by animals and microorganisms. A promising approach to this problem has arisen out of recent work in several laboratories[4, 5] on the *in vitro* biosynthesis of the complex capsular polysaccharides of gram-positive cocci. In addition, advances[6–8] in structural analysis of the lipopolysaccharides of gram-negative bacilli have stimulated considerable interest in the mechanism of biosynthesis of these very complex heteropolymers. The cell-wall lipopolysaccharides of the enteric bacteria determine the specific somatic (O) antigenicity of these organisms, and form the basis of their serological classification. The lipopolysaccharides of *Salmonella* may be composed of as many as seven different sugars, including such rare sugars as aldoheptose and 3,6-dideoxyhexoses,[6] and may account for as much as ten per cent of the dry weight of the cell. The present communication describes an enzyme system, derived from a mutant strain of *S. typhimurium*, which appears to be involved in the biosynthesis of the cell-wall lipopolysaccharide.

Mutants of *Escherichia* and *Salmonella* lacking the enzyme UDP-galactose-4-epimerase characteristically produce an abnormal cell-wall lipopolysaccharide, which is distinguished from the wild type by the absence of galactose as well as certain other components of the normal polysaccharide.[9, 10] Fukasawa and Nikaido[10] have shown that the defect in cell-wall biosynthesis is primarily the result of the inability of the mutant to synthesize the precursor of cell-wall galactose, UDP-galactose, in the absence of an exogenous supply of the sugar. The block in UDP-galactose synthesis can be bypassed by growth in the presence of galactose, and under these conditions the composition of the lipopolysaccharide is similar to that of the wild type. We have confirmed these observations with an epimeraseless mutant of *Salmonella typhimurium*. The lipopolysaccharide of the wild type has been shown[6] to contain glucose, galactose, mannose, rhamnose, abequose (3,6-dideoxy-D-galactose), and an unidentified aldoheptose. In contrast, only glucose and the aldoheptose can be detected in hydrolysates of the mutant cell wall. Glucosamine is also present in both wild type and mutant but appears to be associated exclusively with the lipid component of the lipopolysaccharide. A particulate enzyme system isolated from extracts of the mutant strain catalyzes the transfer of galactose from UDP-galactose into a material which appears to be identical with the cell-wall lipopolysaccharide. Some properties of this UDP-galactose-lipopolysaccharide transferase and partial characterization of the product of the reaction are reported here. The system resembles that recently isolated from *S. enteriditis* by Nikaido.[11]

Materials and Methods.—1. *Chemicals:* Galactose-C^{14}, uniformly labeled, was obtained from

Dr. Gerard Milhaud of the Pasteur Institute, Paris, France, and was chromatographically pure.

UDP-galactose was isolated from the epimeraseless strain *E. coli* C7M by the method of Wiesmeyer and Jordan.[12] UDP-galactose-C^{14} was prepared by a modification of this method, in which the bacteria were exposed to 3×10^{-4} *M* nonradioactive galactose for 90 min prior to incubation with 1×10^{-4} *M* galactose-C^{14} (5×10^6 cpm per μmole). Approximately 50% of the added radioactivity was recovered as UDP-galactose-C^{14}.

Galactose 1-phosphate-C^{14} was prepared enzymatically with *E. coli* galactokinase and isolated by the method of Diedrich and Anderson.[13] Aldoheptoses were kindly provided by N. K. Richtmyer of the National Institutes of Health. Other chemicals were commercial products.

2. *Isolation and characterization of UDP-galactose-4-epimeraseless mutant:* The mutant employed in this work was isolated from wild type *S. typhimurium* LT2 by a technique based on two observations of Fukasawa and Nikaido:[10, 14] (1) that epimeraseless strains are resistant to bacteriophage to which the wild type is sensitive, and (2) that these strains undergo lysis in galactose medium. Overnight cultures of UV-irradiated cells were infected with a virulent mutant of phage PLT22 at a multiplicity of 10. Surviving bacteria were plated on nutrient agar and replicated onto EMB-galactose. Galactose-negative isolates were screened for sensitivity to galactose-induced lysis, and final identification of epimeraseless strains was based on assay of the enzymes of the galactose pathway.

3. *Preparation and assay of the UDP-galactose-lipopolysaccharide transferase:* Bacteria were grown in tryptose-phosphate medium with vigorous aeration, harvested when growth had reached 2–3 $\times 10^9$ cells/ml, and stored at $-18°$. Five gm of frozen cells were suspended in 25 ml of 10^{-2} *M* tris (hydroxymethyl) aminomethane (Tris) buffer, pH 7.5, and sonicated for 6 min in a 10 kc Raytheon sonic oscillator. After removal of unbroken cells by centrifugation at $1,200 \times g$ for 15 min, the turbid supernatant material was centrifuged at $12,000 \times g$ for 30 min, and the supernate was discarded. The pellet was resuspended in 25 ml of 10^{-2} *M* Tris buffer, pH 7.5, and the low and high speed centrifugation repeated. The final particulate fraction was suspended in 10 ml of 10^{-2} *M* Tris buffer, pH 7.5, and stored at $-18°$. The enzyme activity was stable for at least one month.

The routine assay for galactose transfer was based on incorporation of radioactivity from UDP-galactose-C^{14} into acid-insoluble material. The incubation mixture contained 15 μmoles of Tris buffer, pH 8.5, 40 μg of chloramphenicol, 1 μmole of EDTA, 3 μmole of $MgCl_2$, 0.02 μmole of UDP-galactose-C^{14} (5×10^5 cpm per μmole), and 0.05 to 0.25 mg of enzyme protein in a volume of 0.25 ml. After incubation for 10 min at 37°, 1.5 ml of cold 5% trichloroacetic acid (TCA) were added. The precipitate was recovered by centrifugation, and washed twice with 1.5 ml of cold 5% TCA. The washed precipitate was suspended in 50% aqueous ethanol containing 0.5% NH_3, plated, dried, and counted in a windowless gas flow counter. Controls in which TCA was added at zero time were included in each set of assays. Essentially identical results were obtained when the lipopolysaccharide was isolated directly from the reaction mixture by phenol extraction and dialysis.

Isolation of lipopolysaccharide: Lipopolysaccharide was isolated from cell-wall preparations by a modification of the method of Westphal and co-workers.[15] Frozen cells were suspended in 5 volumes of 10^{-2} *M* Tris buffer, pH 7.5, and 0.3 *M* KCl and agitated for 15 min with glass beads in a Waring Blendor. The broken cell suspension was centrifuged at $1,200 \times g$ for 30 min to remove intact cells, and then at $12,000 \times g$ for 30 min. The pellet, containing the cell walls, was stirred for 30 min at room temperature with the Tris-KCl buffer and centrifuged at $12,000 \times g$ for 30 min. The washed cell-wall fraction was extracted twice with 45% phenol for 10 min at 68°, and the extracts cooled to 5°. The pooled aqueous layers were washed with ether to remove residual phenol, and the lipopolysaccharide was precipitated by the addition of $MgCl_2$ to a concentration of 0.025 *M*. Under these conditions the lipopolysaccharide of the epimeraseless mutant is quantitatively precipitated, while contaminating RNA remains in solution. The lipopolysaccharide precipitate was suspended in 0.02 *M* EDTA, and dialyzed 24 hr at 4° against 100 volumes of H_2O. The C^{14}-galactose labeled product of the UDP-galactose-lipopolysaccharide transferase reaction was isolated from large scale incubation mixtures by a similar procedure. The labeled product was precipitated from the reaction mixture with 5% TCA, washed twice, and resuspended in H_2O. The suspension was neutralized, and the C^{14}-labeled lipopolysaccharide isolated by phenol extraction and Mg^{++} precipitation as described above.

Results.—1. *Characterization of the UDP-galactose-4-epimeraseless mutant:* The activities of galactokinase and UDP-glucose-galactose-1-phosphate transferase were similar in extracts of the mutant strain of *S. typhimurium* and the wild type, but UDP-galactose-4-epimerase activity could not be detected in the mutant. Addition of the mutant extract did not result in significant inhibition of the epimerase activity of the wild type extract.

It was originally reported[9, 10] that the cell walls of epimeraseless mutants of *Salmonella* lack not only galactose, but also all other normal polysaccharide components except glucose. In agreement with these findings, no trace of galactose could be detected in cell-wall or lipopolysaccharide hydrolysates of the *S. typhimurium* mutant, either chromatographically or by enzymatic assay with galactose oxidase. Mannose, rhamnose, and abequose were also absent. Chromatography of the hydrolysates in several solvent systems revealed only a single component with R_f values identical to those of glucose. However, the spectrum obtained with the mutant lipopolysaccharide in the cysteine-sulfuric acid reaction[16] showed not only a peak at 412 mμ, characteristic of hexoses, but also an additional peak at 505 mμ, which suggested the presence of an aldoheptose component. Aldoheptose has previously been found in the lipopolysaccharide of a number of wild type *Salmonella*, including *S. typhimurium*, by Westphal and his co-workers.[6] Chromatography of the mutant hydrolysates in 95% acetone[17] resulted in separation of the second component ($R_{glucose} = 0.84$); identification of this component as heptose was confirmed by the spectrum of the chromatographically isolated sugar in the cysteine-sulfuric acid reaction. Experiments to be reported elsewhere indicate that the heptose is L-glycero-D-mannoheptose, which has previously been isolated from other enteric bacteria.[18, 19] The identification of the lipopolysaccharide hexose as glucose was confirmed by quantitative enzymatic assay of the isolated sugar with glucose oxidase.

2. *Enzymatic transfer of galactose into the cell wall:* Sonic extracts of the epimeraseless mutant catalyze the transfer of galactose-C^{14} from UDP-galactose-C^{14} into an acid-insoluble fraction. As shown in Table 1, the reaction is specific for the nucleotide sugar. No significant incorporation of free galactose-C^{14} or of galactose-1-phosphate-C^{14} is observed, nor is the incorporation of radioactivity from UDP-galactose-C^{14} appreciably inhibited by UDP-glucose or glucose-6-phosphate. The enzymatic activity is localized in a particulate fraction. Approximately 75% of the activity of the sonicate is recovered in the fraction sedimenting between 1,200 $\times$ *g* and 12,000 $\times$ *g*, and essentially all of the remaining activity can be sedimented at 105,000 $\times$ *g*. The active particulate fraction contains about 0.05 mg of carbohydrate per mg of protein (exclusive of nucleic acid pentose). This endogenous polysaccharide acts as acceptor of the transferred galactose, and appears to be identical with the cell-wall lipopolysaccharide, as discussed below. Efforts to solubilize the enzyme system, or to separate the bound acceptor from the enzyme, have not been successful. Addition of purified lipopolysaccharide to the incubation mixture does not increase either the rate or maximal yield of galactose transfer; this may be related to the insolubility of both the purified lipopolysaccharide and the enzyme system.

The transfer reaction can be detected only with preparations derived from the epimeraseless mutant. No significant incorporation of galactose into cell-wall

TABLE 1

REQUIREMENTS FOR GALACTOSE INCORPORATION

Exp.	Omissions	Additions	Gal incorporation mμmoles/10 min
1	None	...	1.37
	UDP-galactose-C^{14}	Galactose-C^{14}	0.02
	"	Galactose-1-P-C^{14}	0.10
	None	UDP-glucose	1.30
	None	Glucose-6-P	0.93
2	None	...	1.83
	EDTA	...	1.23
	None	ATP	1.81
3	None	...	0.55
	None	RNase	0.53
4	None	..	0.75
	None	UDP	0.42
	None	UMP	0.39

The standard incubation mixture was employed, with omissions as indicated. Where added, other components were present in the following concentrations: galactose-C^{14} and galactose-1-phosphate-C^{14}, 8×10^{-5} *M* (5×10^5 cpm/μmole); UDP-glucose and glucose-6-phosphate, 2.5×10^{-4} *M*; ATP, 1×10^{-3} *M*; RNase, 20 μg/ml; UDP and UMP, 2×10^{-3} *M*. The following amounts of enzyme were added: Exp. 1, 0.29 mg; Exp. 2, 0.31 mg; Exp. 3, 0.12 mg; and Exp. 4, 0.17 mg.

TABLE 2

RELATION OF ENZYMATIC ACTIVITY TO COMPOSITION OF ENDOGENOUS LIPOPOLYSACCHARIDE

Exp.	Source of enzyme	Lipopolysaccharide galactose content	Galactose-C^{14} incorporation mμmoles/mg/10 min
1	Epimeraseless mutant	−	5.4
	Wild type	+	0.1
2	Epimeraseless mutant	−	5.3
	Epimeraseless mutant, galactose grown	+	0.8
	Wild type	+	0.1
	Rough mutant	+	0.1

The enzyme fractions were prepared as described in *Materials and Methods*. Where specified, the culture of the epimeraseless mutant was exposed to galactose for approximately 1 generation (60 min) before harvesting as described under *Methods*. The standard assay procedure was employed. For assay of preparations from the epimeraseless mutant, 0.08 to 0.24 mg of enzyme protein was used; the activity of the wild type and rough strains was tested at several levels of enzyme from 0.2 to 1.5 mg.

material is observed with the particulate fraction (or whole sonicates) prepared from the wild type organism, or from a rough variant of the wild type (Table 2). Rough mutants have been shown[20] to contain a defective lipopolysaccharide which lacks mannose, rhamnose, and abequose, but still contains galactose. Similarly, the activity of the epimeraseless mutant is markedly decreased by growth of the cells in the presence of galactose for one generation. A lipopolysaccharide resembling that of the wild type is formed during growth in galactose[10, 14] Thus, the *in vitro* transfer of galactose appears to be specifically related to the state of the endogenous lipopolysaccharide. The inactivity of the wild type and rough preparations is also ascribed to the absence of unfilled galactose acceptor sites in the lipopolysaccharide, rather than to inactivation of the UDP-galactose transferase *per se*.

Properties of the enzyme system: Transfer of galactose into the cell walls is stimulated by Mg^{++} or Mn^{++} ions (Fig. 1). Although Mn^{++} is more effective than Mg^{++} at low concentrations, maximal activity is obtained with $10^{-2}M$ Mg^{++}. The reaction is somewhat stimulated by low concentrations of EDTA, as shown in Table 1, but no other cofactors appear to be required. Neither ATP nor UDPG has any effect on the rate or maximal level of galactose incorporation. The reaction is inhibited by relatively high levels of uracil nucleotides.

In view of the possibility that biosynthesis of complex polysaccharides might involve participation of an RNA template, it was of interest to test the effect of

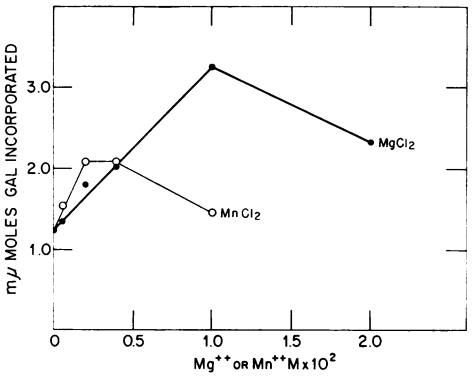

FIG. 1.—Metal requirement of the UDP-galactose-lipopolysaccharide transferase. The standard incubation mixture was employed (0.42 mg of protein) except that $MnCl_2$ or $MgCl_2$ were added at the concentrations indicated.

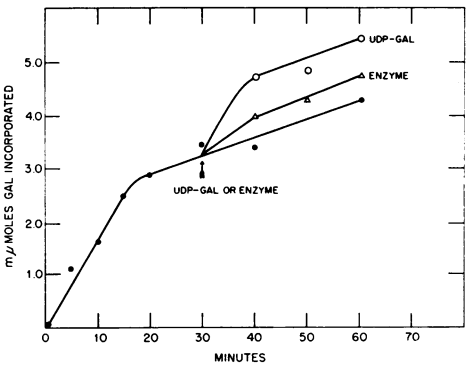

FIG. 2.—Time course of galactose incorporation. The standard incubation mixture (0.28 mg of enzyme protein) was employed, except that 0.04 μmole of UDP-galactose-C^{14} was added at zero time. At 30 min, an additional 0.04 μmole of UDP-galactose-C^{14} was added to one set of tubes, and 0.28 mg of enzyme to a second set.

RNase on the present system. Neither addition of RNase to the incubation mixture nor preincubation of the enzyme with RNase had any effect on the incorporation of galactose (Table 1). However, these results do not rule out the possibility that RNA may be involved in specifying monosaccharide sequence in the highly ordered, antigenically active regions of the complete polysaccharide, which contains mannose, rhamnose, and abequose in addition to galactose.

The rate of galactose incorporation is proportional to enzyme concentration up to about 0.3 mg of protein. Determination of the K_m for UDP-galactose by the method of Lineweaver and Burk[21] yielded a value of $6.2 \times 10^{-5} M$.

The time course of the reaction is illustrated in Fig. 2. The rate of galactose transfer is constant for 10 to 15 min, but thereafter decreases markedly. Addition of more enzyme at 30 min has little effect, but addition of UDP-galactose-C^{14} results in a significant increase in galactose incorporation. The maximal level of galactose incorporation achieved corresponds to 1 galactose residue per 10–20 glucose residues in the endogenous polysaccharide. It is not yet known whether this value represents saturation of the existing galactose acceptor sites, or whether other factors limit the enzymatic transfer of the sugar in the relatively crude system used.

Nature of the reaction product: The conclusion that the defective mutant lipopolysaccharide acts as acceptor of the transferred galactose is based on isolation and degradation of the lipopolysaccharide and the C^{14}-labeled product. The lipopolysaccharide of the epimeraseless mutant is quantitatively released from crude cell-wall preparations by phenol extraction, and can be recovered in 80–90% yield by precipitation with Mg^{++}, as described under *Materials and Methods*. Mg^{++} precipitation has proved to be definitely superior to the more usual techniques of ultracentrifugation and solvent fractionation for purification of the mutant lipopolysaccharide. The material is recovered in high yield, contains virtually no RNA, and is also free of bacterial glycogen. Analytical data on the purified lipopolysaccharide and the results of degradation studies will be reported in detail

in a separate communication; the present discussion will be confined to a summary of evidence bearing directly on the nature of the product of the UDP-galactose transferase system.

Carbohydrate analyses of the lipopolysaccharide show the presence of glucose, heptose, and hexosamine, in a molar ratio of approximately 1:1:0.3. Hydrolysis of the acid-insoluble lipopolysaccharide in 0.01 N H_2SO_4 or 1% acetic acid for 2 hr at 100° results in liberation of over 90% of the glucose and heptose in a soluble form. Hexosamine, on the other hand, is recovered essentially quantitatively in the insoluble lipid residue. Glucose and heptose are released into the acid-soluble fraction at identical rates. On paper electrophoresis at pH 3.5 or 5.0, the bulk of the carbohydrate migrates toward the anode as a single broad band; in addition, a small amount (10–15%) of a neutral component is observed which consists primarily of free glucose. The degraded polysaccharide, as isolated electrophoretically, contains phosphate, heptose, and glucose, in a ratio of approximately 1:1:0.75. Some indication that the material is heterogeneous with respect to size and glucose: heptose ratio has been obtained on further fractionation by paper chromatography, ionophoresis, and by column chromatography on Dowex-1-Cl and charcoal; however, all fractions contain both glucose and heptose in addition to phosphate. The present evidence indicates that the isolated polysaccharide is a phosphorylated heteropolymer containing both sugars, and is not simply a mixture of separate glucose and heptose polymers. It is worth noting that these phosphorylated oligosaccharides or polysaccharides are very difficult to detect on paper with common carbohydrate spray reagents, such as $AgNO_3$–NaOH; they are readily visualized, however, by the periodate-ammoniacal $AgNO_3$ technique.[22]

The available evidence strongly suggests that the product of the UDP-galactose transferase contains galactose bound to the glucose-heptose-phosphate polysaccharide. When the endogeneous lipopolysaccharide of the enzyme preparation is isolated after exposure to UDP-galactose-C^{14}, 80–90% of the radioactivity originally incorporated into acid-insoluble material is recovered in the final, purified lipopolysaccharide fraction. The recovery of radioactivity, therefore, parallels the recovery of lipopolysaccharide closely. The bound radioactivity is nondialyzable, and is not released by treatment with RNase, DNase, or trypsin. On hydrolysis of the purified C^{14}-labeled product in 0.01 N H_2SO_4 or 1% acetic acid, 90–95% of the radioactivity is released into the acid-soluble fraction in parallel with the bulk of the polysaccharide. After electrophoresis or chromatography of the hydrolysate, approximately 75% of the C^{14} is recovered in the major, phosphorylated polysaccharide fraction; free galactose-C^{14} accounts for the remainder of the radioactivity.

The exact site of linkage of galactose is not yet known. Attempts to isolate C^{14}-galactose-containing oligosaccharides have been hindered by the high acid lability of the galactose linkage. Hydrolysis of the C^{14}-galactose labeled polysaccharide in 0.1 N H_2SO_4 at 100° results in rapid liberation of free galactose, as determined by paper chromatography and radioautography. In the radioautogram shown in Fig. 3, approximately 25% of the C^{14} appeared as free galactose after hydrolysis for 10 min. On cochromatography with unlabeled galactose, this area of radioactivity corresponded exactly to that of the carrier galactose. The rest of the C^{14} was still bound to phosphorylated polysaccharide (or oligosaccharide) material, which

remains at the origin in this solvent system. After hydrolysis for 60 min, free galactose accounted for more than 50% of the radioactivity, but in addition, trace amounts of at least three slower moving labeled components were visible. The most rapidly moving of these had an R_f identical to that of lactose. These trace components, which appear to be galactose-containing neutral oligosaccharides, account for only 5% of the total radioactivity, and have not yet been isolated in sufficient quantity for structural analysis.

Discussion.—The characterization of the cell-wall defect in mutants lacking UDP-galactose-4-epimerase by Nikaido[10, 11] has allowed, for the first time, an *in vitro* approach to the study of lipopolysaccharide biosynthesis in *Salmonella*. The data presented here confirm and extend his original observations on the composition of the mutant cell wall and the enzymatic transfer of galactose from UDP-galactose into the wall lipopolysaccharide. The hypothesis that the incomplete lipopolysaccharide acts as specific acceptor of galactose transferred from UDP-galactose is supported by the finding that the enzyme activity is localized in the cell-wall membrane fraction, and that activity is observed only with preparations derived from strains having a galactose-deficient lipopolysaccharide. In addition, the properties of the reaction product appear to be identical with those of the lipopolysaccharide. Bound C^{14}-galactose is recovered essentially quantitatively in the purified lipopolysaccharide fraction, and after separation of the polysaccharide from the lipid moiety, the labeled product is indistinguishable from the bulk of the polysaccharide in chromatographic and electrophoretic properties. However, our present evidence is by no means conclusive, and final identification of the galactose acceptor must be based on detailed structural analysis of the product and the original mutant polysaccharide. Such studies are now in progress.

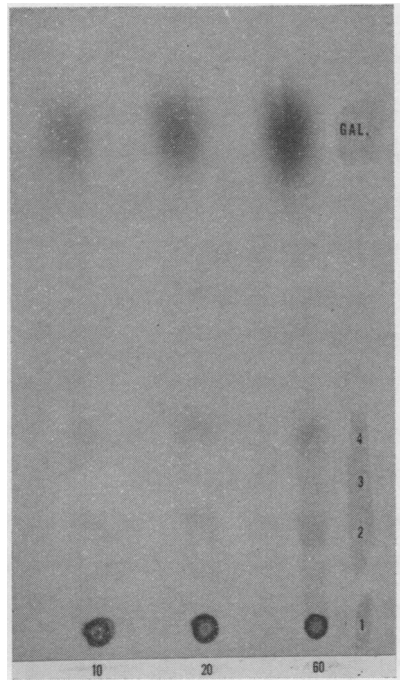

FIG. 3.—Chromatography and radioautography of the C^{14}-labeled product after partial acid hydrolysis. The product was purified as described in *Materials and Methods*, and hydrolyzed for 2 hr in 0.01 *N* H_2SO_4. The acid soluble, radioactive polysaccharide was isolated by paper electrophoresis in pyridine: acetic acid: H_2O (1:10:289), pH 3.5, and subjected to partial hydrolysis in 0.1 *N* H_2SO_4 at 100° for 10, 20, and 60 min. The hydrolysates were neutralized with $BaCO_3$, and chromatographed in *n*-butanol:pyridine:H_2O (6:4:3). Approximately 6,000 cpm of each hydrolysate (equivalent to 0.03 μmole of galactose) were applied to the paper. The radioautogram was obtained by exposure of the chromatogram to Kodak No-Screen X-ray film for 10 days.

In order to account for the nature of the defect in the lipopolysaccharide of epimeraseless strains, Nikaido[11] proposed that galactose forms essential branch points linking a polyglucose core structure to the complex antigenic side chains of the normal polysaccharide. Although preliminary in nature, our evidence indicates that heptose and phosphate, as well as glucose, are integral components of the core structure. It is hoped that further development of nucleotide sugar lipopolysac-

charide transferase systems will provide a means of testing these hypotheses, complementary to the classical methods of structural analysis. For example, it would be predicted that the enzymatic transfer of antigenic side chain components, e.g., mannose or rhamnose, into the incomplete polysaccharide should be dependent upon prior incorporation of galactose. Extension of the present studies to the incorporation of sugars other than galactose should also provide insight into the biosynthetic mechanisms responsible for the specificity of sequence characteristic of the complex polysaccharides carrying O-antigenic specificity.

* This work was supported by grants from the U.S. Public Health Service and the National Science Foundation.

† Research Career Development Awardee of the U.S. Public Health Service.

‡ Postdoctoral Fellow of the National Science Foundation.

§ Aided by Grant No. A-1431 from the U.S. Public Health Service.

[1] Leloir, L. F., J. M. Olavarría, S. H. Goldemberg, and H. Carminatti, *Arch. Biochem. Biophys.*, **81**, 508 (1959).

[2] Hehre, E. J., *J. Biol. Chem.*, **163**, 221 (1946); *J. Am. Chem. Soc.*, **75**, 4866 (1953).

[3] Leloir, L. F., M. A. R. deFekete, and C. E. Cardini, *J. Bicl. Chem.*, **236**, 636 (1961).

[4] Markovitz, A., J. A. Cifonelli, and A. Dorfman, *J. Biol. Chem.*, **234**, 2343 (1959); Markovitz, A., and A. Dorfman, *ibid.*, **237**, 273 (1962).

[5] Smith, E. E. B., G. T. Mills, and H. P. Bernheimer, *J. Biol. Chem.*, **236**, 2179 (1961).

[6] Kauffman, F., O. Lüderitz, H. Stierlin, and O. Westphal, *Zentr. Backteriol. Parasitenk. Abt. I. Orig.*, **178**, 442 (1960).

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

[8] Robbins, P. W., and T. Uchida, *Biochemistry*, **1**, 323 (1962).

[9] Fukasawa, T., and H. Nikaido, *Nature*, **184**, 1168 (1959).

[10] Nikaido, H., *Biochim. et Biophys. Acta*, **48**, 460 (1961); Fukasawa, T. and H. Nikaido, *ibid.*, **48**, 470 (1961).

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

[12] Wiesmeyer, H., and E. Jordan, *Anal. Biochem.*, **2**, 281 (1961).

[13] Diedrich, D. F., and L. Anderson, *Anal. Biochem.*, **2**, 68 (1961).

[14] Fukasawa, T., and H. Nikaido, *Virology*, **11**, 508 (1960).

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

[16] Dische, Z., *J. Biol. Chem.*, **204**, 983 (1953).

[17] Davies, D. A. L., *Biochem. J.*, **67**, 253 (1957).

[18] Slein, M. W., and G. W. Schnell, *J. Biol. Chem.*, **203**, 837 (1953); *Proc. Soc. Exp. Biol. Med.*, **82**, 734 (1953).

[19] Weidel, W., *Z. Physiol. Chem.*, **299**, 253 (1955).

[20] Lüderitz, O., F. Kauffman, H. Stierlin, and O. Westphal, *Zentr. Backtericl. Parasitenk. Abt. I. Orig.*, **179**, 180 (1960).

[21] Lineweaver, H., and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

[22] Hough, L., and J. K. N. Jones, in *Methods in Carbohydrate Chemistry*, ed., R. L. Whistler and M. L. Wolfrom (New York: Academic Press, 1962), vol. I, p. 28.